Palomino • Leão • Ritacco

TUBERCULOSIS 2007

From Basic Science to Patient Care

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Palomino – Leão – Ritacco

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Preface

This book is the result of a joint effort in response to the Amedeo Challenge to write and publish a medical textbook on tuberculosis. This non-profit-making initiative is particularly attractive due to several reasons. First, the medium chosen for dissemination: the book will be readily available on the internet and access will be free to anyone. Second, its advantage over books published via traditional media is the ease to update the information on a regular basis. Third, with the exception of Spanish and Portuguese, no copyright is allocated and the translation of Tuberculosis 2007 to all other languages is highly encouraged.

These innovations in the way of publication were translated to the organization of the chapters in the book. This is not a classical textbook on tuberculosis diagnosis, management, and treatment. On the contrary, it is a multidisciplinary approach addressing a full range of topics, from basic science to patient care. Most authors are former members of RELACTB – a Tuberculosis Research Network for Latin America, the Caribbean and Europe sponsored by the United Nations University – and have worked on collaborative projects since 1995.

Classical knowledge about the disease is focused on chapters dedicated to the history of tuberculosis, microbiology of the tubercle bacillus, description of the disease caused by *Mycobacterium tuberculosis* complex members in adults, children, and HIV/AIDS patients, conventional epidemiology, diagnostics, biosafety, and treatment.

More recent findings, which have changed our knowledge about tuberculosis in the last years, are detailed in chapters on the molecular evolution of the *M. tuberculosis* complex, molecular epidemiology, host genetics, immune response and susceptibility to tuberculosis, studies on the pathogenesis of tuberculosis in animal models, and new diagnostic and drug resistance detection approaches.

Perspectives for future research relevant to fighting the disease have also been included in chapters focusing on the "omics" technologies, from genomics to proteomics, metabolomics and lipidomics, and on research dedicated to the development of new vaccines and new diagnostic methods, and are discussed in the last chapter.

Nowadays, medical science should not be limited to academic circles but readily translated into practical applications aimed at patient care and control of disease. Thus, we expect that our initiative will stimulate the interest of readers not only in solving clinical topics on the management of tuberculosis but also in posing new questions back to basic science, fostering a continuous bi-directional interaction of medical care, and clinical and basic research.

Juan Carlos Palomino, Sylvia Cardoso Leão, Viviana Ritacco

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Abbreviations

2-DE: two dimensional electrophoresis
ADA: adenosine deaminase
ADC: albumin, dextrose, catalase
AFB: acid fast bacilli
AIDS: acquired immunodeficiency syndrome
AMTD: Amplified *Mycobacterium tuberculosis* Direct Test
BAC: bacterial artificial chromosome
BCG: bacille Calmette-Guérin
bp: base pair
cAMP: cyclic adenosine monophosphate
CAS: Central-Asian (or Delhi)
CD4+: cluster of differentiation 4 glicoprotein
CD8+: cluster of differentiation 8 glicoprotein
CDC: Centers for Disease Control and Prevention
cfu: colony forming units
CMI: cell mediated immunity
CPC: cetylpyridinium chloride
CPF-10: culture filtrate protein 10
CR: complement receptor
CRISPR: clustered regularly interspersed palindromic repeats
CTL: cytotoxic T lymphocyte
DARQ: diarylquinoline
DAT: diacyl trehalose
DC-SIGN: dendritic cell-specific intercellular-adhesion-molecule-grabbing non-integrin
DNA: desoxyribonucleic acid
DOTS: directly observed therapy short-course
DR: direct repeat
DST: drug susceptibility test
DTH: delayed type hypersensitivity
EAI: East-African-Indian
EDTA: ethylenediaminetetraacetic acid
ELISA: enzyme-linked immunosorbent assay
ELISPOT: enzyme-linked immunospot for interferon-gamma
EMB: ethambutol
ESAT-6: 6 kDa early secretory antigenic target
ETH: ethionamide
Fc: crystallizable fraction of the Ig molecule
FDA: Food and Drug Administration
FGF-2: fibroblast growth factor 2

G+C: guanine plus cytosine
GLC: gas-liquid chromatography
GLP: good laboratory practices
HAART: highly active anti-retroviral therapy
HEPA: high efficiency particulate air
HIV: human immunodeficiency virus
HLA: human leukocyte antigen
HPLC: high-performance liquid chromatography
Hsp: heat-shock protein
IATA: International Air Transportation Association
ICAT: isotope-coded affinity tag
IFN-γ: interferon-gamma
IFN-γR: interferon-gamma receptor
InhA: enoyl acyl carrier protein reductase
Ig: immunoglobulin
IL: interleukin
INH: isoniazid
iNOS: inducible nitric oxide synthase
IS*6110* RFLP: restriction fragment length polymorphism based on insertion sequence IS*6110*
ITS: internal transcribed spacer
IUATLD: International Union Against Tuberculosis and Lung Disease
KasA: beta-ketoacyl ACP synthase
KatG: catalase-peroxidase enzyme
kDa: kiloDalton
LC: liquid chromatography
LSP: large sequence polymorphism
mAG: mycolyl-arabinogalactan
MALDI: matrix assisted laser desorption/ionization
MBL: mannose-binding lectin
MCP-1: monocyte chemoattractant protein-1
MDR: multidrug-resistant
MGIT: Mycobacteria Growth Indicator Tube
MHC: major histocompatibility complex
MIC: minimal inhibitory concentration
MIRU: mycobacterial interspersed repetitive units
MLST: multilocus sequence typing
MODS: microscopic observation broth-drug susceptibility assay
mRNA: messenger ribonucleic acid
MS: mass spectrometry
MSMD: mendelian susceptibility to mycobacterial diseases
MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

NEMO: NF-κB essential modulator
NF-κ-B: nuclear factor kappa B
NO: nitric oxide
NRP-1: microaerobic stage of nonreplicating persistence
NRP-2: anaerobic state
NRAMP1: natural resistance-associated macrophage protein 1
nsSNP: non-synonymous single nucleotide polymorphism
NTM: non-tuberculous mycobacteria
OADC: oleic acid, albumin, dextrose, catalase
OD: optical density
ORF: open reading frame
oriC: origin of replication
PANTA: polymyxin B, amphotericin B, nalidixic acid, trimethoprim, azlocillin
PAS: para-aminosalycilic acid
PAT: penta-acyl trehalose
PCR: polymerase chain reaction
PDIM: phthiocerol dimycocerosate
PE: proteins that have the motif Pro-Glu
PGE: prostaglandin E
PGG: principal genetic groups
PGL: phenolic glycolipids
PGRS: polymorphic guanine-cytosine rich sequences
pI: isoelectric point
***pks*:** polyketide synthase gene
PNB: para-nitrobenzoic acid
PPD: purified protein derivative
PPE: proteins that have the motif Pro-Pro-Glu
PRA: PCR-restriction enzyme analysis
PTFE: polytetrafluoroethylene
PZase: pyrazinamidase enzyme
PVNA: polymyxin B, vancomycin, nalidixic acid and amphotericin B
rBCG: recombinant BCG
RCF: relative centrifugal force
rDNA: ribosomal desoxyribonucleic acid
RD: regions of difference
REMA: resazurin microtiter assay
RFLP: restriction fragment length polymorphism
RIF: rifampicin
RNA: ribonucleic acid
RNAse: ribonuclease
rRNA: ribosomal ribonucleic acid

***rrn* operon:** ribosomal ribonucleic acid operon
RvD: DNA region deleted from H37Rv genome
SCG: SNP cluster group
SCID: severe combined immunodeficiency
SL: sulfolipid
SLC11A1: solute carrier family 11, member 1
SM: streptomycin
SNP: single nucleotide polymorphism
SpolDB4: fourth international spoligotyping database
sSNP: synonymous single nucleotide polymorphism
ST: shared-type in SpolDB4
STAT1: signal transducer and activator of transcription 1
TACO: tryptophan aspartate coat protein
TB: tuberculosis
TbD1: *M. tuberculosis* specific deletion 1
TCH: thiophene-2-carboxylic acid hydrazide
TGF-β: transforming growth factor beta
Th1: T helper 1 lymphocyte
Th2: T helper 2 lymphocyte
TL7H11: thin layer 7H11 agar
TLC: thin-layer chromatography
TLR: Toll-like receptor
TNF-α: tumor necrosis factor alpha
TOF: time of flight
TST: tuberculin skin test
US: United States
UV: ultraviolet
VDR: vitamin D receptor
VNTR: variable number tandem repeats
WHO: World Health Organization
XDR: extensively drug resistant

Content

Chapter 1: History ***25***

1.1. Primeval tuberculosis 25

1.2. Phthisis/consumption 29

1.3. The White Plague 30

1.4. The discovery of the tubercle bacillus 32

1.5. Sanatorium and initial therapies 34

1.6. 19th and 20th centuries 38

1.7. A global health emergency 45

References 49

Chapter 2: Molecular Evolution of the Mycobacterium tuberculosis Complex ***53***

2.1. A basic evolutionary scheme of mycobacteria 53

2.2. *M. tuberculosis* complex population molecular genetics 57

2.3. Co-evolution of *M. tuberculosis* with its hosts 58

2.4. *M. tuberculosis* through space and time 61

2.5. Looking for robust evolutionary markers 62

2.6. Why repeated sequences were so useful at the beginning 63

2.7. Regions of differences (RDs) and SNPs in *M. tuberculosis* 63

2.8. Looking for congruence between polymorphic markers 69

2.9. Main lineages within the *M. tuberculosis* species 72

2.10. When did the bovine-human switch of *M. tuberculosis* take place? 78

2.11. Comparative genomics and evolution of tubercle bacilli 79

2.12. Short-term evolutionary markers and database building 80

2.13. Conclusion and Perspectives 81

References 83

Chapter 3: The Basics of Clinical Bacteriology ***93***

3.1. The tubercle bacillus: a continuous taxon 93

3.2. Microscopic morphology 95

3.3. Cell wall structure 97

3.4. Nutritional and environmental requirements for growth 102

3.5. Generation time 105

3.6. Metabolic and biochemical markers 106
3.7. Resistance to physical and chemical challenges 107
References 109

Chapter 4: Genomics and Proteomics ***113***

4.1. Impact of new technologies on *Mycobacterium tuberculosis* genomics 113
4.2. *M. tuberculosis* genome 115
4.3. Gene expression in *M. tuberculosis* 127
4.4. *M. tuberculosis* proteome 135
4.5. An insight into *M. tuberculosis* metabolomics 143
4.6. Concluding remarks 146
References 147

Chapter 5: Immunology, Pathogenesis, Virulence ***157***

5.1. Immune response against *Mycobacterium tuberculosis* 157
5.2. Tuberculosis pathogenesis and pathology related to the immune response 171
5.3. Latency and maintenance of the immune response 183
5.4. Immunotherapy for tuberculosis 184
5.5. Concluding remarks 189
References 189

Chapter 6: Host Genetics and Susceptibility ***207***

6.1. The difficulty in proving a genetic component for human susceptibility 207
6.2 Search for mutations and polymorphisms that increase susceptibility 219
6.3. Candidate genes in common tuberculosis 225
6.4 Genes from mouse genetic susceptibility studies 237
6.5. The good, the bad and the maybe, in perspective 244
References 250

Chapter 7: Global Burden of Tuberculosis ***263***

7.1. Global epidemiology of tuberculosis 263
7.2. Tuberculosis and the interaction with the HIV epidemic 269
7.3. Progress of the DOTS strategy 272

7.4. The new Stop TB strategy 275
References 279

Chapter 8: Tuberculosis caused by Other Members of the M. tuberculosis Complex ***283***

8.1. *Mycobacterium bovis* disease in humans 283
8.2. The BCG vaccine: adverse reactions 290
8.3. *Mycobacterium africanum* subtypes 293
8.4. *Mycobacterium microti* disease 295
8.5. *Mycobacterium caprae* and *Mycobacterium pinnipedii* 297
8.6. Identification of species within the *M. tuberculosis* complex 301
References 305

Chapter 9: Molecular Epidemiology: Breakthrough Achievements and Future Prospects ***315***

9.1. Introduction 315
9.2. Historical context 317
9.3. Infectiousness of tuberculosis patients 319
9.4. DNA fingerprinting, contact investigation and source case finding 320
9.5. Transmission of drug resistant tuberculosis 323
9.6. Resistance and the Beijing genotype 325
9.7. Genetic heterogeneity of *M. tuberculosis* and multiple infections 326
9.8. The new standard genetic marker: VNTR typing 329
9.9. DNA fingerprinting to monitor eradication of tuberculosis 331
9.10. Future prospects 332
References 333

Chapter 10: New Vaccines against Tuberculosis ***341***

10.1. Introduction 341
10.2. Historical view 342
10.3. Genetic diversity between BCG vaccines 344
10.4. New vaccines: from the bench to clinical trials 345
10.5. Subunit vaccine candidates 348
10.6. Subunit vaccines for boosting BCG 350
10.7. Recombinant BCG vaccines 350
10.8. Live vaccines based on attenuated *M. tuberculosis* 352

10.9. Conclusions 354
References 356

Chapter 11: Biosafety and Hospital Control ***361***
11.1. Biosafety in the hospital 361
11.2. Biosafety in the laboratory 372
References 396

Chapter 12: Conventional Diagnostic Methods ***401***
12.1. Introduction 401
12.2. Specimen handling 402
12.3. Smear staining 406
12.4. Adenosine deaminase activity 409
12.5. Culture 410
12.6. Identification 416
References 420

Chapter 13: Immunological Diagnosis ***425***
13.1. Historical Overview 425
13.2. Current methods of tuberculosis diagnosis 427
13.3. Basis of immunological diagnosis 428
13.4. Serological assays 431
13.5. T cell based assays 433
13.6. Conclusions and Perspectives 437
References 438

Chapter 14: New Diagnostic Methods ***441***
14.1. Introduction 441
14.2. Automated culture methods 441
14.3. Nucleic acid amplification methods 450
14.4. Genetic identification methods 461
14.5. Non-conventional phenotypic diagnostic methods 472
References 479

Chapter 15: Tuberculosis in Adults ***487***

15.1. Introduction 487
15.2. The initial lesion 487
15.3. The inflammatory response 489
15.4. Tuberculosis infection 490
15.5. Tuberculosis disease 492
15.6. Diagnostic approaches 508
15.7. Treatment of latent tuberculosis infection 516
15.8. Contact tracing and control 518
15.9. The limits between infection and disease 519
References 519

Chapter 16: Tuberculosis in Children ***525***

16.1. Introduction 525
16.2. Etiology, transmission and pathogenesis 526
16.3. Primary pulmonary tuberculosis 530
16.4. Non-respiratory disease 533
16.5. Congenital tuberculosis 536
16.6. Diagnosis 537
16.7. Pediatric tuberculosis treatment 544
16.8. Vaccination 552
16.9. Prognosis of pediatric tuberculosis 553
References 554

Chapter 17: Tuberculosis and HIV/AIDS ***559***

17.1. Epidemiological background 559
17.2. Interactions between *M. tuberculosis* and HIV infection 560
17.3. Clinical characteristics 561
17.4. Multidrug-resistant tuberculosis and HIV/AIDS 568
17.5. Treatment of tuberculosis in HIV/AIDS patients 574
17.6. Immune reconstitution inflammatory syndrome 579
17.7. Treatment of latent tuberculosis infection in HIV/AIDS patients 580
17.8. Mycobacteriosis in AIDS patients 581
References 585

Chapter 18: Drugs and Drug Interactions ***593***
18.1. Introduction 593
18.2. Overview of existing treatment schemes 594
18.3. Drugs: structure, pharmacokinetics and toxicity 601
18.4. Drug resistance mechanisms 612
18.5. Drug interactions 617
18.6. New drugs for tuberculosis 621
18.7. Useful links 627
References 627

Chapter 19: Drug Resistance and Drug Resistance Detection ***635***
19.1. Introduction 635
19.2. Drug resistance surveillance 635
19.3. Methods for detection of drug resistance 640
References 655

Chapter 20: New Developments and Perspectives ***661***
20.1. The scenario 661
20.2. Bacillus and disease under the light of molecular epidemiology 662
20.3. New perspectives in diagnosis 665
20.4. The problem of drug resistance detection 669
20.5. On drug development 670
20.6. On vaccine development 671
20.7. Global management of research & development resources 673
20.8. Useful links 674
References 675

Chapter 1: History

Sylvia Cardoso Leão and Françoise Portaels

> *Nowhere in these ancient communities of the Eurasian land mass, where it is so common and feared, is there a record of its beginning. Throughout history, it had always been there, a familiar evil, yet forever changing, formless, unknowable. Where other epidemics might last weeks or months, where even the bubonic plague would be marked forever afterwards by the year it reigned, the epidemics of tuberculosis would last whole centuries and even multiples of centuries. Tuberculosis rose slowly, silently, seeping into homes of millions, like an ageless miasma. And once arrived, it never went away again. Year after year, century after century, it tightened its relentless hold, worsening whenever war or famine reduced the peoples' resistance, infecting virtually everybody, inexplicably sparing some while destroying others, bringing the young down onto their sickbeds, where the flesh slowly fell from their bones and they were consumed in the years-long fever, their minds brilliantly alert until, in apocalyptic numbers, they died, like the fallen leaves of a dreadful and premature autumn.*
>
> The Forgotten Plague:
>
> How the War against Tuberculosis was Won - and Lost
>
> Frank Ryan, 1992

Tuberculosis (TB) has a long history. It was present before the beginning of recorded history and has left its mark on human creativity, music, art, and literature; and has influenced the advance of biomedical sciences and healthcare. Its causative agent, *Mycobacterium tuberculosis,* may have killed more persons than any other microbial pathogen (Daniel 2006).

1.1. Primeval tuberculosis

It is presumed that the genus *Mycobacterium* originated more than 150 million years ago (Daniel 2006). An early progenitor of *M. tuberculosis* was probably contemporaneous and co-evolved with early hominids in East Africa, three million years ago. The modern members of *M. tuberculosis* complex seem to have originated from a common progenitor about 15,000 - 35,000 years ago (Gutierrez 2005).

TB was documented in Egypt, India, and China as early as 5,000, 3,300, and 2,300 years ago, respectively (Daniel 2006). Typical skeletal abnormalities, including Pott's deformities, were found in Egyptian and Andean mummies (Figure 1-1) and were also depicted in early Egyptian and pre-colombian art (Figure 1-2).

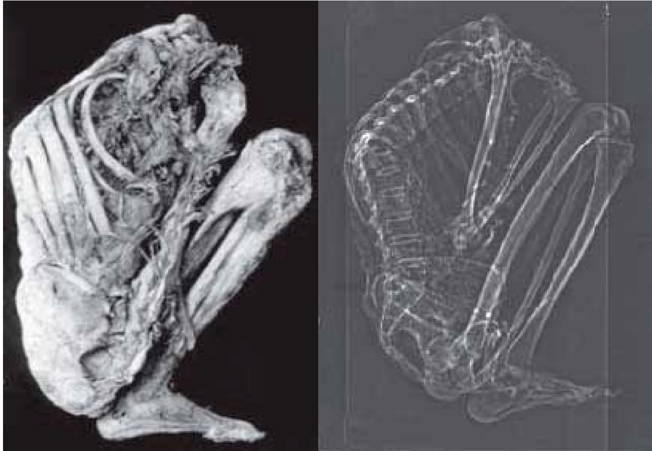

Figure 1-1: Left: Mummy 003, Museo Arqueológico de la Casa del Marqués de San Jorge, Bogota, Colombia. Right: Computerized tomography showing lesions in the vertebral bodies of T10/T11 (reproduced from Sotomayor 2004 with permission).

Figure 1-2: Representation of a woman with pronounced gibbus (Pott´s disease?). Momil culture, 200 BC to 100 AD, Sinú River, Colombia (reproduced from Sotomayor 1992, with permission).

Identification of genetic material from *M. tuberculosis* in ancient tissues has provided a powerful tool for the investigation of the incidence and spread of human TB in historic periods. It also offers potential new insights into the molecular evolution and global distribution of these microbes (see Chapter 2). Research on ancient DNA poses extreme technical difficulties because of the minute amounts of DNA remains, their oxidation/hydrolysis, and the extremely high risk of contamination with modern DNA. For this reason, stringent criteria of authenticity for analysis of ancient DNA were recently proposed, among them: work in physically isolated areas, strict protocols to prevent contamination with modern DNA, the use of negative controls, evaluation of reproducibility in different laboratories, cloning and sequencing, and the study of associated remains (Coper 2002).

Mycobacteria are assumed to be better preserved than other bacteria due to the resistant lipid-rich cell wall and the high proportion of guanine and cytosine in their DNA, which increases its stability. *M. tuberculosis* are found only in the tissues of an infected host, and the characteristic pathology induced by this strictly mammalian pathogen tends to show residual microbial DNA contained in localized lesions. These bacteria are, therefore, ideal microorganisms for studying ancient DNA and were the first to be pursued. These investigations have answered important questions. They proved that TB is an ancient disease with a wide geographical distribution. The disease was widespread in Egypt and Rome (Zink 2003, Donoghue 2004); it existed in America before Columbus (Salo 1994, Konomi 2002, Sotomayor 2004), and in Borneo before any European contact (Donoghue 2004). The earliest DNA-based documentation of the presence of *M. tuberculosis* complex organisms was accomplished in a subchondral articular surface from an extinct long-horned Pleistocene bison from Wyoming, US, which was radiocarbon-dated at 17,870 +/- 230 years before the present (Rothschild 2001).

Another important achievement of the studies on ancient DNA was the confirmation of the TB diagnosis in human remains that showed the typical pathology. Mycobacterial DNA was detected in bone lesions in the spine of a male human skeleton from the Iron Age (400-230 BC), found in Dorset, United Kingdom (Taylor 2005); skin samples from the pelvic region of Andean mummies, carbon-dated from 140 to 1,200 AD (Konomi 2002); and calcified pleura from 1,400 year-old remains, found in a Byzantine basilica in the Negev desert (Donoghue 1998). DNA techniques have also shown the presence of mycobacterial DNA, at a lower frequency, in bones with no pathological changes, suggesting either dissemination of the TB bacilli immediately prior to death or chronic milliary TB (Zink 2003).

Molecular methods other than PCR have also been used to demonstrate the presence of the tubercle bacillus in ancient remains, including mycolic acid analysis by

high performance liquid chromatography (HPLC), which is used for authentication of positive PCR findings in calcified pleura remains (Donoghue 1998). Spoligotyping is a PCR-based technique used for identification and typing of *M. tuberculosis* complex bacteria (see Chapter 9). It is a valuable tool for the study of archeological material, especially when the DNA is highly fragmented, because fragments as small as 55-60 bp long are sufficient to provide a positive result (Donoghue 2004). Spoligotyping was the method used to study the Plesitocene remains of a bison (Rothschild 2001) and was also applied to a subculture of the original tubercle bacillus isolated by Robert Koch, confirming its species identification as *M. tuberculosis* rather than *Mycobacterium bovis* (Taylor 2003).

Until recently, the search for mycobacterial DNA in human archeological specimens failed to find evidence of the presence of *M. bovis,* a member of the *M. tuberculosis* complex with a remarkably wide spectrum of susceptible mammalian hosts, and once considered a putative ancestor of *M. tuberculosis* (Donoghue 2004). In an up to date publication, the identification of *M. bovis* DNA in South Siberian human remains was confirmed by amplification of *pncA* and *oxyR* genes and analysis of regions of difference (RD) (for a comprehensive review on differentiation of species belonging to the *M. tuberculosis* complex, see Chapters 2 and 8). These findings were obtained from remains that showed skeletal evidence of TB. Carbon-dated from 1,761 to 2,199 years ago, they seem to indicate that this population was continuously exposed to wild or domesticated animals infected with *M. bovis*, which could have been reservoirs for human infection (Taylor 2007).

1.2. Phthisis/consumption

The patients suffer from a latent fever that begins towards evening and vanishes again at the break of day. It is accompanied by violent coughing, which expels thin purulent sputum. The patient speaks with a hoarse voice, breathes with difficulty and has hectically flushed cheeks. The skin on the rest of the body is ashen in color. The eyes have a weary expression, the patient is gaunt in appearance but often displays astonishing physical or mental activity. In many cases, wheezes are to be heard in the chest, and when the disease spreads, sweating is seen on the upper parts of the chest. The patients lose their appetite or suffer hunger pangs. They are often also very thirsty. The ends of the fingers swell and the fingernails curve greatly.

Caelius Aurelianus, 5th century AD (Herzog 1998)

The term phthisis (meaning consumption, to waste away) appeared first in Greek literature. Around 460 BC, **Hippocrates** identified phthisis as the most widespread disease of the times. It most commonly occurred between 18 and 35 years of age, and was almost always fatal (www.tuberculosistextbook.com/link.php?id=1). He even warned physicians against visiting consumptives in advanced stages of the disease, to preserve their reputation! Although **Aristotle** (384-322 BC) considered the disease to be contagious, most Greek authors believed it to be hereditary, and a result, at least in part, of the individual's mental and moral weaknesses. **Clarissimus Galen** (131-201 AD), the most eminent Greek physician after Hippocrates, defined phthisis as an ulceration of the lungs, chest or throat, accompanied by coughs, low fever, and wasting away of the body because of pus. He also described it as a disease of malnutrition (Pease 1940).

The initial tentative efforts to cure the disease were based on trial and error, and were uniformly ineffective. Heliotherapy was advocated as early as the 5th century AD by Caelius Aurelianus. Roman physicians recommended bathing in human urine, eating wolf livers, and drinking elephant blood. In the Middle Ages, it was believed that the touch of the sovereigns of England and France had the power to cure sufferers of the King’s Evil or scrofula (scrophula or struma) - the swellings of the lymph nodes of the neck, frequently related to TB. Depending upon the time and country in which they lived, patients were urged to rest or to exercise, to eat or to abstain from food, to travel to the mountains or to live underground.

1.3. The White Plague

Yet the captain of all these men of death that came against him to take him away was consumption, for it was that that brought him down to the grave.

The life and death of Mr. Badman, presented to the world in a familiar dialogue between Mr. Wiseman and Mr. Attentive

John Bunyan, 1680

The TB epidemic in Europe, later known as the "Great White Plague", probably started at the beginning of the 17th century and continued for the next 200 years. Death from TB was considered inevitable and, by 1650, TB was the leading cause of mortality. The high population density and poor sanitary conditions that characterized the enlarging cities of Europe and North America at the time, provided the necessary environment, not met before in world history, for the spread of this airborne pathogen. The epidemic spread slowly overseas by exploration and colonization.

TB existed in America before Columbus' arrival but was rare among the natives. The major outbreaks of TB among the native people of North America began in 1880, after they were settled in reservations or forced to live in barracks in prison camps. Death rates increased rapidly, and by 1886, reached 9,000 per 100,000 people (Bates 1993).

TB was also rare among Africans who lived in small remote villages. When exposed to the disease by contact with Europeans, these populations experienced a high mortality rate. Africans taken as slaves were free from TB on arrival to the Americas. Then, cases of sub-acute fatal TB developed among them. After their liberation from slavery and movement into the cities, TB morbidity and mortality rose quickly, reaching 700 per 100,000 in 1912 (Bates 1993).

There is also evidence of the presence of the disease in pre-historic Asia, but it was only toward the end of the 19th century that peaks in incidence were observed in India and China.

In the 18th century, TB was sometimes regarded as vampirism. These folk beliefs originated from two observations: firstly, following the death from consumption of a family member, household contacts would lose their health slowly. This was attributed to the deeds of the recently deceased consumptive, who returned from the dead as a vampire to drain the life from the surviving relatives. Secondly, peo-

ple who had TB exhibited symptoms similar to what people considered to be vampire traits, such as red, swollen eyes, sensitivity to bright light, pale skin, and a blood-producing cough. They "wasted away" and "lost flesh" and at the same time remained active, and conserved a fierce will to live. This dichotomy of lust and "wasting away" was reflected in the vampires' desire for "food", which forced them to feed off living relatives, who, in turn, suffered a similar wasting away (Sledzik 1994).

Precise pathological and anatomical descriptions of the disease began to appear in the 17th century. **Franciscus Sylvius de la Böe** of Amsterdam (1614-1672) was the first to identify the presence of actual tubercles as a consistent and characteristic change in the lungs and other areas of consumptive patients. In his *Opera Medica,* published in 1679, he also described the progression of the lesions from tubercles to ulcers and cavities. The Latin word *tuber* means all kinds of degenerative protuberances or tubercles.

The English physician **Richard Morton** (1637-1698) confirmed that tubercles were always present in TB of the lungs. He believed that the disease had three stages: inflammation (tubercle formation), ulceration, and phthisis. Both Sylvius de la Böe and Morton regarded the disease as hereditary, although Morton did not rule out transmission by intimate contact.

Gaspard Laurent Bayle (1774-1816) definitely proved that tubercles were not products, or results, but the very cause of the illness. The name 'tuberculosis' appeared in the medical language at that time in connection with Bayle's theory. More precisely, the name 'tuberculosis' was coined in 1839 by the German professor of Medicine **Johann Lukas Schönlein** (1793-1864), to describe diseases with tubercles; but he considered scrofula and phthisis to be separate entities. These ideas were also acknowledged by **Giovanne Battista Morgagni** in Padua (1682-1771) and **Rudolf Virchow** in Berlin (1821-1902) (Herzog 1998). In contrast, **René Théophile Hyacinthe Laënnec** (1781-1826) from Paris, inventor of the stethoscope, and the Viennese **Karl von Rokitansky** (1804-1878) emphasized the unitary nature of both conditions.

The earliest references to the infectious nature of TB appeared in 17th century Italian medical literature. An edict issued by the Republic of Lucca in 1699 stated that, *"... henceforth, human health should no longer be endangered by objects remaining after the death of a consumptive. The names of the deceased should be reported to the authorities and measures undertaken for disinfection"* (Herzog 1998).

1.4. The discovery of the tubercle bacillus

Not bad air, not just a weakness of the infected human body's immune system, not any of the myriad theories that had filled the puzzled heads of his audience all of their working lives...but a bacterium. Not just a bacterium, but a bacillus the like of which had never been even suspected before, a most singular life form, with a frightening propensity to infect every cat and chicken, pigeon and guinea pig, the white mice and rats, oxen and even two marmosets, into which Koch had injected it.

The Forgotten Plague: How the War against Tuberculosis was Won - and Lost

Frank Ryan, 1992

The book *De Morbus Contagiosus,* written in 1546 by **Girolamo Fracastoro** (1478-1553), explained the contagious nature of TB. He pointed out that bed sheets and clothing could contain contagious particles that were able to survive for up to two years. The word "particles" may have alluded to chemicals rather than to any kind of living entity.

In his publication *A New Theory of Consumptions,* in 1720, the English physician **Benjamin Marten** (1704-1722) was the first to conjecture that TB could be caused by "minute living creatures", which, once they had gained entry to the body, could generate the lesions and symptoms of phthisis. He further stated, that consumption may be caught by a sound person by lying in the same bed, eating and drinking or by talking together so close to each other as to "*draw in part of the breath a consumptive patient emits from the lungs*".

In 1865, the French military doctor **Jean-Antoine Villemin** (1827-1892) demonstrated that consumption could be passed from humans to cattle, and from cattle to rabbits. On the basis of this revolutionary evidence, he postulated that a specific microorganism caused the disease. At this time **William Budd** (1811-1880) also concluded from his epidemiological studies that TB was spread through society by specific germs.

On the evening of March 24, 1882, in Berlin, before a skeptical audience composed of Germany's most prominent men of science from the Physiological Society, **Robert Koch** (1843-1910) (www.tuberculosistextbook.com/link.php?id=2) made his famous presentation *Die Aetiologie der Tuberculose*. Using solid media made of potato and agar, Koch invented new methods of obtaining pure cultures of bacteria. His colleague **Julius Richard Petri** (1852-1921) developed special flat dishes (Petri dishes), which are still in common use, to keep the cultures. Koch also de-

veloped new methods for staining bacteria, based on methylene blue, a dye developed by **Paul Ehrlich** (1854-1915) (www.tuberculosistextbook.com/link.php?id=3), and counterstained with vesuvin. "*Under the microscope the structures of the animal tissues, such as the nucleus and its breakdown products are brown, while the tubercle bacteria are a beautiful blue*", he wrote in the paper that followed his dramatic presentation that March evening (Koch 1882).

He had brought his entire laboratory with him: his microscopes, test tubes, small flasks with cultures, and slides of human and animal tissues preserved in alcohol. Showing the presence of the bacillus was not enough. He wanted his audience to note that bacteria were always present in TB infections and could be grown on solidified serum slants, first appearing to the naked eye in the second week. Then, he showed that, by inoculating guinea pigs with tuberculous material obtained from lungs, intestines, scrofula or brains of people and cattle that have died from TB, the disease that developed was the same, and cultures obtained from the experimental animals were identical on the serum slopes. Koch continued his speech, proving that whatever the dose and/or route he used, no matter what animal species he inoculated, the results were always the same. The animals subsequently developed the typical features of TB. He concluded saying that "*…the bacilli present in tuberculous lesions do not only accompany tuberculosis, but rather cause it. These bacilli are the true agents of tuberculosis*" (Kaufmann 2005).

Koch fulfilled the major prerequisites for defining a contagious disease that had, in fact, been proposed by his former mentor **Jacob Henle** (1809-1885). The reknowned Koch's postulates (or Henle-Koch postulates) were then formulated by Robert Koch and **Friedrich Loeffler** (1852-1915) in 1884, and finally polished and published by Koch in 1890. The postulates consist of four criteria designed to establish a causal relationship between a causative microbe and a disease:

- The organism must be found in all animals suffering from the disease, but not in healthy animals
- The organism must be isolated from a diseased animal and grown in pure culture
- The cultured organism should cause disease when introduced into a healthy animal
- The organism must be re-isolated from the experimentally infected animal.

In 1890, at the 10th International Congress of Medicine held in Berlin, Koch announced a compound that inhibited the growth of tubercle bacilli in guinea pigs

when given both pre- and post-exposure. It was called 'tuberculin' and was prepared from glycerol extracts of liquid cultures of tubercle bacilli. Clinical trials using tuberculin as a therapeutic vaccine were soon initiated. The results were published in 1891 and revealed that only few persons were cured, at a rate not different from that of untreated patients. But, although results for treatment were disappointing, tuberculin was proven valuable for the diagnosis of TB (Kaufmann 2005).

One of Koch's papers (Koch 1891), describing the preparation and partial purification of tuberculin served as the first description of the production of the partially purified derivative (PPD) of tuberculin, presently used in the Mantoux test, also known as the Tuberculin Skin Test, Pirquet test, or PPD test (see Chapter 13).

1.5. Sanatorium and initial therapies

> *...not for nothing was it famous far and wide. It had great properties. It accelerated oxidization, yet at the same time one put on flesh. It was capable of healing certain diseases which were latent in every human being, though its first effects were strongly favorable to these, and by dint of a general organic compulsion, upwards and outwards, made them come to the surface, brought them, as it were, to a triumphant outburst.*
>
> *- Beg pardon -- triumphant?*
>
> *- Yes; had he never felt that an outbreak of disease had something jolly about it, an outburst of physical gratification?*

The Magic Mountain [Der Zauberberg]

Thomas Mann, 1924

Translated from the German by H. T. Lowe-Porter, 1953

Dialogue between Hans Castorp and consul Tienappel

The introduction of the sanatorium cure provided the first widely practiced approach to anti-tuberculosis treatment. **Hermann Brehmer** (1826-1889) a Silesian botany student suffering from TB, was instructed by his doctor to seek out a healthier climate. He traveled to the Himalayas where he studied the mountain's flora. He returned home cured and began to study medicine. In 1854, he presented his medical dissertation *Tuberculosis is a Curable Disease*. Brehmer then opened an in-patient hospital in Gorbersdorf, where patients received good nutrition and

were continuously exposed to fresh air. This became the model for all subsequent sanatoria, including the one depicted in Thomas Mann’s *The Magic Mountain.*

A young doctor named **Edward Livingston Trudeau** (1848-1915) established the most famous sanatorium in the United States at Saranac Lake, in New York's Adirondak Mountains (http://www.trudeauinstitute.org/info/history/history.htm). He also suffered from TB and, in 1882, became aware of Koch's experiments with TB bacteria and of Brehmer's sanatorium. Trudeau established the Saranac Laboratory for the Study of Tuberculosis. It was the first institution devoted to TB research in the United States (US).

Sanatoria, increasingly found at that time throughout Europe and the US, provided a dual function. Firstly, they protected the general population by isolating the sick persons, who were the source of infection. Secondly, they offered TB patients bed-rest, exercise, fresh-air, and good nutrition, all of which assisted the healing process. Many of them improved and returned to "life in the flatland"; many did not. The TB specialist, the phthisiologist, was responsible for the complete physical and mental care of the patient and the separation of TB care from the practicing clinician became commonplace.

Architectural features were essential to early sanatorium design (Figure 1-3). These included deep verandas, balconies, covered corridors, and garden shelters, furnished with reclining couches for the “Cure”, the obligatory two-hour period of rest in the open air that was frequently observed in silence (Figure 1-4). Furniture for TB patients had to be robust, able to be thoroughly cleaned and disinfected, and shaped with a concern for the patient’s anthropometric needs.

Alvar Aalto (1898-1976), **Jan Duiker** (1890-1935) and **Charles-Edouard Jeanneret (Le Corbusier)** (1887-1965) were modernist architects and designers that adapted and interpreted the ideas of functionality and rationality derived from concepts used in the treatment of TB, and their designs for buildings and furniture became icons of modernism. Aalto won the competition of Architecture, Interior Design and Furniture Design for the constuction of the Paimio Tuberculosis Sanatorium in 1928, and Duiker designed the Zonnestraal Sanatorium. The symbolic association of light and air with healing made a profound influence on modernist ideas for design. Flat roofs, balconies, terraces and reclining chairs were subsequently adopted for the design of fashionable buildings in rapidly expanding cities such as Paris and Berlin (Campbell 2005).

Figure 1-3: Sanatorio Pineta del Carso, Trieste, Italy.

Figure 1-4: Sanatorio Pineta del Carso. Bed-rest, fresh air and good nutrition were the hallmarks of sanatorium cure.

Probably, it will never be known whether sanatorium treatment was a success or a failure, because no study was undertaken comparing the rates of mortality of sanatorium patients with those of TB patients who were similar in age, sex, and economic position, but who remained untreated or were treated by other methods.

Nevertheless, physicians with a long and intimate experience with the disease were unanimous in the opinion that open-air treatment was an improvement for the average consumptive (McCarthy 2001).

During the early ’60s, many sanatoria started to close. By the middle of that decade only a few beds remained available for patients suffering from TB. Yet, the real end of the TB sanatorium began even earlier, when the depressing era of helplessness in the face of advanced TB was substituted by active therapy.

The Italian physician **Carlo Forlanini** (1847-1918) discovered that the collapse of the affected lung tended to have a favorable impact on the outcome of the disease. He proposed to reduce the lung volume by artificial pneumothorax and surgery, methods that were applied worldwide after 1913. These and other initial therapies are now considered dangerous and, at least, controversial:

- **Artificial pneumothorax** - pleural cavities were filled with gas or filtered air, with the result of splinting and collapsing that lung (Sharpe 1931).
- **Bilateral pneumothorax** - only parts of the lungs were collapsed in such a way that the patient could still live a relatively normal live. The patient suffered from shortness of breath caused by the reduction in the gas exchange surface.
- **Thoracoplasty** - ribs from one side of the thorax were removed in order to collapse the infected portion of the lung permanently (Samson 1950).
- **Gold Therapy** - **Holger Mollgaard** (1885-1973) from Copenhagen introduced the compound sanocrysin in 1925, which is a double thiosulphate of gold and sodium. He tested the compound on animals and considered it safe for human use. However, it was too toxic even in low doses. A controlled trial, completed in the US in 1934, proved the toxic effects of gold therapy. Within a year, most European countries had ceased to use it (Bedenek 2004).

1.6. 19th and 20th centuries

> *There is a dread disease which so prepares its victim, as it were, for death; which so refines it of its grosser aspect, and throws around familiar looks unearthly indications of the coming change; a dread disease, in which the struggle between soul and body is so gradual, quiet, and solemn, and the result so sure, that day by day, and grain by grain, the mortal part wastes and withers away, so that the spirit grows light and sanguine with its lightening load, and, feeling immortality at hand, deems it but a new term of mortal life; a disease in which death and life are so strangely blended, that death takes the glow and hue of life, and life the gaunt and grisly form of death; a disease which medicine never cured, wealth never warded off, or poverty could boast exemption from; which sometimes moves in giant strides, and sometimes at a tardy sluggish pace, but, slow or quick, is ever sure and certain.*
>
> Nicholas Nickleby
> Charles Dickens, 1870

When, in 1820, the poet **John Keats** (1795-1821) coughed a spot of bright red blood, he told a friend, "*It is arterial blood. I cannot be deceived. That drop of blood is my death warrant. I must die*". He died within a year, at just 25 years of age. Keats never wrote specifically about phthisis, but his life and his works became a metaphor that helped transform the physical disease "phthisis" into its spiritual offspring, "consumption".

The central metaphor of consumption in the 19th century was the idea that the phthisic body is consumed from within by its passions. Spes phthisica (*spes* - hope + *phthisis* - consumption) was a condition believed to be peculiar to consumptives in which physical wasting led to a sense of well-being and happiness, an euphoric blossoming of passionate and creative aspects of the soul. While the body expired from phthisis, the prosaic human became poetic and the creative soul could be released from the fevered combustion of the body. The paleness and wasting, the haunted appearance, the burning sunken eyes, the perspiring skin - all hallmarks of the disease - came to represent feminine beauty, romantic passion, and fevered sexuality (Morens 2002).

In the 19th century, it seemed as if everyone was slowly dying of consumption. The disease became to be viewed in popular terms, first as romantic redemption (Figure 1-5), then as a reflection of societal ills (Figure 1-6) (Morens 2002). In **Alexandre Dumas'** tale "The Lady of the Camellias", the heroine was a courtesan regenerated

by love and made unforgettable by progressive consumption. It was adapted to the theatre and the movies and also inspired **Giuseppe Verdi**'s opera "La Traviata". The plot develops around the consequences of the heroine's scandalous past, which prevents her marriage to an honorable youngster whose father objects to the relationship. Redemption is possible only through death, and, in taking her life, consumption also serves as a vehicle for punishment.

Figure 1-5: Romantic view of TB: "The Lady of the Camellias" represented by Brazilian actress Cacilda Becker under Italian director Luciano Salce, in São Paulo, Brazil (1952).

By 1896, the cause of consumption had been discovered, and TB was definitively linked to poverty and industrial disfigurement, child labor, and sweatshops. A contagious disease and shameful indicator of class, it was no longer easily romanticized in conventional artistic terms. **Giacomo Puccini**'s "La bohème" (1896) portrays TB in a new environment, affecting street artists struggling with poverty and disease (Figure 1-6).

At the end of the 19th century, the association of TB with poor living conditions and hygiene brought to life the differentiation and societal repulsion of diseased persons, considered to be responsible for a social wickedness. Unlike the previous image (sick people as victims), they began to be viewed as dangerous, because they were capable of spreading the disease to those who did not share their living conditions. TB was changed from a social disease to an individual one and the patient was at the same time offender and victim of this social ailment.

A list of famous people and celebrities who had, or are believed to have had TB is available on Wikipedia at www.tuberculosistextbook.com/link.php?id=4 and at www.tuberculosistextbook.com/link.php?id=5

Figure 1-6: Social aspect of TB: Second act of “La bohéme”, showing Quartier Latin, with a great crowd on the street and sellers praising their wares.

After the establishment, in the ’80s, that the disease was contagious, TB was made a notifiable disease. A further significant advance came in 1895, when **Wilhelm Konrad von Röntgen** (1845-1923) discovered X-rays (www.tuberculosistextbook.com/link.php?id=6). After this, the progress and severity of a patient's disease could be accurately documented and reviewed.

At the beginning of the 20th century, public health authorities realized that TB was preventable and that it was not directly inherited. Several associations were set up to educate the community at large. Books educated people about bad food, bad air and unhealthy drinking water. Public health reformers used illustrative posters and stamps (see http://www.nlm.nih.gov/exhibition/visualculture/tuberculosis.html) as a means of communication, advertisement, and persuasion. This new medium quickly became an effective educational and fundraising tool in the widespread campaign against TB.

Centralized official and/or non-governmental agencies for coordination and communication were organized and called for conferences specifically focused on TB. At the Central Bureau for the Prevention of Tuberculosis, which was formalized in Berlin in 1902, **Dr. Gilbert Sersiron** suggested that, as the fight against TB was a crusade, it would be appropriate to adopt the emblem of a crusader, the Duke of

Lorraine. **Godfrey of Bouillon** (1060-1100), Duke of Lorraine, was the first Christian ruler of Jerusalem and his banners bearing the double-barred cross signified courage and success to crusaders. Dr. Sersiron's recommendation was adopted and the double-barred cross became the worldwide symbol of the fight against TB (Figure 1-7).

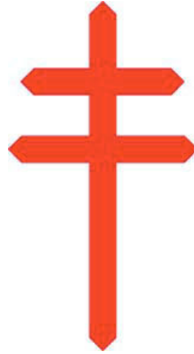

Figure 1-7: double-barred cross, symbol of anti-tuberculosis crusade

Periodic international conferences systematically addressing clinical, research and sociological aspects of TB were held until the outbreak of World War I in 1914. After the war, in 1920, a conference on TB was held in Paris with participation of delegates from 31 countries, among them Australia, Bolivia, Brazil, Chile, China, Colombia, Cuba, Guatemala, Japan, Panama, Paraguay, Iran and Thailand, in addition to those of Europe and North America, thus establishing the International Union Against Tuberculosis and Lung Disease (IUATLD, http://www.iuatld.org/index_en.phtml) in its present form.

With **Edward Jenner**'s (1749-1823) successful invention, showing that infection with cowpox would give immunity against smallpox in humans, many doctors placed their hopes on the use of *M. bovis* – the agent that causes bovine TB – for the development of a vaccine against human TB. However *M. bovis* was equally contagious in humans. From 1908 until 1919, **Albert Calmette** (1863-1933) (http://www.pasteur.fr/infosci/archives/cal0.html) and **Camille Guérin** (1872-1961) (http://www.pasteur.fr/infosci/archives/gue0.html) in France serially passed a pathogenic strain of *M. bovis* 230 times, resulting in an attenuated strain called Bacille Calmette-Guérin or BCG, which was avirulent in cattle, horses, rabbits, and

guinea pigs. BCG was first administered to humans in 1921 and it is still widely applied today (see Chapter 10).

Then, in the middle of World War II, came the final breakthrough, the greatest challenge to the bacterium that had threatened humanity for thousands of years - chemotherapy. In 1943, streptomycin, a compound with antibiotic activity, was purified from *Streptomyces griseus* by **Selman A. Waksman** (1888-1973) (www.tuberculosistextbook.com/link.php?id=7) and his graduate student **Albert Shatz** (1920-2005) (Shatz 1944a). The drug was active against the tubercle bacillus in vitro (Schatz 1944b) and following infection of guinea pigs (Feldman 1944). It was administered to a human patient at the end of 1944 (Hinshaw 1944). Two pioneering clinical studies were conducted on the treatment of TB patients with streptomycin, one in Europe and the other in the US (Medical Research Council 1948, Pfuetze 1955). A considerable improvement in the disease was observed in patients on streptomycin therapy, but after the first months, some patients began to deteriorate and these pioneering studies properly interpreted such treatment failure as a consequence of development of resistance to the drug.

In 1943, **Jörgen Lehmann** (1898-1989) wrote a letter to the managers of a pharmaceutical company, Ferrosan, suggesting the manufacture of the para-amino salt of aspirin because it would have anti-tuberculous properties (Ryan 1992). The Swedish chemist based his theory on published information, stressing the avidity of tubercle bacilli to metabolize salicylic acid. He realized that by changing the structure of aspirin very slightly, the new molecule would be taken up by the bacteria in just the same way, but would not work like aspirin and would rather block bacterial respiration. Para-aminosalicylic acid (PAS) was produced and first tested as an oral therapy at the end of 1944. The first patient treated with PAS made a dramatic recovery (Lehmann 1964). The drug proved better than streptomycin, which had nerve toxicity and to which *M. tuberculosis* could easily develop resistance.

In the late ’40s, it was demonstrated that combined treatment with streptomycin and PAS was superior to either drug alone (Daniels 1952). Yet, even with the combination of the two drugs, TB was not defeated. Overall, about 80 % of sufferers from pulmonary TB showed elimination of their germs; but 20 % were not cured, especially those with extensive disease and cavitation (Ryan 1992).

Two further findings were very important for TB treatment. Firstly, between 1944 and 1948, the action of nicotinamide on the TB bacillus was discovered by two different groups, but this discovery was not widely appreciated at the time. Secondly, in 1949, reports stated that the Germans had treated some 7,000 tuberculous

patients with a new synthetic drug of the thiosemicarbazone series (Conteben), developed by **Gerhard Domagk** (1895-1964) (www.tuberculosistextbook.com/link.php?id=8), the discoverer of the first sulphonamide (McDermott 1969). There is a remarkable similarity between the atomic structures of nicotinamide, Conteben, and PAS. Conteben and PAS both contain a chemical ring of six carbon atoms, the benzene ring, while nicotinamide contains the pyridine ring in which an atom of nitrogen replaces one of the carbon atoms (Fox 1953). Thus, by substituting the benzene ring in thiosemicarbazone by this pyridine ring, a new drug, isoniazid, was developed. By mere coincidence, this was accomplished simultaneously in three pharmaceutical companies – one in Germany (Bayer) and two in the US (Squibb and Hoffman La Roche). Isoniazid was soon submitted for clinical testing and because of the favorable impact of its administration on disease evolution, the lay press headlines already told the story of the "wonder drug" before any scientific paper was published (Ryan 1992). However, none of the three pharmaceutical companies could patent the new drug, because it had already been synthesized back in 1912 by two Prague chemists, **Hans Meyer** and **Joseph Mally**, as a requirement for their doctorates in chemistry. Nevertheless, while clinical studies were still underway, six studies showed that *M. tuberculosis* readily became resistant to isoniazid (Ryan 1992).

In the view of many doctors in those early stages of chemotherapy, the role for drug therapy was to bring the disease under sufficient control to allow surgeons to operate the diseased organs. **John Crofton** (1912-) (www.tuberculosistextbook.com/link.php?id=9), working at the University of Edinburgh, developed a protocol that resulted in a breakthrough in TB treatment and control. With his "Edinburgh method" based on meticulous bacteriology and application of the available chemotherapy, a 100 percent cure rate for TB was a reasonable objective. With the success rate obtained by using three drugs together, (streptomycin, PAS, and isoniazid) TB was completely curable, making surgical treatment redundant.

Dr. Crofton believed that the conquest of the disease would also imply other measures, such as pasteurization of milk, tuberculin testing in cattle, BCG vaccination, mass radiography screening for early diagnosis of disease, isolation of infectious cases, and general population measures, including reduction of overcrowding and general improvement of the standard of living.

The "Madras Experiment" was carried out in India in 1956 to test a totally different concept of therapy, by comparing the results of treatment in a sanatorium with treatment at home with daily PAS and isoniazid for a year. After a 5-year period of

follow up, the proportion of persons clear of disease in the two groups was similar and approached 90 %.

The spirit of optimism that followed was encouraged by the discovery of a series of new anti-tuberculosis drugs. The drug company Lepetit discovered that the mold *Streptomyces mediterranei* produced a new antibiotic, Rifamycin B. Chemical manipulation of this compound by CIBA resulted in the production of rifampicin, which has a remarkable potency against *M. tuberculosis*. Other compounds with anti-tuberculosis activity were discovered: pyrazinamide, ethambutol, cycloserine, and ethionamide.

At the end of the '70s, the primary care of TB patients moved from specialized institutions to general hospitals and ambulatory care services. At that time, many hospitals were reluctant to assume such responsibility for fear of spreading the disease to other patients and to hospital personnel. To overcome their apprehension, rational safety measures were introduced for the provision of primary care to TB patients in those settings. Earlier studies on TB transmission performed by Wells and Riley provided an insight into the characteristics of TB transmission and set the basis for its containment (Gunnels 1977, see Chapter 11). By applying the experimental design of his mentor **William Firth Wells, Richard Riley** pioneered the study that first documented the role of the droplet nuclei in the transmission of TB (Riley 1962). The experiments were carried out using guinea pigs lodged in chambers above wards where TB patients were hospitalized. Only particles small enough to be carried by the air reached the animals, which, as a result of the inhalation of these particles, became infected with the same strains as those infecting the patients. This could be confirmed by comparison of drug susceptibility patterns.

Indeed, the conclusions of those investigations still stand strong. During coughing, sneezing, talking or singing, sputum smear-positive TB patients can eliminate large or small droplets of moisture containing viable bacilli. Large droplets tend to settle quickly onto the floor and, if inhaled, are trapped in the upper airways and destroyed by local mucocilliary defenses. Smaller droplets (1-10 µm) remain suspended in the air for prolonged periods of time. Evaporation of moisture leaves a residue – the droplet nucleus. This frequently contains only one or a few bacteria, which are the infectious units of TB. It was thus established that the risk of TB transmission is proportional to the concentration of droplet nuclei in the environment.

Infectivity was also found to be associated with environmental conditions and the characteristics of the disease in each individual case, such as the bacillary content of sputum, the presence of cavitation, the frequency of cough, and the presence of

laringeal TB (see Chapter 11). Therapy with anti-tuberculosis drugs was identified as the most effective measure for controlling patient´s production of infectious particles and thus readily reversing infectivity (Gunnels 1977). Therefore, patients should only require isolation while they were sputum positive and before initiation of specific therapy. Hospitalization was either abolished or reduced to a few weeks for most patients (Kaplan 1977). Once a patient´s diagnosis and treatment program had been defined, physicians who had no particular expertise in chest medicine could maintain a quality treatment program in most instances. That was the end of the phthisiologist´s era.

1.7. A global health emergency

- *Someone in the world is newly infected with TB bacilli every second.*
- *Overall, one-third of the world's population is currently infected with the TB bacillus.*
- *5-10 % of people who are infected with TB bacilli (but who are not infected with HIV) become sick or infectious at some time during their life. People with HIV and TB infection are much more likely to develop TB.*

World Health Organization
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Revised March 2006

In Europe and in the US, the general improvement in public health helped to reduce the burden of TB well before the arrival of specific drugs. TB program activities, reinforced by successful chemotherapy, resulted in a pronounced reduction of infection and death rates. The disease became greatly controlled but it never quite disappeared. Then, in around 1985, cases of TB began to rise again in industrialized countries. Several inter-related forces drove this resurgence, including increase in prison populations, homelessness, injection drug use, crowded housing and increased immigration from countries where TB continued to be endemic. Above all, the decline in TB control activities and the human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) epidemic were two major factors fueling each other in the reemergence of TB.

TB programmes had become loose in industrialized countries because the disease was considered close to elimination. A study performed in 1991 showed that 89 %

of 224 patients discharged on TB treatment were lost to follow-up and failed to complete therapy. More than a quarter were back in hospital within a year, still suffering from TB (Brudney 1991). This study reflected the occurrence of inconsistent or partial treatment, which was going on everywhere (Clancy 1990). Patients cease to take all their medicines regularly for the required period for different reasons: they start to feel better, doctors and health workers prescribe the wrong treatment regimens, or the drug supply is unreliable. Uncompliance frequently results in the emergence of bacteria resistant to drugs and ultimately in the emergence of a "superbug", resistant to all effective drugs (Iseman 1985). Multidrug-resistant TB, or MDR-TB, refers to *M. tuberculosis* isolates that are resistant to at least both isoniazid and rifampicin, the two most powerful anti-tuberculosis drugs. MDR-TB takes longer to treat with second-line drugs, which are more expensive and have more side-effects (see Chapters 18 and 19).

In the early '90s, an extensive outbreak of highly resistant TB affected more than 350 patients in New York City. The strain was resistant to all first-line anti-tuberculosis drugs and almost all patients had HIV/AIDS. The hospital environment was the setting where more than two thirds of the patients acquired and transmitted the infection. As a consequence, this outbreak affected mainly HIV-infected patients and health care workers (Frieden 1996). At that time, New York City became the epicenter of drug-resistant TB, where one in three new cases were found resistant to one drug and one in five to more than one drug. Important HIV/AIDS related hospital outbreaks of MDR-TB similar to the one occurred in New York were described also in non-industrialized countries like Argentina (Ritacco 1997).

Indeed, the HIV/AIDS epidemic has produced a devastating effect on TB control worldwide. While one out of ten immunocompetent people infected with *M. tuberculosis* will fall sick in their lifetimes, among those with HIV infection, one in ten per year will develop active TB. In developing countries, the impact of HIV infection on the TB situation, especially in the 20-35 age group, is overwhelming.

While wealthy industrialized countries with good public health care systems can be expected to keep TB under control, in much of the developing world a catastrophe awaits. In poorly developed countries, TB remains a significant threat to public health, as incidences remain high, even after the introduction of vaccination and drug treatment (Murray 1990). The registered number of new cases of TB worldwide roughly correlates with economic conditions: highest incidences are seen in the countries of Africa, Asia, and Latin America with the lowest gross national products (see Chapter 7).

Supervised treatment, including sometimes direct observation of therapy (DOT), was proposed as a means of helping patients to take their drugs regularly and complete treatment, thus achieving cure and preventing the development of drug resistance. The Directly-Observed Treatment, Short-course (DOTS, http://www.who.int/tb/dots/whatisdots/en/index.html) strategy was promoted as the official policy of the WHO in 1991 (see Chapter 7).

The World Health Organization estimates that eight million people get TB every year, of which 95% live in developing countries. An estimated two million people die yearly from TB. World Health Organization (http://www.who.int/tb/en) declared TB a global health emergency in 1993 (World Health Organization 2006).

In 1998, the IUATLD joined with the WHO and other international partners to form the Stop TB Initiative, a defining moment in the re-structuring of global efforts to control TB. The original Stop TB Initiative has evolved into a broad Global Partnership, Stop TB Partnership (http://www.stoptb.org), with partners gathered in Working Groups to accelerate progress in seven specific areas: DOTS Expansion, TB/HIV, MDR-TB, New TB Drugs, New TB Vaccines, New TB Diagnostics, and Advocacy, Communications and Social Mobilization.

The World Health Assembly of 2000 endorsed the establishment of a Global Partnership to Stop TB and the following targets:

- By 2005: 70% of people with infectious TB will be diagnosed and 85% of them cured.
- By 2015: the global burden of TB disease (deaths and prevalence) will be reduced by 50% relative to 1990 levels.
- By 2050: The global incidence of TB disease will be less than one per million population (elimination of TB as a global public health problem)

In spite of these global efforts, TB continues to pose a dreadful threat. A notorious example is the sudden emergence in 2005, in a rural hospital located in Kwa-Zulu-Natal, a South African province, of a deadly form of TB associated with HIV/AIDS. This outbreak illustrates the devastating potential of what came to be called extensively drug resistant TB (XDR-TB) (Gandhi 2006). XDR-TB was defined as MDR-TB with further resistance to second-line drugs (see Chapter 19). XDR-TB can develop when these second-line drugs are also misused or mismanaged and, therefore, also become ineffective (Raviglione 2007). The menace of XDR-TB is not restricted to that remote African setting. A recent survey, performed by 14 supra-national laboratories, on drug susceptibility testing results from 48 countries confirmed this. From 19.9 % of identified MDR-TB isolates, 9.9 %

met the criteria for XDR-TB. These isolates originated from six continents, confirming the emergence of XDR-TB as a serious worldwide public health threat (Shah 2007).

Nowadays, treating TB is feasible and effective, even in low income countries, if based on reliable public health practice, including good laboratory infrastructure, appropriate treatment regimens, proper management of drug side-effects and resources to maintain adherence and prevent spread. The emergence of XDR-TB should stimulate the improvement of these basic control measures.

It is also crucially important to intensify research efforts devoted to developing effective TB vaccines, as well as shortening the time required to ascertain drug sensitivity, improving the diagnosis of TB, and creating new, highly effective anti-tuberculosis medications. Without supporting such efforts, we still run the risk of losing the battle against TB.

Disease names related to different clinical forms of TB

Name	Clinical form
Phthisis	Original Greek name for TB
Lung Sickness	TB
Consumption	TB
Lupus vulgaris	TB of the skin
Mesenteric disease	TB of the abdominal lymph nodes
Pott’s disease	TB of the spine
Scrofula	TB of the neck lymph nodes
King´s evil	TB of the neck lymph nodes
White Plague	TB especially of the lungs
White swelling	TB of the bones
Milliary TB	Disseminated TB

Acknowledgement: This chapter is dedicated to Professor Pino Pincherle (1893-1996), radiologist, founder and director of the Sanatorio Pineta del Carso in Trieste, Italy. Since the establishment of the Sanatorium, in 1933, Professor Pincherle was responsible for all physiotherapy treatment and radiologic exams, but after only five years he was compelled to sell his part in the Sanatorium due to racial laws. In 1939 the family emigrated to Brazil. He is the grandfather of Sylvia Cardoso Leão, author of this chapter.

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Chapter 2: Molecular Evolution of the *Mycobacterium tuberculosis* Complex

Nalin Rastogi and Christophe Sola

2.1. A basic evolutionary scheme of mycobacteria

Mycobacteria are likely to represent a very ancient genus of bacteria. Probably, the *Mycobacterium* genus originates from a common ancestor whose offspring specialized in the process of colonizing very different ecological niches. The evolutionary relationships between organisms of the genus *Mycobacterium* have been investigated on the basis of the analysis of derived similarities ("shared derived traits", synapomorphies).

Since no contemporary living species may directly stem from another contemporary species, it is advisable to speak of «common ancestors», by building cladograms rather than genealogical trees when comparing a monophyletic group. Such cladistic analysis (the word clade is derived from the ancient Greek κλάδος, klados, meaning branch) forms an ideal basis for modern systems of biological classification. Cladograms so generated are invariably dependent on the amount of information selected by the researcher.

An ideal approach takes into account a wide variety of information in order to form a natural group of organisms (**clade**) which share a unique ancestor that is not shared with other organisms on the tree, i.e., each clade comprises a series of characteristics specific to its members (**synapomorphies**), and absent from the group of organisms from which it diverged. Such distinction involves the notion of **outgroups** (organisms that are closely related to the group but not part of it). The choice of an outgroup constitutes an essential step, since it can profoundly change the topology of a tree. Similarly, much attention is needed to distinguish between **characters** and **character states** prior to such analysis (e.g., "blue eyes" and "black eyes" are two character states of the character "eye-color"). A character state of a determined clade which is also present in its outgroups and its ancestor is designated as **plesiomorphy** (meaning "close form", also called ancestral state). The character state which occurs only in later descendants is called an **apomorphy** (meaning "separate form", also called the "derived" state). As only synapomorphies are used to characterize clades, the distinction between plesiomorphic and synapomorphic character states is made by considering one or more outgroups.

A collective set of plesiomorphies is commonly referred to as a **ground plan** for the clade or clades they refer to; and one clade is considered **basal** to another if it

holds more plesiomorphic characters than the other clade. Usually, a basal group is very species-poor in comparison to a more derived group. Thus, conservative (apomorphic) branches, defined as **anagenetic branches** represent species whose characteristics are closer to those of the ancestor than others.

Possibly, the founder of the genus *Mycobacterium* was a free-living organism and today's free-living mycobacterial species (and also some saprophytic species?) represent the conservative branches of founding mycobacteria. The more distant organisms are probably the ones that live in association with various multicellular organisms. It has been suggested that the mycobacteria that created a long-lasting association with marine animals (probably placoderms) are at the root of this phylogenetic branch. Thus, *Mycobacterium marinum* would stem from the conservative branch, whereas other vertebrate-associated mycobacteria would build the anagenetic branch. Grmek speculates that the association of a mycobacterial species with a marine vertebrate may have occurred during the superior Devonian (300 million years ago) (Grmek 1994). Figure 2-1 shows the phylogenetic position of the *Mycobacterium tuberculosis* complex species within the genus *Mycobacterium* based on a tree of the gene coding for the 16S ribosomal ribonucleic acid (rRNA).

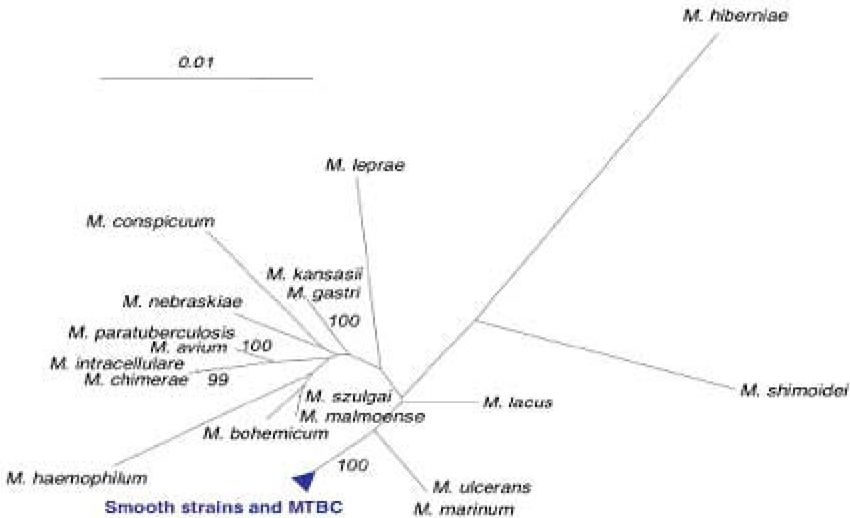


Figure 2-1: Phylogenetic position of the tubercle bacilli within the genus *Mycobacterium* (reproduced with permission from Gutierrez et al. 2005)

In the past, mycobacterial systematics used to rely on phenotypic characters; more recently, however, genetic techniques have boosted taxonomic studies (Tortoli 2003). The first natural characters used to distinguish between mycobacterial species were growth rate and pigmentation. Rapid growers (< 7 days) are free, environmental, saprophytic species, whereas slow growers are usually obligate intracellular, pathogenic species. The slow-fast grower division, which virtually always relies on the possession of one or two rRNA operons (*rrn* operon) (Jy 1994), was shown to be phylogenetically coherent (Stahl 1990, Devulder 2005).

In the '50s, the hypothesis of co-evolution, or parallel evolution, between hosts and mycobacteria looked no more likely than the alternative hypothesis of «multiple, casual (furtive) introductions» of various saprophytes into different hosts. The traditional epidemiological belief for tuberculosis (TB) is that the anthropozoonosis due to *M. tuberculosis* may find its origin in a zoonotic agent, i.e., *Mycobacterium bovis* (Cockburn 1963). This view is still sustained by some authors (Smith 2006a). However, genetics brought some new clues into the debate (Brosch 2002). For example, the sequencing of the *Mycobacterium leprae* genome, by its defective nature, confirmed the previous history-driven hypothesis that *M. leprae* was a younger pathogen than *M. tuberculosis* (Cole 1998, Cole 2001). In the case of the *M. tuberculosis* complex, comparative genomics has also shown that the *M. bovis* genome is smaller than the *M. tuberculosis* genome, opening the way to a new scenario for the evolution of the tubercle bacillus (Brosch 2002). *M. bovis* genomic reduction (loss of genes) indeed suggests that it could be a younger pathogen than *M. tuberculosis* or, in other words, that human TB disease preceded bovine disease (Brosch 2002, Cockburn 1963). Figure 2-2 shows that the common ancestor of members of the *M. tuberculosis* complex is close to three of its branches: "*Mycobacterium canettii*", *Mycobacterium africanum* and the ancestral East-African-Indian (EAI) clade. However, according to Smith et al., "*until it is demonstrated that strains of M. africanum subtype I can be maintained in immunocompetent cells, the host-association of the most recent common ancestor of the M. tuberculosis complex remains unsolved"* (Smith 2006b).

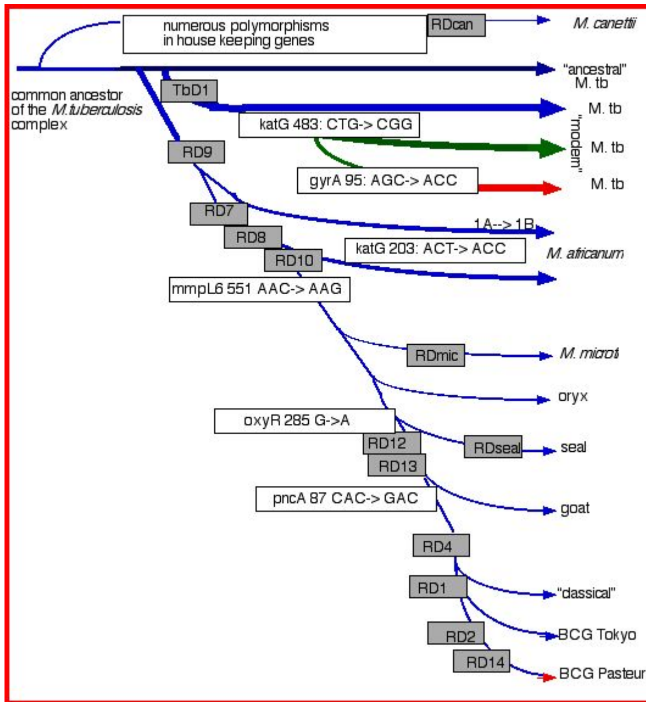


Figure 2-2: Scheme of the proposed evolutionary pathway of the *M. tuberculosis* bacilli illustrating successive loss of DNA in certain lineages (reproduced with permission from Brosch *et al.* 2002)

Ancient humans, bovids and mastodons experienced erosive diseases caused by *M. tuberculosis*. As an alternative to the classical hypothesis of TB spread being driven by human migration, bovids, mastodons, or simply diet might well be considered to be the natural epidemiological vehicle of TB. In this way, a poorly pathogenic environmental or animal *Mycobacterium* spp. would have progressively acquired some human-specific virulence traits (Rotschild 2001, Rotschild 2006a). The association of hyperdisease and endemic stability may have promoted a smooth and long-term transition from zoonosis to anthropozoonosis (Coleman 2001, Rotschild 2006b). Other complex anthropological parameters, such as the history of agriculture and livestock domestication, may also have been mediators of TB spread (Smith 1995, Bruford 2003). In this sense, it is also logical to compare the pathogenicity of the various *M. tuberculosis* complex members in various host species. Interestingly, it has been observed that *M. africanum* apparently elicits a more attenuated T cell response to the 6 kiloDalton (kDa) early secreted antigen

(ESAT-6) than *M. tuberculosis* in patients with TB. *M. africanum* could thus be considered to be an opportunistic human pathogen. If confirmed, these findings are new evidence that strain differences affect human interferon-based T cell responses (de Jong 2006). Strain-related differences in lymphokine (including interferon-gamma) response in mice with experimental infection were also reported in 2003 (Lopez 2003).

2.2. *M. tuberculosis* complex population molecular genetics

Until recently, the question of individual genetic variation within the *M. tuberculosis* complex gained little attention and most research on *M. tuberculosis* was organism- rather than population-centered. The advent of molecular methods, and their widespread use in population studies, introduced both new conceptual and new technological developments. The inference of phylogenies from molecular data goes back to the early '90s with the development of software such as PHYLIP and PAUP (Felsenstein 1993, Swofford 1990, Swofford 1998). In particular, the study of the *M. tuberculosis* complex phylogeny closely followed the development of increasing numbers of sophisticated genotyping methods. The way was opened by *M. tuberculosis* fingerprinting by restriction fragment length polymorphism based on insertion sequence IS*6110* (IS*6110* RFLP) (van Embden 1993). However, the use of IS*6110* RFLP in evolutionary genetics discovery was of limited value for many reasons:

- fast variation rate of this evolutionary marker (de Boer 1999)
- complexity of forces driving its transposition and risk of genetic convergence (Fang 2001)
- nature of experimental data produced which requires sophisticated software for analysis
- difficulty to build large sets of data (Heersma 1998, Salamon 1998)

The discovery in 1993 of the polymorphic nature of the Direct Repeat (DR) locus, and the subsequent development of the spoligotyping method based on DR locus variability, introduced more modern concepts and tools for *M. tuberculosis* complex genotyping (Groenen 1993, Kamerbeek 1997). Our research group bet that the highly diverse signature patterns observed by spoligotyping could indeed contain phylogenetical signals, and the construction of a diversity database was started *de novo* (Sola 1999). Today, a total of 62 *M. tuberculosis* complex clades/lineages are detailed in the Fourth International Spoligotyping Database (SpolDB4) which de-

scribes 1,939 shared-types representing a total of 39,295 *M. tuberculosis* strains from 122 countries (Brudey 2006). This database is available on the internet at SITVIT (http://www.pasteur-guadeloupe.fr:8081/SITVITDemo). Some of the major *M. tuberculosis* complex clades and their spoligotype signatures are described below under section 2.9. The assumption that the DR locus was neutral still remains speculative; however, the finding of other clustered regularly interspersed palindromic repeats (CRISPR) loci in both Archae and Bacteriae has become a hot issue (Jansen 2002, Pourcel 2005, Makarova 2006). Spoligotyping was immediately followed by the discovery of tandem repeat loci in the *M. tuberculosis* complex and the Variable Number of Tandem Repeats (VNTR) genotyping technique (Frothingham 1998). Later, the Mycobacterial Interspersed Repetitive Units (MIRU) technique (Supply 2001) was developed, which is also designated as Multiple Locus VNTR analysis (MLVA). Multi-Locus Sequence Typing (MLST) was introduced as an alternative method (Baker 2004). More recently, systematic Single Nucleotide Polymorphism (SNP) genotyping (Filliol 2006, Gutacker 2006) was described followed by Large Sequence Polymorphism (LSP), the latter performed either by microarray or real-time Polymerase Chain Reaction (PCR) (Mostowy 2002, Tsolaki 2005).

2.3. Co-evolution of *M. tuberculosis* with its hosts

Simulation models reported in 1988 suggested that a social network with a size of 180 to 440 persons is required for TB to occur with endemicity. In such conditions, host-pathogen coexistence would be maintained in populations (McGrath 1988). The concept of endemic stability, already mentioned above, suggests that an infectious disease may reach an epidemiological state in which the clinical disease is scarce, despite high levels of infection in the population (Coleman 2001). Clearly, this concept may apply to TB since it is most likely to have been a vertically transmitted disease before being responsible for large outbreaks.

The question of how many isolated communities of between 180 to 440 persons may have experienced, sequentially or concomitantly, the introduction of one or more founding genotypes of *M. tuberculosis* complex (each one with its own specific virulence), in other words, how TB was “seeded” is of paramount importance. To provide the initial conditions of a dynamic epidemic system we must understand how these early founding genotypes spread in low demographic conditions. Today, we can observe a phylogeographically structured global epidemic, built as a result of millennia of evolution. Some clones are extinct, others have an increased risk of emergence (Tanaka 2006). The evolution rate of TB is likely to have been succes-

sively slow (human and cattle migration and low endemicity or hyperendemicity but little or no disease), then moderate (five centuries of post-Columbus sail-based migration) with important anthropological changes that may have created bursting conditions linked to demographic growth and migration, and lastly, fast (since the introduction of air transportation), i.e. within the five decades of increasing movements of strains and people, concomitantly to new outbreaks in demographically active and resource-poor countries where the great majority of cases is now present.

Consequently, the worldwide bacterial genetic snapshot of the TB epidemic is the result of a combination of slow, medium, and fast evolving superimposition pictures of various outbreak histories. Such a jigsaw puzzle will be difficult, if not impossible, to reconstruct. However, looking for rare and isolated genotypes, which may have undergone a slower evolution, as well as searching for ancient desoxyribonucleic acid (DNA) may constitute two complementary scientific strategies in attempting to reach this goal.

One recent success of the first strategy is exemplified by the finding of a peculiar highly genetically diverse "*M. canettii*" in the Horn of Africa. "*M. canettii*" was likely to be the most probable source species of the *M. tuberculosis* complex, rather than just another branch of it (Fabre 2004). Further results confirm that, despite its apparent homogeneity, the "*M. canettii*" or "*M. prototuberculosis*" genome is a composite assembly resulting from horizontal gene transfer events predating clonal expansion. The large amount of synonymous single nucleotide polymorphism (sSNP) variation in housekeeping genes found in these smooth strains of "*M. prototuberculosis*" suggests that the tubercle bacilli were contemporaneous with early hominids in East Africa, and may have thus been evolving with their human host much longer than previously thought. These results open new perspectives for unraveling the molecular bases of *M. tuberculosis* evolutionary success (Gutierrez 2005).

The second strategy has also provided interesting results that support the notion of TB's ancient origin. The isolation and characterization of ancient *M. tuberculosis* DNA from an extinct bison, dated 17,000 years B.C., suggest the presence of TB in America in the late Pleiostocene (Rotschild 2001). The extensive infection of many individuals of the *Mammut americanum* species with the *M. tuberculosis* agent also suggests that, apart from *Homo sapiens,* mastodons and bovids may have spread the disease during the Pleistocene (Rotschild 2006a, Rotschild 2006b). When looking at human remains, several DNA studies served to trace back the presence of TB to Egyptian mummies, where *M. tuberculosis* and also *M. africanum* genotypes were identified (Zink 2003). Figure 2-3 shows an ancient Egyptian clay arte-

fact with a traditional kyphosis suggestive of Pott's disease. The presence of TB in America before the arrival of the Spanish settlers is also well demonstrated both by paleopathological evidence and studies on ancient DNA (Salo 1994, Arriaza 1995). Recent paleopathological evidence also suggests the presence of leprosy and TB in South East Asian human remains from the Iron Age (Tayles 2004). Taken together, these results may argue that the limited number of different genogroups that we observe today are likely to stem from those that were seeded in the past, have remained isolated by distance during millennia, and have had time to co-evolve independently before gaining reasonable statistical chances to meet.

Figure 2-3: Egyptian clay artefact of an emaciated man with a characteristic angular kyphosis suggestive of Pott's disease (reproduced from TB, Past, Present, 1999, TB Foundation)

2.4. *M. tuberculosis* through space and time

The concept of phylogeography was originally introduced by Avise (Avise 1987), as "*the history of processes that control the geographic distribution of genes and lineages by constructing the genealogies of populations and genes"*. The term was introduced as a way to bridge population genetics and molecular ecology and to describe geographically structured signals within species. This concept might well be applied to studies on the global spread of *M. tuberculosis* through time. If the ancestor of *M. tuberculosis* adapted specifically and slowly to human beings, it may have had the time to develop, via an extreme clonality, a deeply rooted and peculiar phylogeographical structure reflecting both the demographic history and the history of TB spread.

The geographic distribution of bacteriophage types was the only method to detect the geographic subdivision of the *M. tuberculosis* complex species during the '70s and the '80s (Bates 1969, Sula 1973); however, no phylogenetic relationships could be inferred at that time using mycobacteriophages. A numerical analysis of *M. africanum* taxonomy also suggested differences between isolates from West and East Africa (David 1978). The naming of two *M. africanum* variants (subtype I and II) created confusion and the status of *M. africanum* as a homogeneous sub-species of *M. tuberculosis* complex is still uncertain. The existence of some major geographical and epidemiological significant genetic variants of the *M. tuberculosis* complex was also recognized as early as 1982 (Collins 1982). Among these were the Asian, the bovine and the classical variants, in addition to *africanum* I and *africanum* II variants.

Lateral genetic transfer was presumed to be minor in *M. tuberculosis*, and the clonal structure of the *M. tuberculosis* complex was formally demonstrated by the finding of strong linkage disequilibrium within MIRU loci (Supply 2003). Only recently has the issue of *M. tuberculosis* complex lateral genetic transfer gained interest, particularly in regard to its links to genetic diversity and to potential acquisition of virulence (Kinsella 2003, Rosas-Magallanes 2006, Alix 2006). The importance of lateral genetic transfer in one species' history is of primary importance to better understand its specificity. As for the members of the *M. tuberculosis* complex, with the exception of *M. canettii*, there is no evidence for this kind of transfer or for housekeeping gene recombination (Smith 2006a). Indeed, recent evidence argues in favor of the existence of lateral genetic transfer in the precursor of the *M. tuberculosis* complex, and in favor of environmental mycobacteria being the source of certain genetic components in the *M. tuberculosis* complex. These findings reinforce the idea that the ancestor of the *M. tuberculosis* complex was an environ-

mental *Mycobacterium* (Rosas-Magallenes 2006). Another source of exogenous DNA may be plasmids that have been shown to be present in modern species of mycobacteria, and sometimes to carry virulence genes (Le Dantec 2001, Stinear 2000, Stinear 2004). The mosaic nature of the genome of ancestral "*M. prototuberculosis*" species also argues in favor of numerous gene transfer events and/or homologous recombination within ancient species of the *M. tuberculosis* complex (Gutierrez 2005).

2.5. Looking for robust evolutionary markers

When looking for robust evolutionary markers, the evolutionist will first choose markers that are assumedly neutral in order to avoid debates on function or potential selection, whether positive or stabilizing. For the *M. tuberculosis* complex, the very existence of an obligate intracellular life, which provides a stable chemical and metabolic environment, suggests that a classical metabolic selection scheme must have played a minimal role in the evolution of the *M. tuberculosis* complex genome (Musser 2000). Host specialization and niche adaptation may have been more important. Changes towards acquisition of an intracellular life style may also be responsible for loss of function and hence, loss of genes.

Silent mutations in housekeeping genes were the first candidates to be selected as evolutionary markers. However, the amount of genetic diversity found in the genes selected in that original study was unexpectedly low, which led to the hypothesis that TB had spread only recently from a unique precursor. Indeed, the rate of genetically neutral synonymous mutations (sSNP) was shown to be as low as 1/10,000 whereas the rate of non-synonymous mutations (nsSNP) outnumbered sSNPs by almost 2 to 1 (Sreevatsan 1997).

As for spoligo- and MIRU typing, at first glance it seems reasonable to consider these markers as neutral. No evident role for the DR locus, a member of CRISPR sequences, has been proven yet; however, there is an increased interest in CRISPR and the CRISPR-associated genes *cas*, which may mean to the bacterial world what silencing RNAs means for the eukaryotic world (Makarova 2006). Apart from the *sen*X3-*reg*X3 double component system, which was presumably involved in virulence, the function of MIRU loci remains poorly investigated (Parish 2003). In all cases, the phylogenetical information content obtained by studying the DR and the VNTR loci was previously shown to be rich (D. Falush 2003 - Prague, European Concerted Action Meeting, unpublished data).

2.6. Why repeated sequences were so useful at the beginning

The description of repeated sequences goes back to the early age of molecular biology (Britten 1968). Their role in the selection of new vital functions in life is indeed of paramount importance for genetic evolution (Britten 2005). In the *M. tuberculosis* complex, repetitive DNA sequences were used as probes and showed to be useful for fingerprinting strains in epidemiological studies (Eisenach 1988). Shortly after the characterization of the insertion sequence IS*6110* (Thierry 1990), an international consensus method IS*6110* RFLP was adopted almost concomitantly to the World Health Organization declaration of TB as a public health emergency (van Embden 1993). IS*6110* RFLP changed the traditional belief that no more than 10 % of TB cases were due to recent transmission, and sparked a new hope for disease eradication by contributing to the adequate surveillance and prevention of TB transmission (Alland 1994, Small 1994). For diverse reasons, however, the use of IS*6110* was of little help in solving the phylogenetic structure of the *M. tuberculosis* complex because it turned out to be a poor phylogenetic marker (Fleischmann 2002). A rapidly emerging issue was that IS*6110* was ineffective in a large part of the world, including South-East Asia (Fomukong 1994). Another insertion sequence, IS*1081*, was also suggested as an interesting potential phylogenetic marker; however, its generalized use in *M. tuberculosis* complex population genetics was also hampered, among other reasons, by the RFLP format (van Soolingen 1997, Park 2000).

2.7. Regions of differences (RDs) and SNPs in *M. tuberculosis*

One approach to understanding the molecular evolution of the *M. tuberculosis* complex and looking for virulence genes is to identify regions of difference (RD) between *M. tuberculosis* complex genomes (Inwald 2003) or to look for Single Nucleotide Polymorphisms (SNPs). Substractive genomic hybridization was initially used to identify three distinct genomic regions between virulent *M. bovis*, *M. tuberculosis,* and the avirulent *M. bovis* bacille Calmette-Guérin (BCG) strain, designated respectively as RD1, RD2, and RD3 (Mahairas 1996). One of these regions, RD1, was shown to contain important virulence genes including the two immunodominant T-cell antigens ESAT6 and culture filtrate protein 10 (CFP10) (Pym 2002). In another study (Gordon 1999), restriction-digested bacterial artificial chromosome (BAC) arrays of H37Rv strain were used to reveal the presence of 10 regions of difference between *M. tuberculosis* and *M. bovis* (RD1 to 10); 7 of which (RD4-RD10) were deleted in *M. bovis*. The deletion pattern of *M. africanum* is closer to that of *M. tuberculosis* than to the pattern of *M. bovis* (Gordon 1999).

Brosch *et al*. analyzed the distribution of 20 variable regions resulting from insertion-deletion events in the genome of the tubercle bacilli in one hundred strains belonging to all sub-species of the *M. tuberculosis* complex (Brosch 2002). The authors showed that the majority of these polymorphisms resulted from ancient irreversible genetic events in common progenitor cells, the so-called Unique Event Polymorphisms (UEP). Based on the presence or absence of an *M. tuberculosis* specific deletion 1 (TbD1, a 2 kb sequence), *M. tuberculosis* can be divided into "ancient" TbD1 positive and "modern" TbD1 negative strains. This classification superimposes well with the previous principal genetic group (PGG) classification (Sreevatsan 1997); however, only two groups of strains, the EAI and the *M. africanum* strains are TbD1 positive. The RD9 deletion identifies an evolutionary lineage represented by *M. africanum*, *M. microti* and *M. bovis* that diverged from the progenitor of the present *M. tuberculosis* strains before TbD1 occurred (Brosch 2002). These findings contradict the long-held belief that *M. tuberculosis* evolved from a precursor *of M. bovis*, suggesting a new evolutionary scenario of the *M. tuberculosis* complex. Since *M. canettii* and other ancestral *M. tuberculosis* complex strains lack none of these regions, they are supposed to be direct descendants of the tubercle bacilli that existed before the *M. africanum-M. bovis* lineage separated from the *M. tuberculosis* lineage (Brosch 2002). This scenario was confirmed in a follow-up study in which *in silico* and macroarray based hybridization experiments confirmed the existence of a core set of 219 conserved genes shared by *M. leprae* and *M. tuberculosis.* Among these new phylogenetical markers is the *pks* 15/1 gene, which encodes one of the polyketide synthase enzymes required for the lipid metabolism of cell wall building. All modern strains show a 7-base pair (bp) frameshift deletion in this gene that induces a knock-out of the enzyme. *M. canettii,* most PGG1 ancestral EAI, and Beijing strains add two amino acids that do not interfere with *pks* function, whereas strains in the *M. bovis* lineage bear a 6-bp DNA deletion that involves deletion of these two extra amino acids (Constant 2002).

Three recent studies provide landmarks in TB molecular and phylogenetic population studies. The first one suggests the existence of six phylogeographical lineages, each associated with specific sympatric human populations (Gagneux 2006). These observations show that mycobacterial lineages are adapted to particular human populations. Whether these results are considered from either a "splitter" or from a "gatherer" perspective, they endorse the idea that there are probably just a small number of founding genogroups of the *M. tuberculosis* complex. Also, these results support previous results on *M. tuberculosis* complex genetic diversity and our hypothesis that *M. tuberculosis* complex is an ancient pathogen that co-evolved with its hosts (Sola 2001a, 2001b, Sebban 2002).

Two SNP-population-based phylogenies also provided similar results, i.e. a limited number of *M. tuberculosis* complex phylogeographical genogroups (Figure 2-4). According to a study led by Musser's group, eight deeply branching genetic groups (I to VIII) were found; however, this was still not representative of the worldwide genetic diversity of *M. tuberculosis* because of a biased sampling, e.g., lack of Central Asian (CAS) strains (Gutacker 2002). A second study corrected this bias by creating one new subgroup for the CAS lineage (Gutacker 2006). This lineage is close to the root, which suggests that the Indian subcontinent played a major role in TB evolution and expansion.

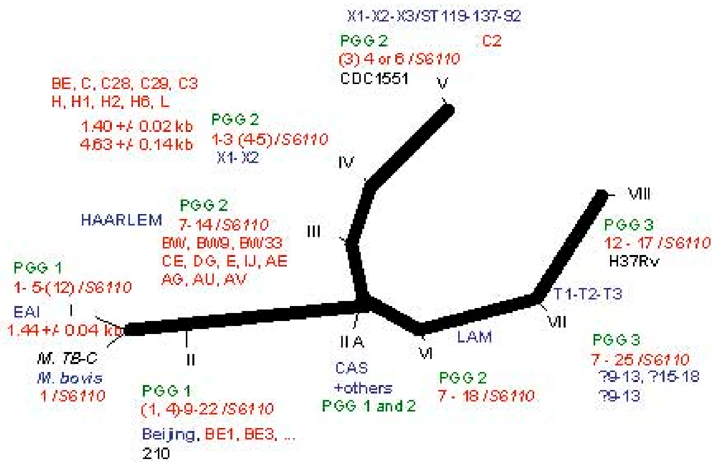
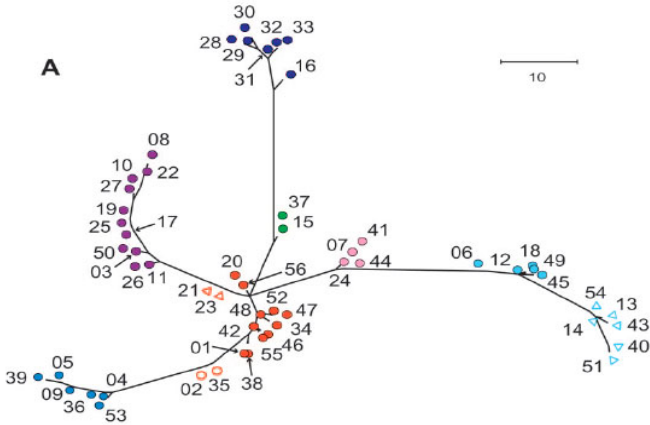


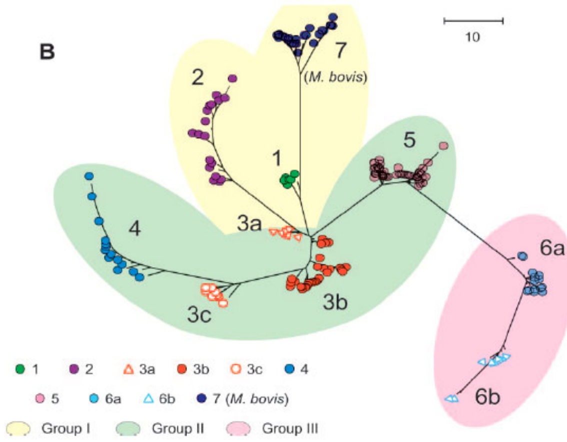
Figure 2-4 Phylogenetic tree obtained on SNPs, adapted from Gutacker *et al*. 2006 and supplemental data. In blue: spoligotyping-based nomenclature or characteristics. In red: IS*6110*-based clade nomenclature with some characteristics IS*6110* copy number or molecular weight data. In green: Musser's principal genetic group (Sreevatsan 1997). In black: SNP-based designation of clades with some characteristics strains (CDC1551, H37Rv, strain 210).

Similar results were obtained independently by Alland *et al*., reinforcing the idea that unrelated lineages may acquire the same number of IS*6110* by homoplasia (Alland 2003). The same group recently analyzed 212 SNPs in correlation with MIRU and spoligotyping on a worldwide representative collection of clinical isolates. Their results are illustrated in Figures 2-5 (A to C). The *M. tuberculosis* complex tree presented four main branches containing six SNP cluster groups (SCG1 to SCG6) and five subgroups as depicted in Figure 2-5 B (Filliol 2006). These results provide good congruence with spoligotyping and, to a lesser extent, with MIRU12,

endorsing the latest genetic diversity studies on spoligotyping (Brudey 2006). Still, it can be argued that in both SNP-based studies, identical bias could have been introduced since the SNPs analyzed in both cases were selected based on the four *M. tuberculosis* complex genome sequences available to date: *M. tuberculosis* strains 210, CDC1551, H37Rv and *M. bovis* strain AF2122.

Figure 2-5, A to C: (From Filliol *et al.* 2006 J. Bacteriol., reproduced with permission). A: a distance-based neighbor-joining tree on 159 sSNPs resolves the 219 *M. tuberculosis* complex isolates in 56 sequence types (ST). STs are indicated by a dot with numerical value and color code for SNP Cluster Group (SCG) belonging. B: Model-based neighbor-joining tree based on a data set with 212 SNPs, which resolves 327 *M. tuberculosis* complex isolates into 182 ST with identical cluster (compare with A). SNP Cluster Groups are indicated by colors. Principal Genetic Groups (1 to 3) are also highlighted. C: distribution of the spoligotype clades on the SNP-based phylogeny.




B
10
7
(M. bovis)
2
1
5
4
3a
6a
3b
3c
6b
1
2
3a
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5
6a
6b
7 (M. bovis)
Group I
Group II
Group III

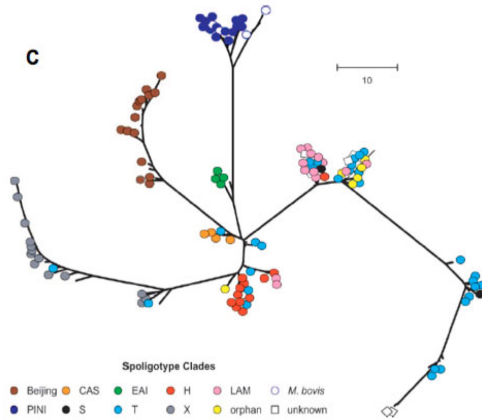

C
10
Spoligotype Clades
Beijing
CAS
EAI
H
LAM
M. bovis
PINI
S
T
X
orphan
unknown

Table 2-1 provides a nomenclature correlation between *M. tuberculosis* complex groups defined by spoligotyping and those defined by sSNPs. As shown in this table, the most ancient clade, EAI defines SCG 1 or sSNP-I according to Alland's or to Musser's designation, respectively. SCG 2 and sSNP-II define the Beijing lineage. SCG 3a or sSNP-IIa defines the CAS or Delhi genogroup. SCG 3b or sSNP-III defines the Haarlem family of strains. SCG 3c and SCG 4, or sSNP-IV and sSNP-V, define the "IS*6110* European low-banders" or X genogroup (Sebban 2002, Dale 2003, Warren 2004). SCG 5 or sSNP-VI is mainly constituted by the Latin American and Mediterranean (LAM) genogroup (Sola 2001a). SCG 6a and SCG 6b (sSNP-VII and sSNP-VIII) define the poorly characterized Principal Genetic group 3 lineage that also includes some ill-defined T genotypes (Filliol 2002). Last but not least, SCG 7 defines the bovine and seal *M. tuberculosis* complex subspecies whereas no counterpart is provided in Musser's classification (Filliol 2006).

Table 2-1: Comparison of spoligotype and SNP terminology

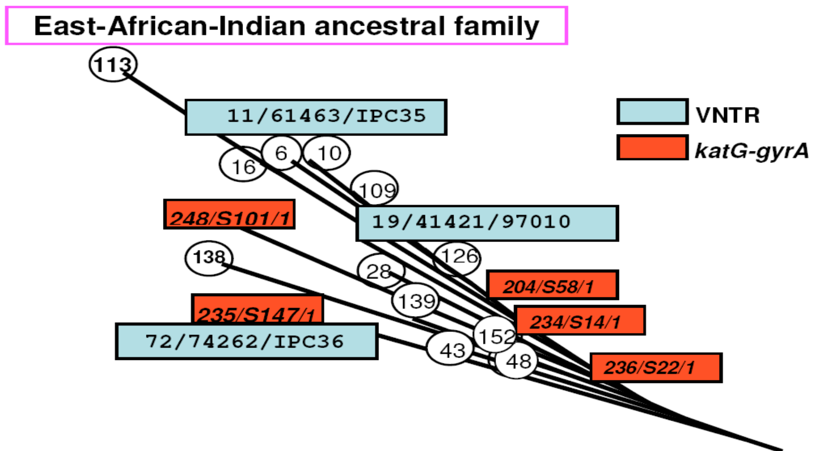
PGG (Sreevatsan 1997)	**Spoligotyping-based (Filliol 2003)**	**SCG-based (Filliol 2006)**	**SNP-based (Gutacker 2006)**
PGG	EAI	SCG 1	sSNP-I
PGG1	Beijing	SCG 2	sSNP-II
PGG1	CAS	SCG 3a	sSNP-IIA
PGG 1	Bovis	SCG 7	*M. tuberculosis* complex
PGG2	Haarlem	SCG 3b	sSNP-III
PGG2	X1	SCG 3c	sSNP-IV
PGG2	X1,X2,X3	SCG 4	sSNP-V
PGG2	LAM	SCG 5	sSNP-VI
PGG3	T (Miscellaneous)	SCG 6	sSNP-VII
			sSNP-VIII

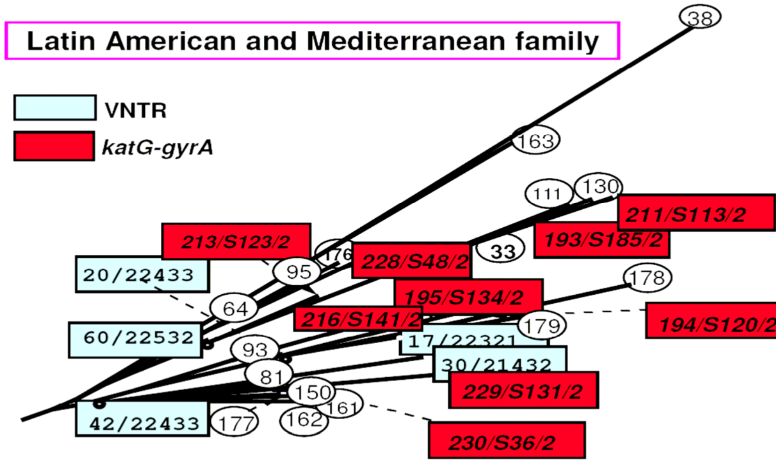
PGG = Principal Genetic Group
SCG = SNP cluster group
SNP = Single nucleotide polymorphism
EAI = East African Indian
CAS = Central Asian (or Delhi)l

2.8. Looking for congruence between polymorphic markers

The concept of molecular clock, attributed to Zuckerkandl and Pauling in 1962, was originally based on hemoglobin evolution and later generalized to DNA evolution (Zuckerkandl 1987). As for *M. tuberculosis*, we are dealing with polymorphic markers, i.e. repeated sequences, which are physically linked to the chromosome and therefore transmitted together with it. Concomitantly, these sequences are evolving at their own pace and hence possess more than one molecular clock. Although the combination of various molecular clocks of different paces in a single analysis may be criticized (Wilson 2003), this approach was used successfully in the past to detect the EAI and the LAM clades by observing congruence between spoligotyping and VNTR data (Figure 2-6, extracted from Sola 2001b).

Figure 2-6 : Close-up on a spoligotyping-based neighbor-joining (NJ) phylogenetical tree, built on SpolDB2 database showing the EAI and LAM branches. The superimposition of spoligotyping, VNTR and Principal Genetic grouping shows congruence between various markers (extracted from Sola 2001b) in blue boxes: Spoligotyping shared-type n°/VNTR allele: ETR-A to E from left to right). In red boxes: SpolDB-shared-type n°/Soini's spoligotyping number (see Soini et al. 2000)/Principal Genetic Group (see Sreevatsan 1997). In the blue boxes of the upper figure, the third number is the strain identification number. In circles: spoligotyping shared-type number.





IS*6110* RFLP was recognized very early to evolve faster than spoligotype since more RFLP than spoligo genotypes are present when a single set is analyzed (Kremer 1999). The mutation rate of IS*6110* was estimated recently to be 0.287 per genome per year for a strain with a typical number of 10 copies (Rosenberg 2003). Using the infinite allele model and the same set of data (Kremer 1999), the relative mutation rate of spoligotype is calculated to be 13.5 % of the rate of IS*6110* (Tanaka 2005). This corresponds to a spoligotype mutation rate of around 0.039 events per year. A more complex model was recently developed, which assumes that the mutation rate of a given spoligotype is proportional to the number of spacer units present in the DR region. This new model allows the detection of emerging strains of *M. tuberculosis* (Tanaka 2006).

Population bottlenecks are important in biology since they create genetic conditions that favor founder effect and speciation. Among many bottleneck hypotheses, the one ascribed to the late Pleistocene is very attractive. It involves volcanic winter and differentiation of modern humans at a time comprised between 50,000 and 15,000-25,000 years ago (Ambrose 1998). These events may have created environmental conditions favoring the spread of *M. tuberculosis*. We may hypothesize on the global spreading of a single clone (Kapur 1994), or of a limited number of clones, based on the expansion of the surviving re-founders, preserved in various small refuges located in tropical areas (Ambrose 1998). The ample human genetic diversity observed today in Africa (as well as the apparently ample *M. tuberculosis* genetic diversity) may be due either to a longer evolutionary period, or to the pres-

ervation of such ample diversity in this continent during the bottleneck event. Consequently, for the *M. tuberculosis* complex, we can hypothesize that the high genetic diversity observed in *"M. prototuberculosis*" could be a remnant of this bottleneck event, with a strong resilience and hence a high preservation of the previous genetic diversity inside these tropical refuges. This ecological perspective is also supported by data suggesting that human beings migrated back to Africa after the demographic expansion into the South-East Asian peninsula (Cruciani 2002). Thus, if demographic and epidemic factors are considered in addition to evolutionary and genetic factors, the modern tubercle bacilli are more likely to find their origin in India or South-East Asia rather than in Africa. The fact that the TbD1 positive East-African-Indian strains, which are likely to have disseminated when adequate demographical conditions were fulfilled, are genetically the closest to the *M. canetti- "M. prototuberculosis*" strains argues in favor of this hypothesis.

Given the astonishingly reduced SNP diversity observed initially in the *M. tuberculosis* complex (Sreevatsan 1997, Musser 2000), the bottleneck hypothesis is seducing. However, the 15,000- to 25,000-year time frame was calculated by computation of synonymous mutation rates based on *Escherichia coli* and *Salmonella* divergence, i.e. based on a uniform calibration rate for nucleotide substitution (the basic molecular clock). This choice of independency from growth rate (doubling time) and other parameters, such as mutation rate and population size, may be criticized. The doubling time of *E. coli* is 20 min and that of *M. tuberculosis* is 20 hours. If we logically assume that sSNPs acquisition is related to DNA metabolism, then, a ratio of 60x should be applied to the computation presented in Kapur's paper, thus providing a much larger time-frame (900,000-1,500,000 years) for the presence of *M. tuberculosis* complex bacilli on earth, an hypothesis that is consistent with the latest results obtained on *"M. prototuberculosis*", which shows an unusually high SNP diversity (Gutierrez 2005).

According to a recent multigenic phylogenetic approach, the speciation process in mycobacteria might have been progressive and relatively homogenous across the whole genome (Devulder 2005). When comparing substitution rates of fast and slow growing mycobacteria by means of a relative rate test, non-significant differences were observed. These findings suggest that the two groups evolved at the same rate. In other words, the evolutionary rate does not necessarily correlate to the number of generations. This framework fits with the strictly clonal evolution of *M. tuberculosis* and the co-evolution hypothesis that suggests adaptation between particular mycobacterial lineages and particular human populations (Supply 2003, Gagneux 2006). However more recent genetic studies using SNPs analysis suggests that some genes such as the ones coding for the PE-PGRS and PPE proteins that

have the motifs Pro-Glu (PE) and Pro-Pro-Glu (PPE), thought to be critical in host-pathogen interactions, are prone to recombination and gene conversion events (Karboul 2006, Liu 2006).

2.9. Main lineages within the *M. tuberculosis* species

Within the scope of this chapter is the description of the results of the molecular population approach that allowed the definition of genetically homogenous clusters of *M. tuberculosis* complex, which are now shown to be preferentially linked to some human hosts (Brudey 2006, Gagneux 2006). Table 2-2 provides the latest description of statistically, epidemiologically or phylogeographically relevant clonal complexes of the *M. tuberculosis* complex based on spoligotyping signatures described in the SpolDB4 database (a high resolution image can be downloaded at: http://www.biomedcentral.com/1471-2180/6/23/figure/F1?highres=y; from Brudey 2006).

2.9.1. Principal lineages of the Genetic group 1

2.9.1.1. The East African-Indian (EAI) lineage

This lineage was first described in Guinea-Bissau (Källenius 1999) and was shown to be frequent in South-East Asia, India, and East Africa (Kremer 1999). This group of strains is characterized by a low number of IS*6110* copies. A subgroup of these strains harboring a single copy of IS*6110* was shown to be widespread in Malaysia, Tanzania, and Oman (Fomukong 1994). In combined datasets (i.e. pooled datasets characterized by one or more methods), this lineage demonstrated congruence between spoligotypes (absence of spacers 29-32, presence of spacer 33, absence of spacer 34), VNTR [exact tandem repeat A (ETR-A) allele ≥4], *kat*G-*gyr*A grouping (Group 1), and later the presence of the TbD1 sequence (Soini 2000, Sola 2001b). More recently, the presence of an *oxyR* C37T transition was shown to be specific to the lineage (Baker 2004). This lineage was shown to belong to cluster group 1 or Cluster I (Filliol 2006, Gutacker 2006). It harbors a specific region of difference, RD239 and was renamed as Indo-Oceanic in the work of Gagneux *et al.* (Gagneux 2006). It is speculated that this lineage, which is endemic in South-East Asia, South-India, and East-Africa, may have originated in Asia, where TB could have historically found favorable spreading conditions. The Manila family was first identified by Douglas in 1997, and was later thoroughly characterized by the same group (Douglas 2003). This genotype was identified based on the prevalence of clustered strains isolated from Philippino immigrants in the

United States (US) and was only later shown to be prevalent in the Philippines. The Manila family bears ST19 as prototypic spoligo-signature and is actually identical to EAI-2 (Filliol 2002). ST89, which defines the Nonthaburi (Thailand) group of strains, is a derived clone (Namwat 1998). In this family, specific variants have been also described for Vietnam (ST139 or EAI-4), Bangladesh (ST591, ST1898 or EAI-6 and 7) and Madagascar (ST109, EAI-6).

We have no precise idea about the prevalence of the EAI lineage in India and China, although it is evident that this genotype is more specifically linked with South-East Asia and South India than with Northern China. This may be due to differences in civilization and agriculture histories between North and South China (Sola 2001b). It is also very difficult to analyze what links these clones may have with strains in the major genetic group 2, given the presence of the spacer 33 in this group of strains (a spacer that is absent in groups 2 and 3). A striking discovery related to these strains was made recently when analyzing medieval human remains discovered in an English parish. TB was confirmed by amplifying multiple *M. tuberculosis* loci and EAI genotypes were apparently identified by spoligotyping (Taylor 1999). Whether these spoligotyping results obtained on medieval remains are reliable or not should be confirmed independently; however, the possibility of the presence of EAI genotypes in 13th century England should not be excluded.

2.9.1.2. The Beijing lineage

The Beijing genotype belongs to the principal genetic group 1 of Sreevatsan, and its specific spoligotype signature (absence of spacer 1-33, presence of spacer 34-43) was discovered in 1995 (van Soolingen 1995). However, a notorious outbreak due to a multidrug resistant clone of one of its offspring (New York W strain) had been characterized earlier, at the beginning of the ’90s (Plikaytis 1994, Bifani 2002). The emergence of this family of related genotypes continues to pose a serious threat to TB control due to its high virulence and frequent association with multidrug resistance. It was hypothesized that this genotype emerged successfully in East Asia due to mass BCG vaccination during the 20th century (van Soolingen 1995, Abebe 2006). However, Beijing should also be considered as a group of variant clones that evolved from a common precursor at an undefined time, maybe during the Genghis Khan reign or before (Mokrousov 2005).

These strains are characterized by the presence of an inverted IS*6110* copy within the DR region, an IS*6110* element at a particular insertion site (within the origin of replication) and one or two IS*6110* copies in a DNA region called NTF (Plikaytis 1994, Kurepina 1998). A characteristic Beijing lineage-defining SNP (G81A in Rv3815c) has been reported by Filliol *et al*. According to SNP analysis, the Beijing

cluster was designated as SCG 2 or sSNP-II (Filliol 2006, Gutacker 2006). Other characteristic sSNPs of the Beijing lineage were described in putative DNA repair genes (Rad 2003).

More recently, new phylogenetically-informative specific LSP markers were found, such as RD105, which is present in all Beijing/W or RD142, RD150 and RD181. It allows a further division of the Beijing lineage into four monophyletic subgroups (Tsolaki 2005). The Beijing lineage was recently renamed as the East Asian Lineage by other authors (Gagneux 2006). Its most frequent VNTR signature is 42435 (Kremer 1999).

Recent evidence points to an early dispersal of the Beijing genotype in correlation to genetic haplotype diversity of the male Y chromosome (i.e. in correlation with human phylogeography). These results suggest that the spreading history of Beijing has a molecular evolutionary history that is much more intricate and more deeply rooted to human history than initially thought. Using the Beijing genotype as a model, and comparing its phylogeography to Y-chromosome-based phylogeography, Mokrousov *et al.* hypothesized that two events shaped the early history of this genotype: (1) its upper Paleolithic origin in the *Homo sapiens sapiens* K-M9 cluster in central Asia, and (2) a primary dispersal of the secondary Beijing NTF:: IS*6110* lineage by Proto-Sino-Tibetan farmers within East-Asia (human O-M214/M122 haplogroup) (Mokrousov 2005).

2.9.1.3. The Central-Asian (CAS) or Delhi lineage

The presence in India of a specific lineage of the *M. tuberculosis* complex was concomitantly and independently reported by two different groups using IS*6110* RFLP and spoligotyping, respectively (Bhanu 2002, Filliol 2003). This lineage was also shown to be endemic in Sudan, other sub-Saharan countries and Pakistan (Brudey 2006). Using IS*6110* RFLP, the Delhi lineage shows a characteristic band pair in the high molecular weight region (12.1 and 10.1 kilobase pairs) and its specific spoligotype signature is formed in the absence of spacers 4-27 and 23-34. This spoligo-signature shows numerous variants and several subgroups such as CAS1-Kili (for Kilimanjaro) and CAS1-Dar (for Dar-es-Salaam), which have already been defined on the basis of new spoligotype-signatures that are specific for each new clonal complex (Mc Hugh 2005, Eldholm 2006). Still, more results using other polymorphic markers should complement these data. VNTR signatures of *M. tuberculosis* complex clinical isolates from South-Asian immigrants in London and native patients in Rawalpindi, Pakistan, were identical (allele combination 42235) and correlated with the CAS spoligotype (Gascoyne-Binzi 2002, Brudey unpublished results).

This genotype family could be the ancestor of the Beijing family since it clusters close to Beijing when analyzed by a combination of MIRU, spoligotyping and VNTR (Sola 2003). In India, its frequency varies from one region to another: it is more prevalent in the North than in the South, where the EAI family predominates (Suresh 2006). An outbreak strain named CH was recently reported in Leicester, United Kingdom. It belongs to the CAS family and harbors a specific deletion (Rv1519). In broth media, this strain was found to grow more slowly and to be less tolerant to acid and H_2O_2 than two laboratory reference strains, CDC1551 and H37Rv. Nevertheless, its ability to grow in human monocyte-derived macrophages was not impaired. This strain induced more anti-inflammatory IL-10, more IL-6 gene transcription/secretion from monocyte-derived macrophages, and less protective IL-12p40 than CDC1551 and H37Rv strains. Thus, this strain seems to compensate the microbiological attenuation by skewing the innate response toward a phagocyte deactivation. The complementation of Rv1519 reversed its ability to elicit anti-inflammatory IL-10 production by macrophages. These results suggest that the Rv1519 polymorphism confers an immune subverting *M. tuberculosis* phenotype that might contribute to the persistence and outbreak potential of this lineage (Newton 2006).

2.9.2. Lineages belonging to the Principal Genetic groups 2 and 3

2.9.2.1. The Haarlem family

The Haarlem family was described in the Netherlands in 1999 (Kremer 1999). On IS*6110* RFLP, these strains harbor a double band at 1.4 kb. Their spoligotype is characterized by the absence of the spacer 31, which is due to the presence of a second copy of IS*6110* in the DR region (Groenen 1993). Due to an asymmetric insertion within the DR locus, this second IS*6110* copy hinders the detection of spacer 31 (Filliol 2000, Legrand 2001). Three main spoligotype-signatures define the variants H1 to H3 (Filliol 2002). However, many Haarlem clonal complexes may harbor other Haarlem-based spoligo-signatures that are, as yet, poorly characterized. Another characteristic of the Haarlem lineage is the frequent VNTR pattern 33233 (Kremer 1999). The Haarlem family is highly prevalent in Northern Europe. It is present in the Caribbean to a lesser extent and is also prevalent in Central Africa, where it is believed to have been introduced during the European colonization (Filliol 2003). This family, which is highly diverse, merits further studies to better understand its evolutionary history. A SNP in the *mgt* gene of the *M tuberculosis* Haarlem genotype was discovered recently (Alix 2006). More SNPs are expected to be specific of the Haarlem lineage.

2.9.2.2. The Latin American and Mediterranean (LAM) family

The LAM family was defined by the finding of linkage disequilibrium between the absence of spacers 21-24 in the spoligotyping and the presence of an ETR-A allele equal to 2 (Sola 2001b). However, this genotype family is more diverse and its study is more complicated than initially thought. Strains belonging to the LAM3/F11 family and the S/F28 family harbor identical spoligotypes of the shared type ST4, revealing the existence of genetic convergence between spoligotypes (Warren 2002). This phenomenon seems, however, to be rare and highly dependent on the structure of the observed spoligotype. The absence of spacers 21-24 may also have occurred more than once in tubercle bacilli evolution although no genetic evidence has suggested such a convergence event until now. Many sub-motifs - LAM1 to LAM12 - have been suggested according to the latest international spoligotype database project SpolDB4 (Brudey 2006). However, the phylogenetic significance of the common absence of spacers 23-24 has not been demonstrated in this lineage. In this sense, some genotypes that show strong geographical specificity (for example the LAM10-Cameroon or the LAM7-Turkey) were initially labeled as LAM, although there is no evidence of their phylogenetical relation to other LAM spoligo-signatures (Niobe-Eyangoh 2003, Zozio 2005). Recently, a specific deletion designated as RD^{RIO} was shown to be linked to certain LAM spoligo-signatures present in Rio de Janeiro, Brazil (L Lazzarini, R Huard, JL Ho personal communication).

The LAM clade is frequent in Mediterranean countries and its presence in Latin America is supposed to be linked to the Lusitanian-Hispanian colonization of the New World. Conversely, it may have been endemic in Africa and/or in South America, spreading to Europe later. At this stage, we must highlight that paleopathological and ancient DNA data support the existence of TB before the arrival of Spanish settlers to Latin America in the 15th century (Arriaza 1995, Salo 2001).

2.9.2.3. The X family: the European IS*6110* low banders

The X family of strains is defined by two concomitant features, a low number of IS*6110* copies and the absence of spacer 18 in the spoligotyping (Sebban 2002). This latter is indeed an important characteristic common to at least three spoligotype shared types: ST119, ST137, and ST92. Both characteristics are present in the CDC1551 strain, which was once suggested to be highly virulent. The X family was also the first group identified in Guadeloupe (Sola 1997) and the French Polynesia (Torrea 1995). Specific epidemic variants of this genotype family were described in South Africa (Streicher 2004). The absence of spacer 18 bears phylogenetical significance because it is improbable that this spacer was deleted more than

once in the evolution of *M. tuberculosis*. The distribution of the X family appears to be linked to Anglo-Saxon countries (Dale 2003). It is also highly prevalent in South Africa and to a lesser extent in the Caribbean. Currently, it is only poorly documented in India. The strong presence of this genotype family in Mexico could be explained by its close proximity to the USA.

2.9.2.4. The T families and others

The «ill-defined» T group is characterized «by default». It includes strains that miss spacers 33-36 and can hardly be classified in other groups. This is a general characteristic of strains belonging to the principal genetic groups 2 and 3, together with the absence of an intact *pks* 15/1 gene (Marmiesse 2004). The presence of intact polyketide synthase genes, active in the synthesis of the specific lipid complex of the *M. tuberculosis* complex is now known to be linked to virulence (Constant 2002). Conversely, the 7 bp frameshift deletion in *pks*15/1 may be considered as a phylogenetical marker specific for the modern *M. tuberculosis* strains (Gagneux 2006) and may define the recently designated Euro-American lineage. It is expected that the combination of spoligotype and improved MIRU signatures will be the best way to precisely define epidemiological clonal complexes (Supply 2006). Alternatively, RDs and/or SNPs may also improve the taxonomic definition of these clones.

Table 2-2 shows the nomenclature correspondence between the main spoligotyping-based *M. tuberculosis* complex lineages and those recently described by MLST-SNPs (Baker 2004) and LSP (Gagneux 2006). As shown, spoligotyping appears to be more discriminative than the other two typing systems since it is able to resolve clinical isolates within the branch of the modern strains that are not solved by LSP. Specific RDs are described for many individual spoligotype-signatures; however, no Table is yet available for LSP and/or SNP synthetic correspondence with spoligotype.

Even if there is consensus in the fact that the main branches of the genetic tree of the *M. tuberculosis* complex have now been found, many uncertainties still remain with regard to the chronology of the evolution of the *M. tuberculosis* complex. For example, Gagneux *et al*. suggest that West African 2 diverged from an ancestral branch of *M. bovis,* whereas West African 1, characterized by a deletion of RD711, did not (Gagneux 2006).

Table 2-2 Comparison of spoligotype, Multi Locus Sequence Typing (MLST) and Large Sequence Polymorphism (LSP) nomenclature

Spoligotyping-based (Filliol 2003)	MLST (Baker 2004)	LSP (Gagneux 2006)	Comment
East-African-Indian (EAI)	IV	Indo-Oceanic	Prevalent in South East Asia, East Africa and South India
Beijing	I	East-Asian	Prevalent in China, Japan, South East Asia, Russia
Central-Asian (CAS)	III	East-African-Indian	Prevalent in North India, Pakistan, Libya, Sudan
X, Haarlem, LAM	II	Euro-American	Ubiquitous
M. africanum	NA	West African 1	Nigeria, Ghana
M. africanum	NA	West African 2	Senegal, Gambia

Recent results in our laboratory have shown that, in certain cases, it should be possible to reconstruct the past evolutionary history of some modern clones of the *M. tuberculosis* complex belonging to the principal groups 2 and 3. As an example, a striking identity was found recently between the MIRU typing results of the main LAM7-Turkey clonal complex (Zozio 2005) and the Japanese group T3-OSA (Ano 2006) (Millet *et al*. unpublished results). The meaning of this identity is under investigation and there is no reason to believe that it is due to convergence. Similarly, an endemic clone found in Nunavik (Nguyen 2003) was shown to be related to a clone found to be prevalent in central Europe (Poland and Germany) (Sola *et al.* unpublished results). Once again, we are trying to analyze how and when such movement of strains took place and whether they are representative of a deeply rooted anthropological structure or from modern outbreaks.

2.10. When did the bovine-human switch of *M. tuberculosis* take place?

The question of the molecular evolution of *M. bovis* provides an interesting framework for comparison with that of *M. tuberculosis* (Smith 2006a). In particular, Smith *et al*. discuss in detail how population bottlenecks and selective sweeps deeply affect the population structure of strictly clonal pathogens, such as members of the *M. tuberculosis* complex. Using the genetic diversity of *M. bovis* in the United Kingdom as a model, these authors demonstrate that all *M. bovis* genotypes derive from a single clonal complex that is likely to have emerged as a result of the actions of bovine TB control programs, which have been in force for the last 100 years. These authors also suggest that comparative genomics between two selected genomes that have gone through very different selection pressures (H37Rv and *M.*

bovis AF2122) may have wrongly suggested that *M. bovis* is an offspring clone of *M. tuberculosis*. As Brosch *et al*. identified deletions in *M. bovis* by comparing it with the only *M. tuberculosis* chromosome sequence available at that time, it was inevitable to conclude that *M. bovis* was the terminal group in the lineage (Smith 2006a). The assumption that the RD9-deleted lineage (including *M. bovis*) descended from an *M. tuberculosis*–like ancestor also implies, by parsimony, that the most recent common ancestor of these strains was adapted to humans. The exact host-association of *M. africanum* subtype I strains has not been examined so far. There is some evidence that *M. africanum*, which is less virulent than other *M. tuberculosis* complex genotypes, is currently extinct in settings where it was the most prevalent strain only three decades ago. Instead, it is being replaced by imported, more virulent genotypes (V. Vincent, unpublished results). The genetic susceptibility of the indigenous African population to TB during World War I is a well-known fact which supports the idea that TB caused by a more virulent genotype evokes a different, acute and even fatal disease, very different from that produced by *M. africanum*.

2.11. Comparative genomics and evolution of tubercle bacilli

The wealth of completed genome sequences, the development of microarray technology, and the decreasing cost of sequencing have enabled scientists to thoroughly study the significance of strain to strain variation in bacteria such as *Streptococcus agalactiae* and to define the "pan-genome" concept (Tettelin 2005). According to this concept, any species is made up of a common and a strain-specific genetic pool. Depending on the population structure of the studied organism and on the levels of lateral gene transfer, the relative part of these two pools may vary significantly. The **core genome** contains genes present in all strains, and the **dispensable genome** contains genes present in two or more strains as well as genes unique to single strains. Given that the number of unique genes is vast, the pan-genome of a bacterial species might be orders of magnitude larger than any single genome (Medini 2005).

LSP analysis is of particular interest in the *M. tuberculosis* complex, given the low level of sSNPs (Sreevatsan 1997, Kato-Maeda 2001, Alland 2007). Figure 2-7 shows the non-randomness of deletions in the 16 clinical isolates that were tested by microarray against the H37Rv genome. Some isolates contained unique deletions whereas other deletions were shared by many isolates. This study was extended to 100 different and unique IS*6110* RFLP types representing the global genetic diversity of the *M. tuberculosis* complex observed in San Francisco over

12 years (Tsolaki 2004). LSP size varied between 105 and 11,985 bp, with eight deleted sequences larger than 5,000 bp. LSPs tend to occur in genomic regions that are prone to repeated insertion-deletion events and may be responsible of a high degree of genomic variation in the *M. tuberculosis* complex (Alland 2007). Chapter 4 provides an exhaustive review on the comparative genomics of members of the *M. tuberculosis* complex.

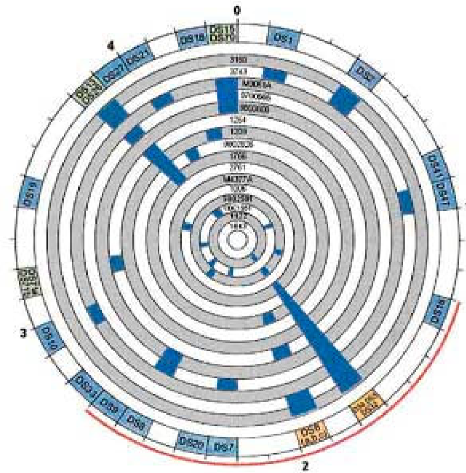


Figure 2-7: Circular map of genomic deletions among *M. tuberculosis* showing that the pattern of deletions differs between clones and is not spatially random. The outer numbers show the scale in mega base pairs (O=replication origin). In blue: genomic locations of deleted sequences. The outer circle summarizes the sum of all detected deletions. Color code (blue, orange, green) is linked to number of deletions (respectively 1, 2 and 3 deletions). The thin red line spans the genomic region of the genome where the number of deletions detected is greater than expected by chance alone. CDC1551 appears as the third ring on this picture. (Reproduced with permission from M. Kato-Maeda and P. Small)

2.12. Short-term evolutionary markers and database building

There are also ongoing debates about the true status of *"M. prototuberculosis"* (Gutierrez 2005). Whereas some consider *"M. prototuberculosis"* to be the most likely common ancestor to all *M. tuberculosis* complex members (Brisse 2006), others do not believe in the fact that these smooth variants of the tubercle bacilli are the true ancestors of today's tubercle bacilli (Smith 2006b). According to Smith, the computation providing a 3 million-year time frame is not reliable and there is no reason to believe that *"M. prototuberculosis*" is a more likely ancestor to the *M.*

tuberculosis complex than any animal pathogen still to be characterized. There is agreement, however, that the gene mosaicism found in *"M. prototuberculosis"* is real. Also, it is widely acknowledged that further studies on the genetic diversity of *"M. prototuberculosis"* will allow light to be shed on lateral genetic transfer and homologous recombination events in the *M. tuberculosis* complex.

Research on the molecular evolution of the *M. tuberculosis* complex is today addressed by exploiting multiple markers such as the DR locus, insertion sequences, deletion regions, mini-satellites, and SNPs, etc. However, in order to data-mine these large polymorphism databases better, newer methods of data analysis are needed in order to discover intelligible rules and to eliminate noisy data. Simplified decision rules are also needed to distinguish emerging pathogenic clones from those in extinction or from others reflecting ongoing TB transmission. A practical consequence of such studies would be a simplification of typing methods, which in turn, would result in a reduction of experimental constraints and an increase in the number of samples processed. At the Institut Pasteur of Guadeloupe, a new version of the spoligotyping database is currently incorporating MIRU-VNTR alleles and will be released for web-based consultation in 2007. In the future, similar websites will add new markers, allowing the performance of combined searches, including country of isolation, country of origin and ethnicity of the patient, multiple genotyping data, as well as a fine analysis of their geographical distribution. Further links of such databases to geographic information systems (GIS) for real-time map construction and clinical expression of the disease might help to shed new light on a stable association between populations of tubercle bacilli and their human hosts over time and across environments, as well as providing brand new tools to tackle the multifactorial nature of the variable clinical expression of the disease.

2.13. Conclusion and Perspectives

The description of the main branches of phylogeographically specific *M. tuberculosis* clonal complexes and the incipient unraveling of the molecular evolution of the *M. tuberculosis* complex took very long and there are reasons to believe that the task has just started. Some of the reasons are to be found within the complexity of the problem itself. A likely ancient TB pathogen may have had the time to create a large number of population-adapted genetic variants. Other challenges may lie in the slow development of efficient methods to characterize the intra-species genetic diversity of the *M. tuberculosis* complex. Also, we may invoke the recent introduction of new concepts, such as statistical phylogeography, whose application to TB will require the construction of an adequate dataset and even more time for the

requisite reconstruction (Knowles 2004). However, the increasing human mobility worldwide is expected to blur the picture of the history of spread of the *M. tuberculosis* complex.

Lastly, a more precise understanding of the evolutionary genetic network of all *M. tuberculosis* complex clonal complexes may also emerge thanks to new studies using the recently standardized MIRU format (Supply 2006). Figure 2-8 illustrates the minimum spanning tree approach, built on polymorphisms of 24 MIRUs, found in a cosmopolitan sample including *"M. prototuberculosis*" isolates. The dotted lines represent some doubtful links (for example, the ancestral position of Beijing, relatively to CAS and EAI is totally speculative since this type of graph does not represent phylogenetical links).

The longer a clone takes to evolve, the more extensive the observed genetic diversity will be. In view of the assumedly ancient origin of TB, much work remains to be done to unravel the true genealogy of the numerous clonal complexes of the *M. tuberculosis* complex that have been described so far. Many others remain to be discovered since the sampling is still very small compared to the extent of diversity that is likely to exist.

Most of the scientific contributions reviewed in this chapter find an echo best translated by Douglas Young's concluding remarks in the lecture "Ten years of research progress and what's to come" (Young 2003): "*Armed with powerful new molecular tools and renewed momentum, laboratory-based researchers are beginning to tackle the fundamental questions of persistence and pathogenesis of human TB that have frustrated previous generations. Progress in fundamental understanding of disease process poses the exciting challenge of translating new ideas into practical tools that will assist in the global control of TB*". It is quite satisfying to see that the research conducted in the last 12 years is clearly advancing towards a better understanding of the tubercle bacillus and its interaction with the host, the mechanisms of pathogenicity involved, and the co-evolution of the bacterium and its host through time and space.

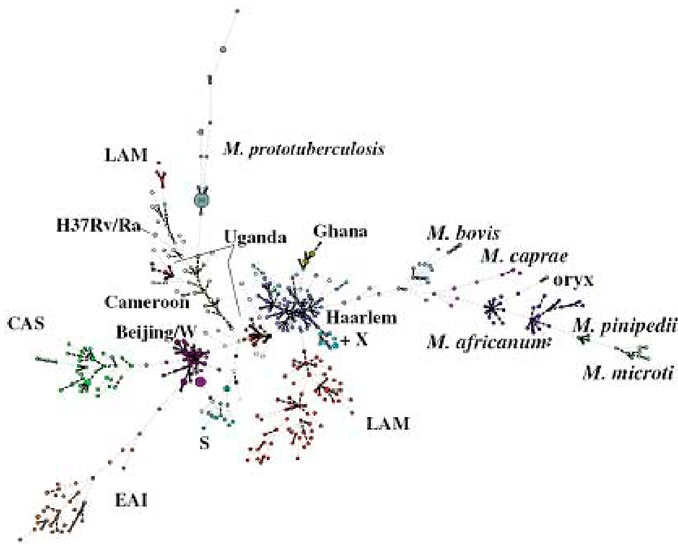


Figure 2-8: Minimum spanning tree based on MIRU-VNTR relationships among tubercle bacilli. Circles correspond to the different types identified by the set of 24 loci among the 494 *M. tuberculosis* isolates from cosmopolitan origins, and 35 *"M. prototuberculosis"*. The corresponding species names and spoligotype family designations (except T types) are indicated. Linkage by a single, double, or triple locus variation is boldfaced. EAI = East-African Indian (Indo-Oceanic in Gagneux's 2006 terminology), CAS = Central Asian (East-African-Indian in Gagneux's 2006 terminology), Beijing/W (East-Asian in Gagneux's 2006 terminology) LAM = Latino-American and Mediterranean, X = European IS*6110* low-banders, S = Sicily-Sardinia clade (all these clades are designated as Euro-American lineages in Gagneux's 2006 terminology) (Reproduced from Supply 2006 with authorization)

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Chapter 3: The Basics of Clinical Bacteriology

Lucía Barrera

3.1. The tubercle bacillus: a continuous taxon

Bacteria of the genus *Mycobacterium* are non-motile and non-sporulated rods. They are grouped in the suprageneric rank of actinomycetes that, unusually, have a high content (61-71 %) of guanine plus cytosine (G+C) in the genomic desoxyribonucleic acid (DNA), and a high lipid content in the wall, probably the highest among all bacteria. *Mycobacterium* and other closely related genera (i.e. *Corynebacterium, Gordona, Tsukamurella, Nocardia, Rhodococcus* and *Dietzia*) have similar cell wall compounds and structure, and hence show some phenotypic resemblance. Several mycolic acids in the envelope structure distinguish the mycobacteria. These quirky lipids may act as carbon and energy reserves. They are also involved in the structure and function of membranes and membranous organelles within the cell. Lipids constitute more than half of the dry weight of the mycobacteria. However, the lipid composition of the tubercle bacillus may vary during the life cycle in culture, depending on the availability of nutrients. The waxy coat confers the idiosyncratic characteristics of the genus: acid fastness, extreme hydrophobicity, resistance to injury, including that of many antibiotics, and distinctive immunological properties. It probably also contributes to the slow growth rate of some species by restricting the uptake of nutrients.

Even exhibiting this common badge, the species within the genus *Mycobacterium* show great diversity in many aspects. Most of them live and replicate freely in natural ecosystems and seldom, if ever, cause disease. Only a few mycobacteria became successful pathogens of higher vertebrates, preferentially inhabiting the intracellular environment of mononuclear phagocytes. The host-dependent mycobacteria that cannot replicate in the environment are *Mycobacterium leprae, Mycobacterium lepraemurium, Mycobacterium avium* subsp. *Paratuberculosis,* and the members of the *Mycobacterium tuberculosis* complex. Bacteria within the *M. tuberculosis* complex are able to reproduce *in vitro*, in contrast to *M. leprae* and *M. lepraemurium,* which are uncultivable and require the intracellular milieu for survival and propagation.

Comprised within the *M. tuberculosis* complex and generically called the tubercle bacillus, the various etiologic agents of tuberculosis (TB) have distinct hosts, zoonotic potential and reservoirs. *M. tuberculosis,* and the regional variants or subtypes *Mycobacterium africanum* and *"Mycobacterium canettii"* are primarily pathogenic

in humans. *Mycobacterium bovis* and *Mycobacterium microti* are the causative agents of TB in animals, and can be transmitted to humans. Some particular strains isolated from goats and seals have been named *Mycobacterium caprae* and *Mycobacterium pinnipedi,* although sometimes they are identified as *M. bovis* subspecies or variants. It could be expected that the major evolutive shifts involved in adaptation to different hosts would have entailed significant microbiological differentiation. However, the above mentioned agents of TB together with the vaccine bacille Calmette-Guérin (BCG) strains rank close to each other along a phenotypically continuous taxon (David 1978, Wayne 1982, Vincent 1992, van Soolingen 1997, van Soolingen 1998, Niemann 2000, Niemann 2002, Sola 2003, Mostowy 2005). Phenotypic differentiation is consistently clear-cut between the extreme species within the taxon, i.e. *M. tuberculosis* and *M. bovis*, but differences between species comprised within these two extremes are much less defined. The close affiliation among the members of the complex is endorsed by high genomic DNA similarity. At the same time, some molecular markers allow species differentiation within the complex (see chapter 2).

Table 3-1: Lineage of the agents of TB.

http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Tree&id=1760&lvl=3&lin=f&keep=1&srchmode=1&unlock

Kingdom	Bacteria
Phylum	Actinobacteria
Class	Actinobacteria
Subclass	Actinobacteridae
Order	Actinomycetales
Suborder	Corynebacterineae
Family	Mycobacteriaceae
Genus	*Mycobacterium*
	unique genus
Species	*M. tuberculosis*
	M. bovis
	M. africanum
	M. microti
	"M. canettii"
	M. caprae
	M. pinnipedii

In general, systematic and clinical mycobacteriologists accept new taxa at a slow pace. This is why the taxonomic status of some new members of the complex is still uncertain (see LPSN, http://www.bacterio.cict.fr and DSMZ, http://www.dsmz.de/microorganisms/bacterial_nomenclature.php). At the same time, the rank and species assignment have been questioned in other cases (Niemann 2003, Niemann 2004). The value of phenotypic and genotypic traits in the definition of a species in the complex should be reconsidered to meet new widely accepted definitions.

3.2. Microscopic morphology

The microscopic appearance does not allow the differentiation of the pathogenic agents of TB, mainly *M. tuberculosis*, from other mycobacteria although some characteristics may be indicative. In smears stained with carbol fuchsin or auramine and examined under light microscope, the tubercle bacilli typically appear as straight or slightly curved rods. According to growth conditions and age of the culture, bacilli may vary in size and shape from short coccobacilli to long rods. A typical curved shape has been described for *M. microti* (van Soolingen 1998). The dimensions of the bacilli have been reported to be 1-10 µm in length (usually 3-5 µm), and 0.2-0.6 µm in width. Therefore, the length of the microorganism is comparable to the diameter of the nucleus of a lymphocyte. Unlike some fast growing mycobacteria and other actinomycetales, *M. tuberculosis* is rarely pleomorphic, it does not elongate into filaments, and does not branch in chains when observed in clinical specimens or culture. In the experimental macrophage infection, intracellular bacilli were described as being significantly elongated compared to broth-grown bacilli and, remarkably, to display bud-like structures (Chauhan 2006).

When numerous and actively multiplying, the bacilli are strongly acid fast and show an evident and distinctive tendency to form hydrophobic bundles (Figure 3-1 and 3-2). Free bacilli can also be seen, though, especially at the border of the swarms. In unlysed host tissue, the bacilli are more numerous within the phagocytic cells.

Once the disease has been controlled, dying bacilli become sparser, often faintly and unevenly colored, due to partial loss of the internal contents. Of course, irregular staining may also be the consequence of technical defectiveness of dyes or staining procedures.

Figure 3-1: Ziehl-Neelsen staining of *Mycobacterium tuberculosis* growing in culture at 1000x magnification.

Figure 3-2: Electron microscopy of *Mycobacterium tuberculosis* growing in culture (*Courtesy of M. Rohde -M. Singh).*

The light microscope examination can not resolve the internal structures of the tubercle bacillus with the exception of some intracellular lipid vacuoles appearing as unstained spherules at regular intervals inside the bacilli (Draper 1982) and deposits of lipophilic material that might have a storage function (Garton 2002). Despite considerable efforts, a more subtle resolution of the ultrastructure of the bacillus has not been achieved. This is probably due to technical problems arising from biosafety, from the minute size of the bacilli, and from the large amounts of

complex lipids existing in their wall. With electron microscopy, some inner dense granules can be identified. They are believed to consist of polyphosphate and might be an energy store in the cell and also the site of oxidation-reduction reactions. In sections of the cell, the plasma membrane is seen to proliferate into vesicular or laminated internal bodies that might supply metabolic activities. Ribosomes, DNA filaments and radial bands, the latter postulated to be remaining scars of cell division, have also been described (Draper 1982, Brennan 1994).

Recently, the initiation of septum formation prior to division was clearly evidenced by tagging the mid-cell rings with green fluorescent protein (Chauhan 2006). Also, impressive images of the surface of *M. bovis* BGG were obtained by atomic force microscopy (Verbelen 2006).

3.3. Cell wall structure

As the most distinctive anatomical feature of the bacillus, the cell envelope has been the main object of research. Progressive chemical, molecular and ultrastructural research has produced robust knowledge on the synthetic pathways and structure of the mycobacterial cell envelope (Draper 1982, Brennan 1994, Draper 2005, Kremer 2005). The envelope, which has been profusely represented by schematic models, is composed of the **plasma membrane**, a **cell wall,** and an **outer capsule like layer**.

The **cytoplasmic membrane** of mycobacteria does not seem to be peculiar except for the presence of some lipopolysaccharides that are anyway shared by all actinomycetales (Mahapatra 2005). This vital interface provides osmotic protection, regulates the traffic of specific solutes between the cytoplasm and the environment, and subsumes the cell house-keeping tasks. The membrane contains proteins with different functions, i.e. sensors measuring the concentration of molecules in the environment, proteins translocating signals to genetic and metabolic machinery in the cytoplasm, enzymes involved in metabolic processes and energy generation, and carriers mediating selective passage of nutrients and ions. The enzymes intervene in cell wall and membrane synthesis, septum formation during cell division, assembly and secretion of extracytoplasmic proteins, and DNA replication. Still, very little is known specifically about the membrane of *M. tuberculosis*.

The membrane is surrounded, as in almost all bacteria, by a **cell wall** that protects the cell contents, provides mechanical support and is responsible for the characteristic shape of the bacterium. The mycobacterial cell wall, however, is unique among prokaryotes. The wall is constituted by an inner peptidoglycan layer, which

seems to be responsible for the shape-forming property and the structural integrity of the bacterium. The structure of this stratum differs slightly from that of common bacteria, as it presents some particular chemical residues and an unusual high number of cross-links. Indeed, the degree of peptidoglycan cross linking in the cell wall of *M. tuberculosis* is 70-80 % whereas that in *E. coli* is 20-30 %.

Covalently bound to the peptidoglycan is a branched polysaccharide, the arabinogalactan, whose outer ends are esterified with high molecular weight fatty acids called mycolic acids. These components are peculiar as the arabinogalactan has unusual components and linkages and the mycolic acids are typically long and branched chains containing 60- to 90-carbon atoms. The genera *Dietzia, Rhodococcus, Nocardia, Gordona,* and *Mycobacterium* have mycolic acids with increasing average numbers of carbon atoms. The arrangements of these mycolic acids are species-specific, a property that allows the identification of many species of mycobacteria by gas-liquid, high-performance liquid or thin-layer chromatography (see chapter 14). The mycolic acids specific to *M. tuberculosis* are alpha, keto and methoxymycolates containing 76 to 82, 84 to 89, and 83 to 90 carbons respectively.

The outer layer of the cell wall presents an array of free lipids such as phthiocerol dimycoserosates (PDIM), phenolic glycolipids (PGL), trehalose-containing glycolipids and sulfolipids (SL). The unusual "*M. canettii*", with its smooth colony morphology, has a unique phenolic glycolipid (van Soolingen 1997). *M. bovis* and *M. bovis* BCG produce sizable amounts of a PGL designated as mycoside B, whereas most *M. tuberculosis* strains are deficient in this component.

Traversing the whole envelope, some glycolipids such as the phosphatidyl-myoinositol mannosides, lipomannan (LM) and lipoarabinomanan (LAM), are anchored to the plasma membrane and extend to the exterior of the cell wall. LAMs are species-specific. The mycobacterial wall also contains interspersed proteins. Some are in the process of being exported, some might be residents. Several of these proteins are responsible for cell wall construction during the life of the bacillus. There are also certain proteins called porins forming hydrophilic channels that permit the passive passage of aqueous solutes through the mycolic acid layer. Mycobacterial porins seem to be different from those of gram-negative bacteria.

While growing in a static liquid culture or within a human cell, *M. tuberculosis* seems to accumulate an unbound **pseudo-capsule**. Apparently, when the medium is disturbed, the capsule separates, leaving the lipophilic surface exposed. In fact, the capsule components have largely been recognized in culture filtrates but its structure and location were resolved rather recently. The capsule contains proteins, polysaccharides and minor amounts of inner lipids, which are apparently in con-

stant turnover. The constituents of the capsule might be shed in vivo within the infected host cells. It has been proposed that the capsule might be protective and bioactive. In addition, a number of envelope-associated substances have been described, mostly lipids and glycolipids.

The tubercle bacillus shares most ultrastructural features with other members of the genus, including non-pathogenic mycobacteria. Its distinctive ability to survive in mammalian hosts, its pathogenicity and its immunogenic properties seem to derive, at least in part, from the nature of some of the molecules of the bacterial wall (Riley 2006, Smith 2003).

The envelope of the tubercle bacillus seems to be a dynamic structure that can be remodeled as the microorganism is either growing or persisting in different environments (Kremer 2005). In fact, in growth conditions interfering with the synthesis of the wall, *M. tuberculosis* may be induced to produce wall-deficient spheroplasts that apparently are not pathogenic unless they revert to being normal bacteria (Ratnan 1976). Cell wall thickening was observed in oxygen-deficient conditions (Cunningham 1998). Besides, the expression of genes that putatively code for porins seems to be up regulated in certain environmental conditions, such as mildly acidified culture medium, as well as inside the macrophage vacuoles (Draper 2005).

3.3.1. Acid fastness

Unlike Gram-negative bacteria, mycobacteria do not have an additional membrane in the outer layers of the cell wall. They are structurally more closely related to Gram-positive bacteria. However, mycobacteria do not fit into the Gram-positive category as the molecules attached to the cell wall are distinctively lipids rather than proteins or polysaccharides. Frequently, they do not retain the crystal violet and appear as "ghosts" after Gram staining. The waxy cell wall of mycobacteria is impermeable to aniline and other commonly used dyes unless these are combined with phenol.

To discover the causative agent of TB, Robert Koch had to develop a specific staining process using alkaline dyes. Soon after, Ehrlich discovered the acid fastness of the tubercle bacillus, which has been the prominent characteristic of mycobacteria up until now. The expression "acid-fastness" describes the resistance of certain microorganisms to decolorization with acid-alcohol solutions after staining with arylmethane dyes such as carbol fuchsin. This feature is of utmost practical

importance in identifying the tubercle bacillus, particularly in pathological specimens.

In spite of being a hallmark, the wall permeability to alkaline dyes and the mechanisms preventing their removal by acids are still not totally understood in molecular terms. Most of the current knowledge on this phenomenon was disclosed in pioneer experiments. The beading observed inside the cells was interpreted as accumulation of free dye rather than staining of particular structures, which led to the early hypothesis that alkaline stains are retained in the cytoplasm (Yegian 1947). Later, evidence was provided sustaining the role of lipids in trapping the dyes. Indeed, there is a parallelism between the increasing degree of acid fastness displayed by microorganisms in the genera *Corynebacterium*, *Nocardia,* and *Mycobacterium,* and the increasing length of mycolic acid chains in their walls. This correspondence suggests that the chemical binding of the dye to these molecules might be a determinant for acid fastness.

Bacilli suspended in aqueous solution retain the acid fastness for a long time, even after heating. However, the property is absolutely dependent on the integrity of the bacillus. Unimpaired mycolic acids are required to hinder the penetration of water-soluble dyes and bleaching acids (Goren 1978). The acid fastness of the bacillus is obliterated by cell trauma or autolysis (Baisden 1942), infection by specific mycobacteriophages (Gangadharam 1976) or treatment with antibiotics targeting cell-wall synthesis, such as isoniazid (INH) (Mohamad 2004). Acid fastness seems to also be dependent on nutrients and oxygen tension, as suggested by fluctuations in staining observed in different culture conditions (Nyka 1971). Dormant *M. tuberculosis* bacteria bearing cell wall alterations may remain undetected by the classic Ziehl-Neelsen staining (Seiler 2003).

3.3.2. Cord formation

By microscopic observation, Robert Koch first described the arrangement of bacilli in braided bunches and associated this phenomenon with virulent strains of *M. tuberculosis*. He also detailed the aspect of cultures in blood serum as compact scales which could be easily detached. In general, fresh virulent *M. tuberculosis* bacilli produce rough textured colonies on solid media, expanded gummy veils on the surface of liquid media and serpentines on microscopic smears. In contrast, non-virulent mycobacteria and tubercle bacilli attenuated by prolonged cultures usually develop smooth colonies on solid media, form discrete mats in liquid media and distribute randomly in loose aggregates when smeared. The recognition of these two peculiarities, cording and crumbly colony formation, provides a reliable

and timely clue to the experienced microbiologist for the presumptive distinction of *M. tuberculosis* from other mycobacteria in cultured specimens and even in sputum smears (see chapter 12).

These distinctive characteristics of the virulent bacilli have been attributed to the trehalose 6, 6'-dimycolate. This compound, also known as cord factor, was described as an extractable glycolipid consisting of two mycolic acid molecules loosely bound in the outer layer of the cell wall (Noll 1956). A myriad of biological activities related to pathogenicity, toxicity, and protection against the host response have been attributed to this molecule. However, it does not seem to be essential for bacterial multiplication *in vitro* (Indrigo 2002).

Several models were used to identify the role of the trehalose 6, 6'-dimycolate (TDM) in the microscopic and macroscopic morphology of *M. tuberculosis*. In this way it was demonstrated that beads coated with this substance generate an oriented hydrophobic interaction and aggregate in elongated structures similar to cords (Behling 1993). Later, the molecular packing of TDM was imitated (Almond 1996). Recently, immunohistochemistry was used to investigate the distribution of TDM in *M. tuberculosis* culture pellicles. According to the results of this experiment it was proposed that the TDM released by the microorganism molds a rigid hydrophobic interphase that is responsible for the cultural and microscopic appearance of virulent bacilli (Hunter 2006).

However, this phenomenon is not yet clearly understood. One matter of confusion is the fact that TDM is also present in other non-cording avirulent mycobacteria. Taking this into consideration, the activity of the cord factor in *M. tuberculosis* has been ascribed to a particular surface conformation (Schabbing 1994) and to the large amounts of this molecule released by the tubercle bacilli (Hunter 2006). The localization of DNA sequences encoding cording has not yet been elucidated. Five genes probably associated with cord formation were identified, but their real implication has not been demonstrated (Gao 2004).

So far, the characteristics of the TDM of "*M. canettii*", a human pathogen that produces unusually smooth colonies, have not been described.

3.3.3. Permeability barriers

The tightly packed mycolic acids provide the bacillus with an efficient protection and an exceptional impermeability. In addition to the capsule, an even thicker layer of carbohydrate and protein outside the lipid layer impedes the diffusion of large molecules, such as enzymes, and protects the lipid layer itself. The shell restricts

the permeability to most lipophilic molecules. Other substances can bypass this barrier through the porins, although this mechanism is not very efficient: *M. tuberculosis* possesses a low number of porins compared to other bacteria and the porins admit only small water soluble molecules (Niederweis 2003).

Several experiments have been performed that have provided the rationale for the long believed concept that impermeability is at least one of the determinants for two *M. tuberculosis* characteristics: its slow growth and its intrinsic drug resistance. The penetration rate of β-lactam antibiotics into *M. tuberculosis* was found to be comparable to that of *Pseudomonas aeruginosa* and approximately 100 times lower than that of *Escherichia coli* (Chambers 1995). In recombination experiments, the expression of the *M. smegmatis* porin MspA was followed by increased susceptibility of the tubercle bacillus to β-lactam antibiotics and even to first-line anti-tuberculous drugs. At the same time, the expression of the same porin in *M. bovis* BCG stimulated the uptake of glucose and accelerated growth (Mailaender 2004).

Treatment with some drugs that are known to fray or somehow alter the surface architecture of the cells was shown to increase the susceptibility of *M. tuberculosis* (Verbelen 2006). In effect, at sub-inhibitory concentrations, ethambutol and dimethyl sulfoxide enhanced the activity of anti-tuberculosis drugs against *M. tuberculosis* strains that were originally resistant to these drugs (Jagannath 1995). Similarly, some antidepressants, such as chlorpromazine, have in vitro activity themselves against the tubercle bacillus (Ordway 2003).

3.4. Nutritional and environmental requirements for growth

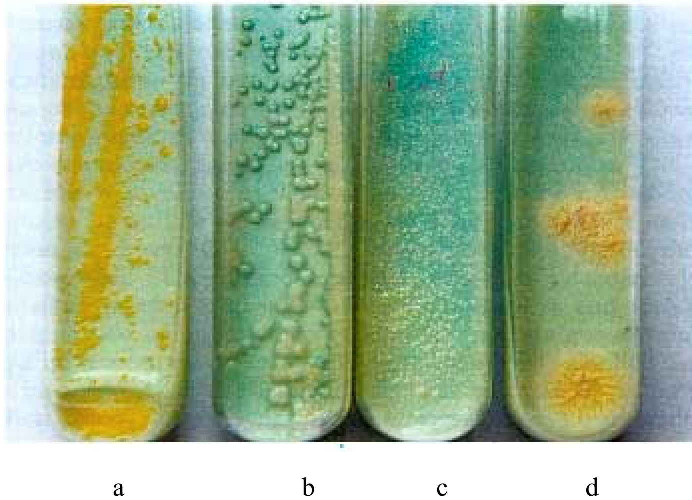
The tubercle bacillus is prototrophic (i.e. it can build all its components from basic carbon and nitrogen sources) and heterotrophic (i.e. it uses already synthesized organic compounds as a source of carbon and energy). The microorganism macromolecular structure and physiological (metabolic) capabilities result in high adaptation to the specific environment. In turn, the nutritional quality of the environment determines the bacillus lifestyle and limitations, either in the natural habitat or in culture media, as do various physical conditions such as oxygen availability, temperature, pH and salinity.

As the environment changes, the bacillus is able to bring into play different physiological pathways in order to survive even in harsh conditions. This is a highly resourceful strategy, not only for pathogenicity but also for species persistence. It has been shown that, during the course of infection in mice, *M. tuberculo-*

sis metabolism may shift from an aerobic, carbohydrate-metabolizing mode to one that is more microaerophilic and utilizes lipids (Segal 1956). These demonstrations, which were reported a long time ago, were supported in recent times by the complete sequencing of the *M. tuberculosis* genome in which an unusually high number of genes putatively involved in fatty acid metabolism were identified. This phenomenon may be related to the ability of the pathogen to grow or persist in host tissues where fatty acids may be the major carbon source (Neyrolles 2006) (see chapter 4).

In vitro, the members of the *M. tuberculosis* complex are not fastidious unless damaged by some noxious agents. In fact, the medium used by Koch to cultivate *M. tuberculosis* was simply sterile coagulated blood serum. The tubercle bacilli can also grow in salt solutions using glycerol as a carbon source, ammonium ions and asparagine as nitrogen sources, and micronutrients. *M. tuberculosis* is able to metabolize glycerol into pyruvate, whereas *M. bovis* is not. Indeed, the genome sequence analysis confirmed that all the genes required for the formation of pyruvate are non-functional in *M. bovis*. Being defective in this metabolic process, *M. bovis* grows much better in the presence of a pyruvate salt as a source of carbon. Albumin, which is normally provided by adding eggs or bovine serum albumin to the culture media, promotes the growth of these microorganisms. Other subsidiary media components may be used, such as Tween 80, a detergent that disperses the bacilli in liquid media. It was postulated that bovine serum albumin may bind the excess of oleate that can be released from the detergent up to toxic amounts. Biotin and catalase have been incorporated to the Middlebrook series media to stimulate the revival of damaged bacilli in clinical specimens (Wayne 1982).

Trace elements found by the microorganism in the water, inorganic ions, small molecules, and macromolecules have either a structural or a functional role in the cell. Magnesium and iron are essential for life. A deficiency in these elements frequently reduces the virulence of bacterial pathogens, including the tubercle bacillus. As iron is usually in the form of insoluble ferric salts in the environment, special iron systems are required to incorporate this element into the cell. Exochelins and mycobactins are the major siderophores used by mycobacteria to perform this function. The former are hydrophilic peptides secreted into the environment for iron gathering. The latter are hydrophobic compounds located within the cell wall to introduce the iron into the cytoplasm. The *mbt* operon is putatively involved in the synthase activities required to produce the mycobactin core (De Voss 2000). The incorporation of mycobactin into culture media can promote the growth of ailing *M. tuberculosis* isolates.



a b c d

Figure 3-3: Mycobacteria growing on Löwenstein-Jensen slants. a. *Mycobacterium gordonae*; b. *Mycobacterium fortuitum*; c. *Mycobacterium avium*; d. *Mycobacterium tuberculosis.*

The tubercle bacillus requires oxygen as a final electron acceptor in aerobic respiration. Molecular oxygen is reduced to water in the last step of the electron transport system. In nature, the bacillus grows most successfully in tissues with high oxygen partial tension, such as the lungs, particularly the well-aerated upper lobes. Carbon dioxide is essential and may be taken from the atmosphere and also from carbonates or bicarbonates. In the laboratory, an atmosphere of 5 to 10 % carbon dioxide favors culture growth, at least during the early stage of incubation. On the other hand, *M. bovis* is microaerophilic, i.e. it grows preferentially at a reduced oxygen tension.

M. tuberculosis is mesophile and neutrophile as its multiplication is restricted to conditions offered by warm-blooded animals: about 37ºC and a neutral pH. The temperature and hydrogen ion concentration ranges, in which the bacillus is able to multiply, are relatively narrow. High saline concentration such as that found in media containing 5 % sodium chloride, inhibits the growth of the microorganism.

3.5. Generation time

Under favorable laboratory conditions, *M. tuberculosis* divides every 12 to 24 hours. This pace is extremely slow compared to that of most cultivable bacteria, which duplicate at regular intervals ranging from about 15 minutes to one hour. Recently, the low multiplication rate of the tubercle bacillus was nicely exposed by Chauhan *et al.* These authors demonstrated the small proportion of cells initiating the septation process prior to division among tubercle bacilli growing either in broth or inside macrophages (Chauhan 2006).

The slow growth rate might be partially determined by the cell wall impermeability that limits nutrient uptake. However, only a minimal stimulus to bacterial multiplication is achieved when the permeability is increased through treatment with some compounds that interact with the cell envelope. Harshey and Ramakrishnan identified ribonucleic acid (RNA) synthesis to be a major factor associated with the long generation time of the tubercle bacillus. They demonstrated that both the ratio of RNA to DNA and the RNA chain elongation rate are ten-fold lower in *M. tuberculosis* compared to *E. coli* (Harshey 1977). Another unusual feature is the existence of a unique operon commanding RNA synthesis. Furthermore, when the tubercle bacillus switches from the stationary to the active multiplying phase, its total RNA content increases only twofold. Consequently, the protein synthesis must be retarded (Verma 1999). The influence of nutrient availability on the ribosome synthesis rate, which is a proxy of metabolic activity, remains controversial (Hampshire 2004).

The low multiplication rate explains the typically sub-acute to chronic evolution of the disease and the long time required to attain visible growth in vitro. Numerous experiences using different nutrients and culture conditions have demonstrated that some factors may abrogate a lag in adaptation of the bacilli in culture media but, once growth is initiated, the replication cycle will still take no less than 12 hours. This limitation in accelerating the tubercle bacillus growth could not be overcome. Instead, the main achievements for diagnosis have been made through the use of tools that enable the detection of a minimal quantity of bacilli in the media. First, transparent agar medium allowing the detection of tiny colonies were introduced; more recently, the addition of biosensors has been adopted to detect redox changes produced by the bacilli metabolism (see chapters 12 and 14).

3.6. Metabolic and biochemical markers

In the laboratory, the classical phenotypic identification, speciation and subspeciation of members of the *M. tuberculosis* complex include key diagnostic tests developed to detect certain metabolic intermediates and the activity of some enzymes that are essential for life and pathogenicity. In addition to some susceptibility tests, the investigation of niacin accumulation, nitrate reductase and urease activity allows the distinction of *M. tuberculosis* complex and species differentiation within the complex (see chapter 8). Most of the information on the structure and function of these metabolites and enzymes has focused on *M. tuberculosis* and, to some extent, on *M. bovis.* Much less is known about these features in other members of the *M. tuberculosis* complex.

Niacin (nicotinic acid) plays a vital role in organic life, as it is involved in the oxidation-reduction reactions of energy metabolism and in the DNA repair processes. Although all mycobacteria produce niacin, most of them employ the majority of the yielded metabolite in the synthesis of co-enzymes. In contrast, *M. tuberculosis* produces and accumulates substantial amounts of niacin as a result of a very active nicotinamide adenine dinucleotide degradation pathway and the inability to process the resultant niacin (Kasarov 1972). In vitro, *M. tuberculosis*, "*M. canettii"*, and some isolates of *M. africanum* excrete water-soluble niacin into the culture media, the detection of which is extremely useful for definitive identification. This is another hallmark that has not been investigated in molecular terms. Again, most of the knowledge existing on this phenomenon and the tools for its detection were produced a long time ago by bacteriological and chemical studies.

Like many aerobes, including other mycobacteria, the tubercle bacillus depends upon certain enzymes to detoxify lethal oxygen radicals, such as peroxides and H_2O_2, which are self-generated during respiration or produced by host phagocytes. The main *M. tuberculosis* antioxidant enzyme that can hydrolyze H_2O_2 is a heat-labile catalase-peroxidase with both catalase and peroxidase activities. The thermal lability of this enzyme is a marker of the *M. tuberculosis* complex. *M. tuberculosis* also has an alternative alkyl-hydroperoxidase, which is postulated to compensate for the lack of catalase activity. Paradoxically, the catalase is not only self-protective but can also be self-destructive as it activates the anti-tuberculous prodrug INH. Mutations in the genes encoding both enzymes (*katG* and *ahpC*) are involved in resistance to INH and thus, have been the subject of active investigation (see chapter 18). Understandably, resistance to INH may be associated with irregular catalase activity. Among the biochemical markers commonly investigated for mycobacteria identification in the clinical microbiological laboratory, this is the only one that may be affected by drug resistance to some extent.

Even though *M. tuberculosis* prefers ammonium and asparagine, it can deficiently utilize nitrate and nitrite as sole sources of nitrogen in vitro. It has been speculated that, in infected hosts, the microorganism might use nitrate as a nitrogen source and/or as a terminal electron acceptor in the absence of oxygen. Whatever the physiological function may be, *M. tuberculosis* has an enzyme bound to the cell membrane that rapidly reduces nitrate and leads to the accumulation of nitrite. Unlike those of other mycobacteria, *M. tuberculosis* nitrate reductase is permanently very active *in vitro* regardless of the culture conditions. Under hypoxic conditions or on exposure to nitric oxide, its activity may even be enhanced by induction of the protein NarK2. This protein is a nitrate transporter that might be able to sense the redox state of the cell and adjust its own activity accordingly (Sohaskey 2005). The reductase activity may be hindered by very high concentrations of INH. Furthermore, some isolates of the tubercle bacillus that are resistant to INH and para-aminosalicylic acid (PAS) were found to be unable to reduce nitrate when growing in minimal media (Hedgecock, 1962). The nitrate reductase activity seems to be encoded by the constitutive *narGHJI* operon (Weber 2000), which is present in both *M. tuberculosis* and *M. bovis*. However, *M. bovis* does not reduce nitrate. It was demonstrated that a single nucleotide polymorphism at position 215 in the promoter of this gene cluster determines different levels of enzyme activity in both species (Sohaskey 2003). "*M. canettii*" and some isolates of *M. africanum* produce detectable amounts of nitrite from nitrate in vitro.

M. tuberculosis is able to produce ammonia from urea by a urease-mediated reaction. The ammonium can be then used by the microorganism for biosynthesis. The urease is coded by the genes *ureABC* (Reyrat 1995) and it might also be important for nitrogen acquisition as its activity increases when nitrogen sources are limited (Clemens 1995). In addition, the consequent alkalinization of the microenvironment by ammonium ions might inhibit the maturation of phagolysosomes and contribute to the defective maturation of major histocompatibility complex class II molecules of host monocytes (Sendide 2004).

3.7. Resistance to physical and chemical challenges

Although the tubercle bacillus is not a spore-forming bacterium, it has a remarkable capacity to endure unfavorable conditions. The bacillus is able to circumvent destruction within the macrophages and to limit the access to the bacterial targets of hydrophilic antiseptics and antibiotics (see Chapters 5, 11, and 18). For example, chloride and bromide salts of cetylpyridium do not impair the viability of the tubercle bacilli for at least 14 days (Tazir 1979, Pardini 2005). Therefore, these salts are

used as preservatives when the processing of specimens is delayed. Likewise, the natural impermeability of the bacterium to common hydrophilic antimicrobial agents is used in the clinical mycobacteriology laboratory. In effect, some broad spectrum antibiotics are added to selective media to isolate the tubercle bacillus.

As already mentioned, *M. tuberculosis* complex organisms multiply within narrow temperature and pH ranges, and at a high oxygen tension, which is indicative of the effect produced by these physical conditions on the rates of enzymatic reactions. However, the tubercle bacilli can withstand conditions far distant from those optimal for propagation. The bacillus survives to some extent in the acid or alkaline microenvironment as a result of its interaction with the defensive mechanism of the host, as well as the acid contents of the stomach. Similarly, a significant proportion of the bacilli population present in clinical specimens can endure a brief treatment with diluted solutions of acids and alkalis such as sulfuric acid or sodium hydroxide. This property is peculiar as most microflora present in the specimens are killed by this treatment; thus, it is exploited to isolate mycobacteria (see chapter 12). The stress generated by a low pH is more severe in a nutrient-limited environment. High levels of magnesium are required for growth in mildly acidic media (Cotter 2003).

The microorganism also withstands very low temperatures. Its viability may be increasingly preserved for a long term between 2-4°C to -70°C. When ultrafrozen, the viability of the bacilli remains almost intact as well as the taxonomic, serologic, immunologic, and pathogenic properties. After thawing, they may require re-adaptation to recover full metabolic activity (Kim 1979). On the other hand, the bacilli are very sensitive to heat, sunlight and ultraviolet (UV) irradiation. In sputum or in aqueous suspension, they progressively lose viability between 30 and 37°C within one week. Exposed to direct UV irradiation, moderate loads of tubercle bacilli die in a few minutes (Huber 1970, Collins 1971).

In addition, *M. tuberculosis* tolerates low oxygen tension as demonstrated in undisturbed liquid culture media where the self-generated microaerophilic sediment contains non-dividing, yet viable, bacilli. The bacilli may survive for many years in this condition but need a minimal concentration of oxygen to induce the switch into a fermentative metabolism (Wayne 1982, Wayne 1984). Adaptation to microaerophilic conditions was further substantiated when it was found that, unlike aerobically-cultured bacilli, those persisting at low oxygen tension were susceptible to metronidazole, a drug that is known to be effective against anaerobic bacteria. Using transmission electron microscopy, Cunningham and Spreadbury demonstrated that the cell wall of the microorganism thickens notoriously in microaerobic and anaerobic cultures, which might be a strategy to endure oxygen depletion

(Cunningham 1998). Under these conditions, a highly expressed and ubiquitous 16 kilo Dalton protein was identified. This heat-shock protein might play a role in stabilizing the cell structures for long-term survival in the dormant state.

The tight structure of the cell wall of the tubercle bacillus is undoubtedly the shield that preserves the posistion and function of the metabolic and replicating machinery, even when inactive. At the same time, a succession of physiological mechanisms, which are still poorly understood, are ready to shift this machinery towards dormancy whenever necessary. This seems to be the main adaptive response of the bacilli to almost all sub-optimal or even harsh conditions, *in vitro, ex vivo,* and *in vivo* (see chapter 5).

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Chapter 4: Genomics and Proteomics

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4.1. Impact of new technologies on *Mycobacterium tuberculosis* genomics

A new wave in the analysis of the physiological secrets of microorganisms started more than a decade ago with the reading of the first complete genome sequence, corresponding to the bacterium *Haemophilus influenzae* (Fleishman 1995). Nowadays, the accessibility to hundreds of bacterial genome sequences has changed our way of studying the bacterial world, including bacterial pathogens such as *M. tuberculosis*.

The overwhelming information displayed by genome sequences started the era of "omics" technologies. These technologies are in accordance to the currently fast times. A quick search in PubMed, limiting results to the last 10 years, showed more than 27,000 papers devoted to "omics" issues: more than three thousand concerning bacteria, and almost three hundred concerning *Mycobacterium tuberculosis*. Up to five different "omics" methodologies have been described so far, all concerning the global study of the target organism, analyzing all its genes, transcriptional products, proteins, etc.

- **Genomics** involves the study of all genes that are present in the genomes
- **Transcriptomics** concerns the analysis of the cellular functions at the messenger ribonucleic acid (mRNA) level
- **Proteomics** refers to the detection and identification of all proteins in a cell
- **Metabolomics** comprises the complete set of all metabolites formed by the cell and its association with its metabolism
- **Fluxomics** compares the cellular networks (Fiehn 2003, Nielsen 2005)

In the tuberculosis (TB) field, only papers concerning genomics, transcriptomics, and proteomics have been published. Integration of data derived from the several "omics" by bioinformatics will probably allow a rational insight into *M. tuberculosis* biology and its interactions with the host, leading to true control of the disease.

Undoubtedly, the biggest step in our knowledge on TB during the last decade was the description of the complete genome sequence of the laboratory reference *M.*

tuberculosis strain H37Rv (Cole 1998a). For example, the identification of genes involved in the bacterial cell wall biosynthesis, the routes for lipid metabolism, the location of insertion sequences and the variability in the PE_PPE genes allowed scientists to merge the fragments of knowledge derived from the pre-genomic era in a more comprehensive way. The sequence of the genome, and its comparison to sequences of other microorganisms reported in several databases, allowed the assignation of precise functions to 40 % of the predicted proteins and the identification of 44 % of orthologues (genes with very similar functions in a different species), leaving 16 % as unique unknown proteins.

The elucidation of complete genome sequences and the development of microarray-based comparative genomics have been powerful tools in the progress of new areas by the application of robotics to basic molecular biology. Comparative genomics and genomic tools have also been used to identify factors associated with the pathogenicity of *M. tuberculosis*, such as virulence factors and genes involved in persistence of the pathogen in host cells. Moreover, these tools allowed a description of the evolutionary scenario of the genus (see Chapter 2).

Structural genomics was the starting point. As more accurate technologies became available, the interest was focused into functional genomics. Thus, information on specific mRNA actively synthesized by bacteria inside macrophages or during in *vitro* starvation, opened ways to the analysis of gene expression. Microarray technology was applied to the detection of global gene activity in *M. tuberculosis* under several environmental conditions. However, bacterial function cannot be understood by looking at the mRNA level alone. A major barrier for genomic studies has been the great number of genes with unknown function that have been identified. Up to 60 % of the open reading frames (ORFs) had unknown functions after the initial annotation of genes (identification of the protein unrevealed by the corresponding ORF's amino acid sequence) (Cole 1998a). The elucidation of protein function was possible with the global analysis of bacterial proteins, giving insights into the functional role of several so far unknown proteins. Thanks to the joint contributions of biochemical techniques and mass spectrometry, up to 1,044 non-redundant proteins were reported in different cellular fractions (Mawuenyega 2005). The upcoming task will be to assign them all a functional role. As more results are obtained from the proteomic analysis, it is expected that the function of more ORFs will be unveiled with the aid of new data on transcriptomics and proteomics.

Genomics and other molecular tools allowed studies on gene expression and regulation, which were unthinkable years ago. *M. tuberculosis* is a restricted human pathogen; therefore it must have developed mechanisms enabling its quick and

efficient adaptation to a variety of "intra-human" environments, which are, in fact, its natural habitat. Understanding how the bacillus regulates its different genes according to environmental changes will probably lead to the comprehension of many interesting aspects of *M. tuberculosis*, including latency and host-adaptation.

This chapter will address the general basics, as well as the state-of-the-art genomics, transcriptomics and proteomics in relation to *M. tuberculosis*. Finally, a general overview will be made on lipids, the most peculiar metabolites of this bacterium.

4.2. *M. tuberculosis* genome

4.2.1. Genomic organization and genes

TB research made huge progress with the availability of the genome sequence of the type strain *M. tuberculosis* H37Rv (Cole 1998a). Expectations were generated on the elucidation of some unique characteristics of the biology of the tubercle bacillus, such as its characteristic slow growth, the nature of its complex cell wall, certain genes related to its virulence and persistence, and the apparent stability of its genome. This first available genome sequence of a pathogenic *M. tuberculosis* strain helped to answer some of these questions and, what is even more stimulating, to open many more. We describe herein the main characteristics of the *M. tuberculosis* genome sequences completed thus far and highlight some of the most interesting questions answered and opened with this advance in TB research.

M. tuberculosis H37Rv (Cole 1998a) was revealed to possess a sequence of 4,411,529 bp, the second largest microbial genome sequenced at that time. The characteristically high guanine plus cytosine (G+C content; 65.5 %) was found to be uniform along most of the genome, confirming the hypothesis that horizontal gene transfer events are virtually absent in modern *M. tuberculosis* (Sreevatsan 1997). Only a few regions showed a skew in this G+C content. A conspicuous group of genes with a very high G+C content (> 80 %) appear to be unique in mycobacteria and belong to the family of PE or PPE proteins. In turn, the few genes with particularly low (< 50 %) G+C content are those coding for transmembrane proteins or polyketide synthases. This deviation to low G+C content is believed to be a consequence of the required hydrophobic amino acids, essential in any transmembrane domain, that are coded by low G+C content codons.

Fifty genes were found to code for functional RNAs. As previously described (Kempsell 1992), there was only one ribosomal RNA operon (*rrn*). This operon was found to be located at 1.5 Mbp from the origin of replication (*oriC* locus).

Most eubacteria have more than one rrn operon located much closer to the *oriC* locus to exploit the gene-dosage effect during replication (Cole 1994). The possession of a single *rrn* operon in a position relatively distant from *oriC* has been postulated to be a factor contributing to the slow growth phenotype of the tubercle bacillus (Brosch 2000a).

One of the most thoroughly studied characteristic of *M. tuberculosis* is the presence and distribution of insertion sequences (IS). Of particular interest is IS*6110*, a sequence of the IS3 family that has been widely used for strain typing and molecular epidemiology due to its variation in insertion site and copy number (van Embden 1993, see Chapter 9). Sixteen copies of IS*6110* were identified in the genome of *M. tuberculosis* H37Rv; some IS*6110* insertion sites were clustered in sites named insertional hot-spots. The same strain was found to harbor six copies of the more stable IS*1081*, an insertion sequence that yields almost identical profiles in most strains when analyzed by Restriction Fragment Length Polymorphism (RFLP) (Sola 2001, Kanduma 2003). Another 32 different insertion sequences were found, of which seven belonged to the 13E12 family of repetitive sequences; the other insertion sequences had not been described in other organisms (Cole 1998b). Virtually all the ISs found in *M. tuberculosis* so far belong to previously described IS families (Chandler 2002). The only exception is IS*1556*, which does not fit into any known IS family (Cole 1999).

Two prophages were detected in the genome sequence; both are similar in length and also similarly organized. One is the prophage PhiRv1, which in the *M. tuberculosis* H37Rv genome interrupts a repetitive sequence of the family 13E12. This prophage is deleted or rearranged in other *M. tuberculosis* strains (Fleischmann 2002). The genome of *M. tuberculosis* possesses seven potential *att* sites for PhiRv1 insertion, which explains the variability of its position between strains (Cole 1999). The second prophage, PhiRv2 has proven to be much more stable, with less variability among strains (Cole 1999).

Regarding protein coding genes, it was determined that *M. tuberculosis* H37Rv codes for 3,924 ORFs accounting for 91 % of the coding capacity of the genome (Cole 1998a). The alternative initiation codon GTG is used in 35 % of cases compared to 14 % or 9 % in *Bacillus subtilis* or *Escherichia coli* respectively. This contributes to the high G+C bias in the codon usage of mycobacteria.

A bias in the overall orientation of genes with respect to the direction of replication was also found. On average, bacteria such as *B. subtilis* have 75 % of their genes in the same orientation as that of the replication fork, while *M. tuberculosis* only has 59 %. This finding has led to the hypothesis that such a bias could also be part of

the slow growing phenotype of the tubercle bacillus (Cole 1999). This conjecture, however, does not take into account the fact that *E. coli*, a bacterium that grows much faster than *M. tuberculosis,* has only 55 % of its genes in the same direction as the replication origin (Li 2005).

From the predicted ORFs, all proteins have been classified in 11 broad functional groups (Table 4-1), more precisely classified into COG functional categories (http://www.ncbi.nlm.nih.gov/sutils/coxik.cgi?gi=135) according to the National Center for Biotechnology Information (NCBI) of the United States (US). The analysis of the codon usage showed a preference for G+C-rich codons. It was also found that the number of genes that arose by duplication is similar to the number seen in *E. coli* or *B. subtilis*, but the degree of conservation of duplicated genes is higher in *M. tuberculosis*. The lack of divergence of duplicated genes is consistent with the hypothesis of a recent evolutionary descent or a recent bottleneck in mycobacterial evolution (Brosch 2002, Sreevatsan 1997, see chapter 2).

From the genome sequence it is clear that *M. tuberculosis* has the potential to switch from one metabolic route to another including aerobic (e.g. oxidative phosphorylation) and anaerobic respiration (e.g. nitrate reduction). This flexibility is useful for survival in the changing environments within the human host that range from high oxygen tension in the lung alveolus to microaerophilic/anaerobic conditions within the tuberculous granuloma. Another characteristic of the *M. tuberculosis* genome is the presence of genes for synthesis and degradation of almost all kinds of lipids from simple fatty acids to complex molecules such as mycolic acids. In total, there are genes encoding for 250 distinct enzymes involved in fatty acid metabolism, compared to only 50 in the genome of *E. coli* (Cole 1999).

Concerning transcriptional regulation, *M. tuberculosis* codifies for 13 putative sigma factors and more than 100 regulatory proteins (see section 4.3 of this chapter).

Among the most interesting protein gene families found in *M. tuberculosis* are the PE and PPE multigene families, which account for almost 10 % of the genome capacity. The names PE and PPE derive from the motifs of Pro-Glu (PE) and Pro-Pro-Glu (PPE) found near the protein N-terminus in most cases. These proteins are believed to play an important role in survival and multiplication of mycobacteria in different environments (Marri 2006). There are about 100 members of the PE family, which is further divided into three sub-families, the most important of which is the polymorphic GC-rich sequences (PGRS) class that contains 61 members. Proteins in this class contain multiple tandem repetitions of the motif Gly-Gly-Ala, hence, their glycine concentration is superior to 50 %. The PE_PGRS proteins

have been found to be exclusive to the *M. tuberculosis* complex (Marri 2006) and resemble the Epstein-Barr virus nuclear antigens (EBNA), which are known to inhibit antigen presentation through the histocompatibility complex (MHC) class I (Cole 1999).

Interestingly, the analysis of the desoxyribonucleic acid (DNA) metabolic system of *M. tuberculosis* indicates a very efficient DNA repair system, in other words, replication machinery of exceptionally high fidelity. The genome of *M. tuberculosis* lacks the MutS-based mismatch repair system. However, this absence is overcome by the presence of nearly 45 genes related to DNA repair mechanisms (Mizrahi 1998), including three copies of the *mutT* gene. This gene encodes the enzyme in charge of removing oxidized guanines whose incorporation during replication causes base-pair mismatching (Mizrahi 1998, Cole 1999).

With the aim of making the information publicly available and the search and analysis of information easier, the Pasteur Institute (http://www.pasteur.fr/recherche/unites/Lgmb/) has created a database system incorporating not only all genes and annotation but other search tools such as Blast or FastA, that allow the user to search for homologue sequences of a query sequence inside the *M. tuberculosis* genome. This database is freely available for use on the Internet and is known as the Tuberculist Web Server http://genolist.pasteur.fr/TubercuList/).

As more information was generated, databases grew bigger, more experimental information became available, and better and more accurate algorithms for gene identification and prediction were released. The initial genome annotation in *M. tuberculosis* H37Rv strain soon became out of date. For this reason, a re-annotation of that genome sequence was published in 2002. This re-annotation incorporated 82 additional genes. The gene nomenclature was not altered; the new genes have the name of the preceding gene followed by A, B or D, for example, two new ORFs were described between *Rv3724* and *Rv3725*, hence, they were named *Rv3724A* and *Rv3724B*. The letter C was not included since it usually stands for "complementary", which means that the gene is located in the complementary strand. As expected, the classes that exhibited the greatest numbers of changes were the unknown category and the conserved hypothetical category (Table 4-1). The re-annotation of the genome sequence allowed the identification of four sequencing errors making the current sequence size change from 4,411,529 to 4,411,532 bp (Camus 2002).

As shown in Table 4-1, the information obtained from a single sequenced genome is enormous. The advances made on the analysis of such information have accelerated TB research.

Table 4-1: Functional classification of *M. tuberculosis* H37Rv and re-annotation*

Class	Function	Number of genes (1998)	Number of genes (2002)
0	Virulence, detoxification, adaptation	91	99
1	Lipid metabolism	225	233
2	Information pathways	207	229
3	Cell-wall and cell processes	516	708
4	Stable RNAs	50	50
5	Insertion sequences and phages	137	149
6	PE and PPE proteins	167	170
7	Intermediary metabolism and respiration	877	894
8	Proteins of unknown function	606	272
9	Regulatory proteins	188	189
10	Conserved hypothetical proteins	910	1,051

* Data taken from Fleischman 2002

4.2.2. Comparative genomics

In recent times, new technologies have been developed at an overwhelming pace, in particular those related to sequencing and tools for genome sequence data management, storage and analysis. As of April 2007, 484 microbial genomes have been finished and projects are underway aimed at the sequencing of other 1,155 microorganisms (http://www.genomesonline.org/gold.cgi). Mycobacteria are not an exception in this titanic genome-sequencing race; since 1998, when the first mycobacterial genome sequence was published (Cole 1998a); many genome projects have been initiated. Until April 2007, 34 projects on the genome sequencing of different mycobacterial species are finished or in-process. Of these, 15 are directed towards *M. tuberculosis* strains, and 5 towards other members of the *M. tuberculosis* complex. This information will be invaluable to improve the knowledge about *M. tuberculosis* in the next few years. Currently, there are only two *M. tuberculosis* (H37Rv and CDC1551) and two *M. bovis* (AF2122/97 and BCG Pasteur) genome sequences annotated and published. For this reason, these are the strains that have been used as reference strains for comparative genomics both *in vitro* and *in silico*.

The pioneer of in vitro assays of comparative mycobacterial genomics involved comparison of restriction profiles using low frequency restriction enzymes and pulsed-field gel electrophoresis (PFGE). These studies allowed a rough analysis of differences among *M. bovis* bacille Calmette-Guérin (BCG) isolates (Zhang 1995) and most importantly, contributed to the construction of the first physical maps, which were essential for the generation of the first genome sequence (Philipp 1996).

The next step in comparative genomics was the use of genomic subtractive hybridization or bacteria artificial chromosome hybridization for the identification of regions of difference among the strains under analysis (Mahairas 1996, Gordon 1999). Mahairas *et al*. (Mahairas 1996) used subtractive hybridization to identify regions of difference that account for the avirulent phenotype of the vaccine strain *M. bovis* BCG. As a result of their studies, they identified three regions of difference (RD1-RD3) in the genome of *M. tuberculosis* H37Rv that appeared to be absent from *M. bovis* BCG. Further studies of these regions showed that RD3 corresponded to the prophage PhiRv1, a sequence that has been shown to vary among *M. tuberculosis* clinical isolates and laboratory strains (see section 4.2.1). RD2 was only deleted in isolates of *M. bovis* BCG that were re-cultured after 1925. Finally, RD1 turned out to be the only sequence deleted from all *M. bovis* BCG strains and present in pathogenic strains. However, complementation assays did not reconstitute the full virulent phenotype in *M. bovis* BCG (Mahairas 1996). The RD1 region contains eight ORFs, including members of the Early Secretory Antigenic Target 6 (ESAT-6) gene cluster (Brosch 2000a). The ESAT-6 proteins have been shown to act as potent stimulators of the immune system (Brodin 2002).The genome of H37Rv contains 23 copies of ESAT-6 family proteins distributed in 11 different regions. Except for *esxQ,* all are clustered in pairs belonging to the ESAT-6 and CFP-10 protein families (Stanley 2003, Gey Van Pittius 2001).

Gordon *et al*. (Gordon 1999) used ordered bacteria artificial chromosome arrays to determine genomic differences between *M. tuberculosis* H37Rv and *M. bovis* BCG. As a result, they identified 10 regions of difference, including the three previously described (Mahairas 1996). Interestingly, two of the newly described regions (RD5 and RD8) also contained members of the ESAT-6 family of proteins. In addition, RD5 contained three genes coding for phospholipase C, a gene with a putative role in mycobacterial pathogenesis (Johansen 1996). Several members of the PE and PPE family proteins were also found in the regions of difference. One copy of IS*1532* was identified in RD6 and one copy of IS*6110* in RD5. Furthermore, the study searched for regions present in *M. bovis* BCG but absent from *M. tuberculosis* H37Rv. Two regions with this characteristic were found and were named RvD1

and RvD2 standing for H37Rv Deleted. Almost all ORFs from these regions code for unknown proteins, so the role of these deletions has not been elucidated.

Until 2002, most studies concerning comparative genomics were based on differences among the strain type *M. tuberculosis* H37Rv and other tuberculous bacilli (Behr 1999, Brosch 1999, Brosch 2002). Different approaches using DNA hybridization techniques, such as microarrays, allowed identification of regions of difference with more accuracy and sensitivity than previous methodologies. In total, 16 regions of difference have been found in *M. tuberculosis* H37Rv that were deleted from *M. bovis* BCG. The basic idea behind the identification of regions of difference between the avirulent strain *M. bovis* BCG and the virulent laboratory strain *M. tuberculosis* H37Rv was the identification of specific deletions in all BCG strains that could be responsible for their lack of virulence. However, nine of the regions of difference were also absent in pathogenic isolates of *M. bovis*.

Other studies have been done comparing *M. tuberculosis* H37Rv to its avirulent counterpart *M. tuberculosis* H37Ra (Brosch 1999), in which other Rv-deleted regions were identified. These regions, named RvD3 to RvD5, were found to be products of homologous recombination of adjacent IS*6110*, as with RvD2. Finally, only RD1 was found to be absent in all *M. bovis* BCG strains and present in other members of the complex.

The regions of difference were used as markers of the molecular evolution of *M. tuberculosis* (Brosch 2002) and are represented in Figure 4-1. The use of deletions as molecular markers has been described in Chapter 2.

Besides the above mentioned deletions, two duplications were identified in the *M. bovis* BCG genome (Brosch 2000b). These duplications, named DU1 and DU2, apparently arose from independent events. DU1 seems to be restricted to the BCG Pasteur strain and comprises the *OriC* locus, indicating that BCG Pasteur is diploid for *OriC* and some other neighboring genes. The DU2 region has been found in all BCG substrains tested and includes the sigma factor *sig*H, which has been related to the heat-shock response (Brosch 2001). Some excellent reviews are available on comparative genomics, made before the publication of the second *M. tuberculosis* genome (Cole 1998a, Brosch 2000a, Brosch 2000c, Brosch 2001, Domenech 2001, Cole 2002a, Cole 2002b).

In 2002, the second *M. tuberculosis* genome sequence was completed, namely the clinical strain CDC1551, which had been previously involved in a TB outbreak. This strain was considered to be highly transmissible and virulent for human beings (Fleischmann 2002). With the sequence of this second strain, a first approach to the bioinformatic analysis of intraspecies variability became possible. In the initial

comparison by sequence alignment, H37Rv presented a total of 37 insertions (greater than 10bp) relative to strain CDC1551; from these, 26 affected ORFs while the remaining 11 were intergenic. On the other hand, CDC1551 presented 49 insertions relative to *M. tuberculosis* H37Rv; 35 affecting ORFs and 14 intergenic. A total of 80 ORFs were inserted in either genome, 25 (31.2 %) of them were hypothetical or conserved hypothetical ORFs, while 36 (45 %) corresponded to the family of PE/PPE proteins, showing the potential role of this family of proteins in antigenic variability and thus in pathogenicity.

Deletion	***M. tuberculosis* H37Rv**	***M. africanum***	***M. microti***	***M. bovis***	***M. bovis* BCG**
RD2					
RD14					
RD1					
RD4					
RD12					
RD13					
RD7					
RD8					
RD10					
RD9					
RvD1					
TbD1					

Figure 4-1: Distribution of deleted regions in *M. tuberculosis* complex members. Dark gray filled cells indicate the presence in all strains tested, light gray indicate the presence in some strains, white is absence from all strains tested. Data taken from (Gordon 1999, Brosch 2002, Brosch 2000b, Marmiesse 2004)

Only one major rearrangement was found, consisting of the PhiRv1 (RD3),which was found in the genome of *M. tuberculosis* H37Rv on coordinates 1,779,312 associated with a protein of the REP13E12 family. On the genome of CDC1551, it was found to be located on the complementary strand at coordinates 3,870,803, also associated with a REP13E12 protein. *M. tuberculosis* CDC1551 was found to have four copies of IS*6110* while *M. tuberculosis* H37Rv had 16. Interestingly, four of the 16 IS*6110* copies found in *M. tuberculosis* H37Rv lacked the characteristic 3 to 4 base pair direct repeat and were adjacent to regions deleted in *M. tuberculosis* H37Rv relative to *M. tuberculosis* CDC1551, which suggests homologous recombination.

Since 2002, a large number of studies has been based on Large Sequence Polymorphisms (LSPs) and Single Nucleotide Polymorphisms (SNPs), identified by the comparison of the first two *M. tuberculosis* genome sequences (Hughes 2002, Gutacker 2002). These studies have been complemented with data obtained from the genome sequence of a third organism of the *M. tuberculosis* complex. The complete genome of *Mycobacterium bovis* AF2122/97, a fully virulent strain isolated from a diseased cow in 1997 in Great Britain, was published in 2003 (Garnier 2003). This genome was composed of 4,345,492 bp with a G+C content of 65.63 %, 3,952 putative coding genes, one prophage (PhiRv2), and four IS elements. As expected, similarity of more than 99.95 % was found with a complete colinearity, without evidence of extensive rearrangements. With regard to LSP, most of them have been described above as regions of difference. Sequencing confirmed the absence of 11 regions of difference, and the presence of only one insertion in comparison to the sequenced *M. tuberculosis* genomes: the region named *M. tuberculosis* specific deletion 1 (TbD1), is a reflection that deletion events relative to *M. tuberculosis* have shaped the *M. bovis* genome. The comparison of the three genomes reflects the high degree of conservation among the members of the *M. tuberculosis* complex, as well as the divergence of *M. bovis* related to *M. tuberculosis* strains.

For specific proteins or genes that vary between *M. bovis* and *M. tuberculosis*, a detailed list can be found in Garnier *et al*. (Garnier 2003). However, it is important to mention that the greatest degree of variation among these bacilli is found in genes encoding cell wall components and secreted proteins. Extensive variations have been found in genes of the PE/PPE family of proteins as well as in genes from the ESAT-6 family, where six of the more than 20 members are absent or altered in *M. bovis*. Some other changes are registered in genes coding for lipid synthesis and secretion as the *mmpL* and *mmpS* family of genes. Deletions responsible for the *M. bovis* requirement of pyruvate as a carbon source were also identified (Garnier 2003).

The analysis of the genome sequence of members of the *M. tuberculosis* complex has led to great advances in the knowledge of the biology and pathogenesis of these bacteria. The sequencing of whole genomes of *Mycobacterium leprae* (Cole 2001), *Mycobacterium avium* subspecies *paratuberculosis* (Li 2005) and of other members of the genus, such as *Mycobacterium smegmatis* and *M. bovis*, has also made huge contributions to the understanding of the lifestyle of mycobacteria. Recently, a report compared the metabolic pathways shared among five of the mycobacterial genomes that have been sequenced (the genome sequence of *M. smegmatis* was not included on this report) (Marri 2006). The characteristics of the sequenced ge-

nomes of organisms in the genus *Mycobacterium* are presented in Table 4-2. The main differences were found in ISs, the PE/PPE gene family, genes involved in lipid metabolism and those encoding hypothetical proteins. The members of the *M. tuberculosis* complex had the highest number of IS elements, which might suggest higher intra-species variability in *M. tuberculosis* compared to other species of mycobacteria.

Table 4-2: Features of sequenced genomes of species belonging to the *Mycobacterium* genus*

Feature	*M. tuberculosis* H37Rv	*M. tuberculosis* CDC1551	*M. bovis* AF2122/97C	*M. leprae*	*M. avium* subsp. *paratuberculosis*	*M. smegmatis*
Genome size (bp)	4,411,529	4,403,836	4,345,492	3,268,203	4,829,781	6,988,209
Protein coding genes	3,927	4,186	3,920	1,604	4,350	6,897
G+C (%)	65.6	65.6	65.6	57.79	69.3	67.40
Protein coding (%)	91.3	~ 91	90.8	49.5	91.5	92.42
Gene density (bp/gene)	1,114	1,052	1,099	2,037	1,112	1,013
Average gene length	1,012	952	995	1,011	1,015	936
tRNAs	45	45	45	45	45	47
rRNA operon	1	1	1	1	1	2

*Data taken from Li 2005, Marri 2006

The comparison of the proteins encoded within the five sequenced genomes revealed a core, or a number of shared proteins, of 1,326 proteins, compared to the 219 core genes described by macroarray and bioinformatic analyses (Marmiesse 2004). Unique genes ranged between 966 (*M. avium* subsp *paratuberculosis*) and 26 (*M. tuberculosis* H37Rv) depending on the genome, and most of these proteins are hypothetical. Regarding the PE/PPE family proteins, it is worth mentioning that *M. tuberculosis* and *M. bovis* contained the highest number of these proteins, while neither *M. leprae* nor *M. avium* subsp *paratuberculosis* have PE_PGRS proteins. Also, a wide variation has been noted in the *mmpL* gene family, known to partici-

pate in lipid transport and secretion. It has been proposed that these variations could be involved in host specificity (Marsh 2005).

4.2.3. Comparing genomes of clinical strains of *M. tuberculosis*

Genome comparison has shown that gene content can vary between strains of *M. tuberculosis*. The analysis of complete genome sequences identified SNPs, LSPs, and regions of difference (RDs) when clinical isolates of *M. tuberculosis* were compared (Fleischmann 2002, Gutacker 2002, Tsolaki 2004, Filliol 2006).

The microarray approach allows the comparison of a large number of genomes, providing information on the diversity, frequency, and phenotypic effects of polymorphisms in the population (Tsolaki 2004). This kind of genomic analysis is also useful for the investigation of outbreaks. Particularly when applied to genomics, DNA microarrays allow the identification of sequences present in the *M. tuberculosis* reference strain, but absent from different clinical isolates. Unfortunately, the microarray technique cannot detect genes present in a clinical isolate that are absent in the reference strain. These changes can originate from small deletions, deletions in homologous repetitive elements, point mutations, genome rearrangements, frame-shift mutations, and multi-copy genes (Ochman 2001, Schoolnik 2002). Fleischeman *et al*. suggested that genetic variation among *M. tuberculosis* strains might denote selective pressure, and therefore might play an important role in bacterial pathogenesis and immunity (Fleischmann 2002). Although associations between host and pathogen populations seems to be highly stable, the evolutionary, epidemiological, and clinical relevance of genomic deletions and genetic variation regions remain ill-defined, as do the molecular bases of virulence and transmissibility (Hirsh 2004).

Up to six *M. tuberculosis* lineages adapted to specific human populations have been described by Gagneux *et al*. using comparative genomics and molecular genotyping tools: the Indo-Oceanic lineage, East-Asian lineage, East-African-Indian lineage, Euro-American lineage, and two West-African lineages (Gagneux 2006, see chapter 2). Specific deletions associated with the hypervirulent Beijing/W strains of *M. tuberculosis* were identified (Tsolaki 2005). Evidently, these differences cannot include sequences present in clinical isolates that are absent from *M. tuberculosis* H37Rv, so they necessarily represent a small part of the total potential genetic variability. Up to 13 complete genome sequences of representative *M. tuberculosis* clinical isolates are currently under progress (http://www.genomesonline.org/gold.cgi). That number accounts for near half of all

the mycobacterial strains that are currently undergoing complete genome sequencing.

All major functional categories are represented among deleted genes in clinical isolates of *M. tuberculosis*. Mobile genetic elements (insertion sequences or prophages) are frequently deleted. DNA loss frequently results from the activity of the insertion sequence IS*6110* (Brosch 1999, Gordon 1999). The rate of deletion in genes involved in intermediary metabolism and respiration, and in cell wall synthesis is surprisingly high. Some of the genes encoding for potential antigens (*plc*A, *plc*D, *lpq*H, *lpp*A, *esx*, or PE/PPE genes) might be deleted under the influence of host selective pressures, which would confer an adaptational advantage during infection or help transmission (Tsolaki 2005). Some of these missing genes (e.g. *esx* genes) encode proteins from the ESAT-6 family (Marmiesse 2004).

The use of microarray-based comparative genomics for the study of the genetic variability of pathogens provides interesting information. Not only the identification of the deleted or absent genes is important, but also the differential hybridization signal between samples is of interest. These differential signals can indicate sequence divergence or a difference in the copy number, which may provide an insight into strain evolution and pathogenesis (Taboada 2005).

4.2.4. Functional genomics

Functional genomics is the analysis of the biological function of the genes and their products within a cell or organism. Unlike genomics and proteomics, functional genomics focus on gene transcription, translation, and protein-protein interactions. Genes operate as long as they are expressed and their expression is regulated at the transcriptional or post-transcriptional level.

Functional genomics uses mRNA expression profiling to provide a picture of the transcriptome in a specific condition or time, in order to identify co-regulated genes that perform common metabolic and biosynthetic functions. A set of co-regulated genes is known as a *regulon*.

Microarrays have additional applications in functional genomics apart from gene expression studies, and other uses have also been reported. Using this new powerful technique, Sassetti *et al*. developed a method to map transposon insertion sites in order to identify essential genes in mycobacteria. The probes were synthesized from a transposon library and then used for hybridization in the array. A number of mutants carrying an insert in each gene were obtained, which were later isolated and identified (Sassetti 2001).

The results of studies on comparative mycobacterial genomics have been validated by functional analysis, involving transcriptomics and proteomics. In fact, gene knock-out followed by transcript analysis and proteome definition seems to be the way to identify essential genes. For example, *M. tuberculosis* genes that encode functions essential for growth are prime choices for further investigation as targets for the development of new drugs or diagnostic methods (Cole 2002b).

Subsequently, research derived from comparative genomic studies was directed towards the study of particular genes. That is the case of the deletion designated as RD750 (corresponding to the *Rv1519* gene) in the genome of the *M. tuberculosis* strain named CH, from a large outbreak that occurred in a community of Indian immigrants in the United Kingdom (Rajakumar 2004) and belonging to the East African-Indian lineage. Complementation and combination of *in vitro* and *in vivo* assay systems indicated the participation of the gene *Rv1519* in the persistence and outbreak potential of this *M. tuberculosis* lineage in human populations (Gagneux 2006, Newton 2006).

Construction and transcription analysis of the appropriate mutant have revealed the functional role of the *Rv3676* gene, a member of the cyclic adenosine monophosphate (cAMP) receptor protein family of transcription factors. This factor is required for virulence of *M. tuberculosis* in the mouse model. The functional map obtained from the transcriptome revealed information about regulatory pathways. The global transcription profiling experiments, comparing the wild type *M. tuberculosis* H37Rv strain and *Rv3676* mutant grown *in vitro,* identified some of the genes that are co-regulated, directly or indirectly, by *Rv3676* in *M. tuberculosis* (Rickman 2005).

4.3. Gene expression in *M. tuberculosis*

4.3.1. Control of gene expression

The ability of *M. tuberculosis* to survive within host cells requires a complex and tightly controlled gene regulation. The genes that are used under different conditions could be readily inferred from the corresponding mRNAs. Thanks to the development of highly specific and sensitive technologies, such as microarrays and quantitative real-time Polymerase Chain Reaction (qRT-PCR), it is now possible to analyze the global expression from both the bacillus and the infected host. Taken together, all this could help us to understand the adaptative machinery of *M. tuberculosis.*

The deciphering of the complete *M. tuberculosis* genome sequence has unveiled its well-equipped machinery, which accounts for its high degree of adaptability. Thirteen putative sigma (σ) factors and 192 regulatory proteins seem to be involved in the control of *M. tuberculosis* gene expression (Cole 1998a). Interchangeable σ factors regulate the function of RNA polymerase, initiating transcription and conferring promoter specificity to the holoenzyme (Kazmierczak 2005, Mooney 2005). To date, consensus promoter sequences have been proposed for six σ factors, besides the housekeeping σ factor, σ^A (for a review, see Rodriguez 2006). Gene expression levels could be further modified by the action of transcriptional activators and repressors: regulatory proteins (Barnard 2004). These regulatory proteins include 11 two-component systems, five unpaired response regulators, seven *wbl* genes, and more than 130 other putative transcriptional regulators (Cole 1998a).

The differential expression of these regulatory gene products throughout different stages of the lifespan of *M. tuberculosis* must be determinant for the pathogen's successful infection and/or persistence within the human host. In recent years, a number of reports have correlated the response of several of these transcriptional regulators to a variety of environmental stresses (for a summary, see Table 4-3 at http://www.tuberculosistextbook.com/pdf/Table 4-3.pdf), such as cold shock, heat shock, hypoxia, iron or zinc starvation, nitric oxide, surface stress and oxidative stress (Manganelli 1999, Raman 2001, Sherman 2001, Shires 2001, Manganelli 2002, Stewart 2002, Park 2003, Rodriguez 2003, Voskuil 2003, Canneva 2005, Geiman 2006). However, the biological signals that stimulate the expression of the majority of them are still poorly recognized. Likewise, the connections between the different regulatory circuits of the complex network that controls gene expression in *M. tuberculosis* are incompletely established. An example of the intricacy of this network is the genetic regulation of *sig*B, which is induced by σ^E in response to surface stress (Manganelli 2001) or by σ^H under heat shock and oxidative stress (Manganelli 2002). The regulation of *sig*B expression seems to be more complex than the above cited, given that σ^F- and σ^L-dependent promoters were identified in the regulatory promoter region of *sig*B; and σ^L-dependent transcription was originated upstream to *sig*B (Dainese 2006). It has been shown that σ^H is also responsible of the induction of *sig*E after heat shock and exposure to diamine (Raman 2001). DNA microarray experiments with *M. tuberculosis* mutants revealed that some σ factors control the expression of their own structural gene (Manganelli 2002, Geiman 2004, Sun 2004, Raman 2004, Dainese 2006). Autoregulation has also been demonstrated for the six two-component systems studied so far in *M. tuberculosis*, which are *sen*X3-*reg*X3 (Himpens 2000), *trc*RS (Haydel 2002), *prr*AB (Ewann 2004), *dos*RS (Bagchi 2005), *mpr*AB (He 2005), and *pho*PR (Gupta

2006). Two-component signal transduction systems are composed of a histidine kinase sensor and a cytoplasmic response regulator that is activated by the cognate histidine kinase (West 2001). One of these systems, *dos*RS, is induced by hypoxia, exposure to ethanol or the nitric oxide donor S-nitrosoglutathione (Sherman 2001, Voskuil 2003, Kendall 2004). This regulon is responsible for the transcriptional changes during oxygen limitation, which is considered an important stimulus for the entry of *M. tuberculosis* into a dormant state (Wayne 2001). For this reason, the genes included under control of *dos*RS are considered members of the *dormancy regulon*. Recently, the induction of *sig*B and *sig*E has been shown to depend on the two-component system MprA/MprB when the bacilli are subjected to surface stress (He 2006).

The transcriptional regulator WhiB3 seems to positively regulate the expression of the housekeeping σ factor named *sig*A, by interacting with the subregion 4.2 of σ factor (Steyn 2002). WhiB3 is encoded by one of the seven *whi*B-like genes described in the *M. tuberculosis* genome (Cole 1998a) and belongs to the *wbl* family of genes, which encodes putative transcription factors, which are unique to actinomycetes (Molle 2000, Soliveri 2000). A recent analysis has demonstrated that the expression of *M. tuberculosis whi*B-like genes is modified in response to antimycobacterial agents and environmental stress conditions (Geiman 2006). Additionally, *whi*B1 transcription is regulated by cAMP levels via direct binding of the activated form of the product of *Rv3676* (CRP protein-cAMP) to a consensus site adjacent to the *whi*B1 promoter (Agarwal 2006).

A post-translational regulation has also been reported for several σ factors. Antagonist proteins, known as anti-σ factors, can negatively regulate some σ factors by sequestering them and preventing their association with RNA polymerase. Many of these anti-σ factors are located downstream of their cognate σ factor-encoding gene and both genes are usually co-transcribed (Bashyam 2004). The functions of five specific anti-σ factors of *M. tuberculosis* have so far been examined: RseA (Rodrigue 2006); RshA (Song 2003); RslA (Hahn 2005, Dainese 2006); RsbW or UsfX (Beaucher 2002); and RskA (Saïd-Salim 2006). Interestingly, RsbW, the σF-specific antagonist, is post-translationally regulated by two anti-anti-σ factors: RsfA and RskB (Beaucher 2002, Parida 2005).

Although the function of many of these mycobacterial transcriptional regulators and signal transduction systems remains poorly defined, recent studies have begun to provide evidence of the biological role of these regulatory circuits throughout each stage of the lifecycle of *M. tuberculosis* inside the human host. The expression of *sig*A, *sig*E and *sig*G (Manganelli 2001, Capelli 2006, Volpe 2006), that of some

two-component systems (Ewann 2002, Haydel 2004, Walters 2006), as well as that of the transcriptional regulator *whi*B3 are induced during macrophage infection. The role of these transcriptional regulators in pathogenesis and virulence became even more evident in animal model experiments, where disruption or deletion of these genes was shown to affect *M. tuberculosis* virulence in mice (Parish 2003a, Parish 2003b, Sun 2004, Manganelli 2004, Raman 2004, Hahn 2005, Walters 2006).

Studies on mutagenesis and the expression profile of several regulators during the growth of *M. tuberculosis* in the macrophages and in organs of experimental animal models are currently underway. These regulators can modify bacterial physiology and are able to modulate host-pathogen interactions in response to environmental signals.

4.3.2. *In vitro* gene expression

M. tuberculosis is an obligate mammalian pathogen that is able to infect many different cells, including macrophages, dendritic cells, alveolar-epithelial cells, and neutrophils. It is also able to reside extracellularly in the lung, inside granulomas. As mentioned previously, the tubercle bacillus adapts its transcriptome to the environment in which it replicates. The adaptation of a bacterium to harsh environments involves the transcriptional activation of genes whose final products help the bacterium to reprogram its physiology, thus ensuring survival. Among the genetic determinants that the bacterium must modulate are those involved in intermediary and secondary metabolism, cell wall processes, stress responses and signal transduction pathways.

By utilizing the microarray technology, quantitative RT-PCR and laboratory generated mutants, studies on *M. tuberculosis* global gene expression have been undertaken using broth cultures, cell cultures, and animal models. None of these models reproduce several key features of TB in the human infection; and, unfortunately, no data is available from human tissues.

Table 4-4 summarizes the most important genes whose expression is modulated by the transcriptional regulators mentioned previously (see section 4.3.1).

Table 4-4: Regulation of cell process genes

Transcriptional regulation	Condition	Cell process genes	
		Up regulated	Down regulated
σC	Growth curve	Two-component systems: *sen*X3, *mtr*A, *hsp*X (α-crystallin), *fbp*C (antigen 85C)	
σD	Exponential growth	r*pf*C (resuscitation factor), *Rv1815*, *Rv3413c*	PE_PGRS family genes
σH	Diamine exposure, heat stress[b]	Heat shock proteins: *hsp*, *clp*, *trx*B2C operon, transcriptional regulators: *sig*E, *sig*B	
σM	Log-phase growth	Esx family genes, PPE1, PPE19	PPE60, *kas*A-*kas*B, *fas*, *pks*2, *pks*3
σE	Exponential growth, SDS exposure	Icl1 (isocitrate lyase), heat-shock proteins, transcriptional regulators: *sig*B, *mpr*AB	
σL		*sig*L-*rsl*A, *pks*10-*pks*7, *mpt*53-*Rv2877c*, *Rv1139c-Rv1138c*	
σF	Stationary-phase growth	HP and CHP family of proteins, transcriptional regulators: *sig*C, *sig*F, and MarA, GntR TetR family, cell envelope: *mur*B	
DosS-DosR	Standing cultures	*hsp*X (α-crystallin), *Rv3130c* (CHP), *Rv1738* (CHP), *Rv0572c* (CHP)	
PhoPR	Exponential growth	Cell envelope components	
MprA	Mid-exponential phase	~95 genes of *M. tuberculosis.* PE/PPE gene family, HP, CHP	

CHP = conserved hypothetical protein

For example, analysis of a mutant of *M. tuberculosis sig*C showed that this σ factor induces the expression of some virulence-associated genes. On the contrary, the genes *hsp*X (encoding the α-crystalline homologue), *sen*X3 (sensor kinase), *mtr*A (response regulator), and *fbp*C (mycolyl transferase and fibronectine binding protein or antigen 85C) were down-regulated in that mutant strain at different times of the growth curve (Sun 2004). Genes induced by σ^D include the resuscitation promoting factor *rfp*C, several chaperone genes and genes involved in lipid metabolism and cell wall processes (Raman 2004). Thirty-nine genes were shown to be under the control of σ^H. These include genes coding for some heat shock proteins

(*hsp* and *clp*), the *trx*B2C operon and some transcriptional regulators (Manganelli 2002). Quantification of mRNA by primer extension under different stresses demonstrated that the transcription of *trx*B2, *dna*K, *clp* and *sig*E could be induced from σ^H-dependent promoters located upstream of these genes (Raman 2001). σ^E seems to regulate the expression of proteins involved in fatty acid degradation, such as the isocitrate lyase (coded by *icl*1), two proteins related to fatty acid degradation (*fad*E23 and *fad*E24), heat shock proteins, and the transcriptional regulators *sig*B and *mpr*AB (Manganelli 2001). Recently, it was reported that σ^M induces the expression of two pairs of secreted proteins of the Esx family and two PPE genes, while it negatively regulates PPE60 expression as well as the expression of several genes involved in surface lipid biosynthesis and transport (Raman 2006).

At least four small operons appear to be directly regulated by σ^L: *sig*L-*rsl*A, *pks*10-*pks*7, *mpt*53-*Rv2877c*, and *Rv1139c-Rv1138c*, which clearly have a σ^L-consensus promoter sequence in their regulatory region (Hahn 2005, Dainese 2006). The *pks* genes are involved in the biosynthesis of phthiocerol dimycocerosate, a component of the cell envelope associated with virulence (Sirakova 2003); and the *mpt* operon contains genes involved in fatty acid transport (Sonden 2005). DNA microarrays of a mutant of *M. tuberculosis* lacking a functional *sig*F, have revealed that this σ factor is able to induce gene expression almost exclusively during the stationary phase of growth, supporting the hypothesis of a major role of σ^F in the adaptation to the stationary phase. Among the σ^F-targeted genes, 50 % coded for hypothetical proteins or proteins of unknown function, some were transcriptional repressor/activators (MarR, GntR and TetR family of DNA binding regulators) and others were found to be involved in the biosynthesis and structure of the cell envelope (Geiman 2004).

A complete genomic microarray analysis has also been performed on *M. tuberculosis* strains mutated in two-component regulatory systems. The dormancy-related two-component system *dos*RS was inactivated in *M. tuberculosis* using different methodologies (Parish 2003b, Park 2003). It was shown that the expression of this two-component system is highly induced under hypoxia (Sherman 2001b, Park 2003). A consensus *dos*R-specific binding motif was reported to be located upstream of hypoxic response genes (Park 2003, Kendall 2004). The microarray expression profiles of mutants in each of the components (*dos*R and *dos*S) showed that DosR is required for the expression of genes usually induced under oxygen limitation, such as *hsp*X gene. Several putative operons with unknown function were also strongly regulated by DosRS.

Up to 30 genes were found to be up-regulated, and another 68 genes down-regulated in a mutant of *M. tuberculosis senX3-regX3*. However, it has not been clearly determined if the changes found in gene expression were directly or indirectly related to the lack of this two-component regulatory system (Parish 2003a). Recently, the global transcriptional profile of the two-component systems PhoP and MprA has been reported. One of these studies provided evidence that the PhoP/PhoR system is a positive transcriptional regulator of genes involved in the synthesis of the cell envelope of *M. tuberculosis* (Walters 2006). On the other hand, MprA regulates *sig*B and *sig*E and many other genes previously reported to be associated to various stress conditions (He 2006).

In order to analyze the mechanisms involved in bacilli intracellular survival, mycobacterial gene expression was determined in *M. tuberculosis* infected macrophages from different sources. Macrophages play a crucial role in TB infection because they represent both the effector cells for bacterial killing and the primary habitat in which the persisting bacilli reside. Macrophages have been investigated at different time points post-infection for the differential expression of various two-component system regulators (*reg*X3, *pho*P, *prr*A, *mpr*A *kdp*E, *tcr*, *dev*R and *tcr*X) (Haydel 2004). More recently, the gene expression profile of *M. tuberculosis* grown in human macrophages compared to that of bacteria growing in synthetic culture medium was published (Capelli 2006). In this work, the authors reported that approximately one-third (32 %) of the genes upregulated by *M. tuberculosis* in macrophages correspond to conserved hypothetical proteins, with unknown function; this finding highlights the considerable gap that still remains in the knowledge of how this bacterium survives intracellularly. Genes involved in cell wall processes (19.5 %), regulation and information pathways (16 %), and PE family proteins (3.6 %) were also upregulated. Interestingly, the authors observed high induction of the sigma factor *sig*G and 13 other putative transcriptional regulators. Upregulation of *sig*A, *sig*E, and *sig*G was also reported in a similar study (Volpe 2006). The *whi*B3 gene was also induced in *M. tuberculosis* during infection of naïve bone marrow-derived macrophages in comparison to bacteria in broth culture mid-log growth (Banaiee 2006).

4.3.3. *In vivo* gene expression

The use of microarrays for profiling transcriptomes of bacteria inside the host cell has been limited by the paucity of bacterial mRNA in samples containing a preponderance of mammalian RNA. Therefore, while significant work has been per-

formed on the gene expression profile of the host, information on *M. tuberculosis* expression inside infected hosts is still limited.

So far, there is only one publication concerning global mycobacterial transcription expression in the animal model, using microarray as the analytical method (Talaat 2004). Differential expression levels of *M. tuberculosis* during infection in Balb/c or Severe Combined Immunodeficiency (SCID) mice were evaluated and compared to the levels found in mycobacteria grown in broth culture. These authors identified up to 40 genes whose expression significantly changed during Balb/c and SCID mouse infection. These genes include *rub*B, *din*F, and *fdx*A. The same genes were also found to be induced 24 hours post-infection in murine bone marrow macrophages (Schnappinger 2003). Additionally, several genes were regulated up or down only in Balb/c mice, such as *pro*Z (transport system permease protein), *aceA*a (probable isocitrate lyase involved in lipid metabolism), and genes encoding for regulatory proteins, such as *sig*K, *sig*E and *kdp*E. The authors concluded that the expression profile of *M. tuberculosis* in SCID mice resembles the profile found in bacilli grown *in vitro*, while the expression profile in Balb/c mice resembles that reported in multiplication within the macrophages (Schnappinger 2003). Exceptionally, some genes were found to be expressed only in Balb/c mice.

A small number of studies applied quantitative RT-PCR to investigate the expression of mycobacterial genes in the animal model. These studies focused on the analysis of a few particular genes. Examination of lungs of infected C57BL/6 mice showed that the transcriptional regulator genes *whi*B3, *fdx*A (electron transfer), *hsp*X (α-crystalline), *acg* (unknown function), *Rv1738* (unknown function), and *Rv2626c* (unknown function) were markedly induced during the course of infection (Banaiee 2006). A gene required for extrapulmonary dissemination (*hbh*A) was also upregulated in the lung but not in the spleen during the early stages of infection (Delogu 2006). While the expression of PE_PGRS16 was up-regulated in the spleens and lungs of infected mice, the expression of PE_PGRS26 was down-regulated (Dheenadhayalan 2006). A study on human lung biopsies revealed a high variability in expression profiles of specific *M. tuberculosis* genes among the specimens analyzed (Timm 2003). The biopsies were obtained from four HIV-negative patients with chronic active TB that was unresponsive to therapy. Although some differences were observed when comparing human and murine lung, the authors admitted that it was difficult to ascertain whether the infection stage in the analyzed human lung specimens could be correlated with the persistent infection in mice.

4.4. *M. tuberculosis* proteome

With the availability of the genomes of *M. tuberculosis* H37Rv*, M. tuberculosis CDC1551*, *M. bovis*, and the ongoing sequencing projects, attention in the coming years must be focused on the interpretation of the sequences determining the structure and function of the proteins. Proteomics, the global study of proteins that are translated in a given physiological state is one of the most important and ambitious goals in *M. tuberculosis* research. The proteome of an organism implies not only an inventory of its gene products but also the transduction rate and the post-transcriptional events that occur in the organism (Betts 2002). Classical studies of proteomics involve two dimensional electrophoresis (2-DE), in which proteins are first separated by the isoelectric point and then by the molecular weight (O'Farrel 1975). Every spot of protein is then isolated, hydrolyzed and subjected to techniques of mass spectrometry (MS), tandem MS (MS/MS); matrix-assisted laser desorption/ionization mass spectrometry (MALDI/MS) and, matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF/MS). For a good review on the different techniques used in protein mapping, readers are referred to Patterson *et al* (2000). Techniques different from two dimensional electrophoresis have also been implemented. For instance, the use of one dimension electrophoresis has been shown to be very useful for the separation of hydrophobic proteins (Simpson 2000). Other approaches that do not involve the use of gels, such as two-dimensional liquid chromatography (LC) and the subsequent analysis by MS (2 DLC/MS), have been shown to be very efficient in the identification of hydrophobic and membrane proteins (Isobe 1991). In 1999, the isotope-coded affinity tag (ICAT) technology was reported (Gygi 1999). In this, mixtures of proteins from bacteria in two different conditions are covalently labeled with isotopically labeled heavy or light forms of the reagents. The samples are combined and subjected to proteolysis. After purification of the labeled peptides through affinity tag, which is part of the reagents, they are analyzed by LC-MS/MS. This new technology has proven to be very useful in the quantitation of complex mixtures of proteins.

Before the disclosure of the *M. tuberculosis* genome, antigens and proteins were identified by one- and two-dimension polyacrilamyde gel electrophoresis, and the use of cumbersome immunological methods (Nagai 1991, Garbe 1996). With the advance in high-resolution 2-DE and analytical chemistry, *M. tuberculosis* proteome is at present a reality. Pioneering studies in the proteomic field included the mapping of 32 N-terminal sequences by MS and the identification of culture filtrate proteins by 2-DE and immunodetection (Sonnenberg 1997).

Very soon after the publication of its genome, bioinformatic tools were applied to predict the proteomic profile of *M. tuberculosis* (Tekaia 1999). The in silico analysis showed characteristics of the tubercle bacillus as the duplication of numerous genes, especially those involved in gene regulation and in lipid metabolism, and those coding for the PE/PPE protein family. The study also showed a reduced repertoire of proteins devoted to transport, which might reflect the intracellular lifestyle. The bioinformatics-predicted proteome was compared to 2-DE protein maps obtained from *M. tuberculosis* whole cell lysates, which were separated in a broad pH range between 2.3 and 11.0. This work demonstrated that proteins with a molecular mass below 10 kDa were not predicted from the genome sequence and also that experimentally basic and high molecular mass proteins could not be resolved by 2-DE (Urquhart 1998). Since 1999, the huge amount of data in proteomics has led to the creation of 2-DE databases, where images generated in different laboratories can be stored and analyzed. These databases are accessible on the internet at: http://web.mpiib-berlin.mpg.de/cgi-bin/pdbs/2d-page/extern/overview.cgi?gel=16 (Mollenkopf 1999) and http://www.ssi.dk/sw14644.asp (Rosenkrands 2000b).

4.4.1. Structural proteomics of *M. tuberculosis*

Thanks to recent technological advances, the subcellular protein profile of *M. tuberculosis* can now be drawn. The global analysis of compartmentalized proteins will shed light on host-pathogen interactions, metabolic pathways and cell communication, just to mention some of the mechanisms related to pathogenesis. In addition, many pathogenic bacteria secrete proteins that are involved in virulence (Finlay 1997) and thus culture filtrates of *M. tuberculosis* could be a source for identification of virulence factors. Cell wall proteins play a fundamental role in cell architecture, resistance of the pathogen to chemical injury and dehydratation, and many other key functions of this microorganism. Thus, the identification of proteins localized in this subcellular fraction may lead, in the near future, to the development of new diagnostic tests and drugs. Membrane proteins demand special attention, because they are involved in host-pathogen interactions, nutrient transport, quorum sensing mechanisms, etc. Knowledge of these proteins could be the clue to the development of novel vaccines. Finally, the identification of cytosol proteins and the intricate network of their interaction will reveal metabolic pathways that can be targets for the design of rational drugs against TB. Even though we are still far from identifying the almost 4,000 genes predicted by genomics, the number of identified proteins increases each year and shows how genomic and proteomic technologies complement each other.

The biochemical methods developed for the separation of the cell wall, membrane and cytosol fractions have facilitated proteomic studies in *M. tuberculosis* (Hirschfield 1990, Lee 1992). Jungblut *et al*. identified 53 proteins from cell lysates and 54 from culture filtrates using 2-DE and MALDI/MS (Jungblut 1999). These authors performed comparative proteomics in *M. tuberculosis*, which will be discussed later in this chapter.

Reference maps of cellular fractions and culture filtrate proteins were constructed using 2-DE, N-terminal sequencing and antibodies against previously identified antigens (Rosenkrands 2000b). As many as 1,184 spot proteins were visualized after silver staining. Only 10 % of them were identified. In order to map less abundant proteins, different methods were applied for their separation, which allowed the identification of 12 novel proteins, five of them with a known function (Rosenkrands 2000b). The study also showed the identification of a protein that was not predicted by genomics and revealed the presence of alternative start codons.

The implementation of immobilized pH gradient for 2-DE and MALDI/MS allowed the identification of 288 proteins (Rosenkrands 2000a). Six proteins were identified, all of them with molecular masses between 13,200 and 7,200 kDa and with isoelectric point (pI) ranges between 4.5 and 5.9. Five of these proteins were correctly identified in the genome of the clinical strain CDC1551 (Jungblut 2001).

In spite of the enormous usefulness of 2-DE in proteomic studies, there are certain disadvantages inherent to its technique, such as the low resolution of proteins with very high or very low molecular masses, or proteins that are very acidic, very basic or hydrophobic. But in particular, the technique is biased towards the preferential identification of the most abundant proteins. Therefore, less abundant proteins, such as transcriptional regulators, are rarely detected when whole cell lysates are analyzed (Gygi 1999). To overcome these inconveniences, alternative techniques have been applied in proteomic studies. For example ICAT reagent method and LC-MS/MS were used as a complement of 2-DE-MS/MS. Using these approaches, 388 *M. tuberculosis* proteins were quantified and identified. Each one of these techniques has been shown to be adequate for the identification of certain classes of proteins. For example, the ICAT method performed better for the identification of cell membrane and high molecular mass proteins, while 2-DE showed better results in the identification of low molecular mass and cysteine-free proteins (Schmidt 2004). Interestingly, none of these techniques allowed the identification of proteins in the following subclasses: cell division, IS elements, repeated sequences, phages, PE/PPE families, cytochrome P450 enzymes, cyclases, and chelatases.

By 2004, only about 400 proteins had been identified in the proteome of *M. tuberculosis*, probably due to the limitations of 2-DE-based separation methods. The use of automated two-dimensional, capillary high-performance liquid chromatography (HPLC) coupled with MS gave a wider and more accurate proteomic profile of *M. tuberculosis* (Mawuenyega 2005). Proteins of the cell wall, membrane and cytosol subcellular fractions could be identified. The number of identified proteins increased to 1,044 non-redundant proteins, 67 % more than those obtained by conventional 2-DE. This study identified proteins in extreme pI ranges, among them the most acidic proteins (PE_PGRS, *Rv3512*) with a pI of 3.89 and the most basic proteins (*rps2*, a 30S ribosomal protein) with a pI of 12.18. Proteins of high molecular mass, such as the 230,621 Da polyketide synthase *ppsC,* were also identified. A total of 705 proteins were identified in the membrane, 306 were localized in the cell wall, and 356 in the cytosol fraction. Forty-seven were present in all analyzed fractions. The study also included a computational analysis of protein networks, one of the most exciting fields in the coming years. Readers are invited to consult the supplementary table of this work (Mawuenyega 2005).

M. tuberculosis is an intracellular pathogen, the bacillus is engulfed by alveolar macrophages where it can survive and grow by altering the intracellular compartments to preclude the normal maturation to phagolysosomes or to prevent fusion of phagosomes to lysosomes (Clark-Curtiss 2003). The interaction between host and pathogen is thought to be mediated by membrane proteins. Therefore, the characterization of membrane proteins is a topic of intensive research. As mentioned before, most of the studies regarding *M. tuberculosis* proteomics have been carried out by 2-DE. However, the number of membrane and membrane-associated proteins has been underestimated by the 2-DE technology due to the hydrophobic nature of this class of proteins and their low solubility. In order to overcome these problems, fractions of cellular membranes were prepared by differential centrifugation and separated by one-dimensional electrophoresis. The separated bands were then excised and hydrolyzed prior to LC and MS/MS (Gu 2003). This approach allowed the identification of up to 739 membrane and membrane-associated proteins. Very hydrophobic proteins, including those with 15 transmembrane helices, were detected in this study. The use of alternative solubilizing agents, such as Triton X-114, has proven to be a good choice for membrane fractionation. The detergent was shown to be useful in the identification of nine novel proteins that have been already incorporated in the *M. tuberculosis* proteome (Sinha 2005). Interestingly, when analyzing the interferon-gamma (IFN-γ) response of BCG-vaccinated healthy individuals from an endemic area to these newly identified proteins, the strongest response was found to be that against ribosomal proteins. Other mem-

brane associated proteins, such as ESAT-6, did not contribute significantly to the T-cell response in these individuals.

4.4.2. Comparative proteomics

The comparative proteomic analysis using 2-DE and MALDI/MS was applied to compare proteins present in two virulent laboratory *M. tuberculosis* strains (H37Rv and Erdman strains) with those present in two *M. bovis* BCG strains (Chicago and Copenhagen BCG strains) (Jungblut 1999). The results showed that, as expected, the two *M. tuberculosis* strains differed from each other in only a few proteins. Of the 18 variant proteins, 16 were identified. L-alanine dehydrogenase (*Rv2780*) was not detected in the Erdman strain, and the protease IV was absent in this strain. On the other hand, the Soj protein and the hypothetical protein *Rv2641* were absent from the *M. tuberculosis* H37Rv proteome. Some of the 18 proteins were overexpressed in one or the other strain, and some shifted their mobility probably due to the presence of amino acid substitutions. The comparison of *M. tuberculosis* H37Rv with *M. bovis* BCG revealed the presence of 13 protein spots exclusive to the tubercle bacilli, six of which were identified. The differential proteins comprised L-alanine dehydrogenase (40 kDa protein), isopropyl malate synthase nicotinate-nucleotide pyrophosphatase (*Rv1596*), MPT64 (*Rv1980c*), and two hypothetical conserved proteins (*Rv2449*c and *Rv0036c*). On the other hand, *M. tuberculosis* H37Rv lacked eight spots compared to *M. bovis* BCG.

In another study using 2-DE and MS, a comparison of the proteins present in *M. tuberculosis* and *M. bovis* BCG revealed the presence of 56 unique protein spots in *M. tuberculosis* and 40 in the attenuated strain BCG (Mattow, 2001). Of these, 32 were identified as exclusive proteins of *M. tuberculosis,* of which 12 had been previously reported to be deleted in *M. bovis* BCG. The remaining 20 spots were newly identified as absent from *M. bovis* BCG.

A third comparative proteomic study of *M. tuberculosis* and *M. bovis* BCG was performed using 2-DE and ICAT technology (Schmidt 2004). This work demonstrated the presence of only three exclusive proteins in *M. tuberculosis* H37Rv. One is *Rv0223c*, a protein belonging to the aldehyde dehydrogenase family. The second is *Rv0570*, a ribonucleotide reductase class II. The third is a hypothetical protein named *Rv1513*.

The studies on comparative proteomics allowed the identification of isopropyl malate synthase exclusively in the *M. tuberculosis* proteome. Recently, this enzyme was included in a new class of virulence factors known as ‘anchorless adhesins’

(Kinhicard 2006) that were absent from the avirulent BCG, thus proving the usefulness of this methodology.

Of special interest in the coming years will be the proteomic comparison between circulating *M. tuberculosis* strains differing in virulence, transmissibility, tissue tropism, and/or ability to acquire drug resistance. As a matter of fact, the proteomic profile of *M. tuberculosis* H37Rv has already been compared with that of the clinical strain CDC1551 at different time points during *in vitro* growth (Betts 2000). Subscribing the low structural DNA polymorphism observed in *M. tuberculosis* (Sreevatsan 1997), the resulting patterns of the protein-spot were found to be both highly reproducible and highly similar between the two strains during growth. One unique protein was identified in *M. tuberculosis* CDC1551, namely *Rv0927c*, a probable alcohol dehydrogenase. Similarly, a spot corresponding to the HisA protein, which is involved in the histidine biosynthetic pathway, was detected in the *M. tuberculosis* H37Rv proteome but was absent from the *M. tuberculosis* CDC1551 protein profile. Oddly enough, both genes were found to be present in both *M. tuberculosis* strains. Thus, the described proteomic differences between H37Rv and CD1551 might be ascribed to post-translational events or to degradation during the manipulation of the specimens. Another interesting feature in the same study was the mobility variations of the transcriptional regulator MoxR, which the authors attributed either to amino acid changes or to post-translational modifications. A BlastP analysis of both genomes showed a single amino acid substitution of histidine in *M. tuberculosis* H37Rv to asparagine in *M. tuberculosis* CDC1551 that might explain the variation in mobility.

Transcriptional regulation differences between strains might be the key to understanding how virulence factors are involved in a variety of roles, including host-cell invasion, survival within the host cell, and long-term persistence. Therefore, comparative proteomic studies are of special interest in the post genomic era, helping to understand the manifestation of disease produced by different strains involved in the current TB epidemic.

4.4.3. Environmental proteomics

The information obtained by genomic studies is static, because DNA is not essentially affected by the environment. In contrast, the proteomic profile of an organism in a particular physiological situation complements and helps to decipher its interaction with the environment. The study of the *M. tuberculosis* proteome in different physiological states is one of the most fascinating fields of research. Being an intracellular pathogen, the bacillus is challenged by a variety of environmental

changes. Inside the mammalian macrophage, the microorganism is subjected to a series of different weapons. Inside the granuloma, it faces low oxygen tension, starvation, low pH, reactive nitrogen, and reactive oxygen species, among other offenses (Schnappinger, 2006). The bacillus has developed adaptive mechanisms for survival and persistence in these hostile environments. The identification of proteins expressed under such conditions is a matter of demanding research.

M. tuberculosis has another outstanding characteristic: it is able to persist for years in its host causing latent infection, in a state known as dormancy. The mechanisms governing this state are still not fully understood and the protein expression profile in models mimicking the dormant state is an issue of intense research. Different *in vitro* models have been developed, aimed at simulating the *in vivo* conditions inducing dormancy (Wayne 1996, Betts 2002, Voskuil 2003). In the Wayne's model, which has been applied in proteomic studies, the level of oxygen is gradually depleted due to bacterial growth, defining two non-replicating stages: a microaerophilic stage NRP-1 (non-replicating persistance-1), followed by an anaerobic stage NRP-2. Still, the evidence linking human *M. tuberculosis* latent infection with *M. tuberculosis* dormant stages attained *in vitro* remains merely circumstantial.

Hypoxia is among the most conspicuous conditions encountered by the tubercle bacilli in the central part of the granuloma, where bacilli are considered to remain dormant. The hypoxic response of *M. tuberculosis* in the Wayne's model was investigated by performing a proteomic study of cell lysates and culture filtrates (Rosendkrands, 2002). The comparison of the protein content between aerobic and anaerobic cultures identified up to seven proteins that were more abundant in hypoxic conditions. The main proteins characterized were fructose biphosphate aldolase (in culture filtrate only), hypothetical protein *Rv0569*, and alpha-crystallin protein, also known as HspX. Other proteins identified included hypothetical proteins *Rv2623* and *Rv2626c*, L-alanine dehydrogenase (only in culture filtrates), and BfrB, a bacterioferritin involved in iron uptake and storage.

Using a modified Wayne's model, Stark *et al*. (Stark 2004) visualized 13 unique and 37 more abundant spots under hypoxia, revealed by 2-DE and MALDI-TOF/MS. Of these 50 spots, 16 proteins were identified, including some that had not been previously detected such as GroEL2, KasB, Ef-Tu, ScoB, TrxB2, and CmaA2. Among the hypothetical proteins found were *Rv2005c*, with similarity to universal stress proteins, *Rv0560c*, *Rv2185c*, and *Rv3866*.

Applying the ICAT technology to the comparison of the physiological NRP-1/NRP-2 states *versus* active growth (logarithmic phase), the number of newly identified proteins increased up to 875 (Cho 2006). A total of 586 proteins were

identified and quantified in the microaerobic stage of nonreplicating persistence stage (NRP-1), 628 others were detected in the anaerobic dormancy state (NRP-2), and 339 were common to both non-replicating persistence stages. Proteomic comparison between the NPR-1 and the logarithmic phase of growth showed that 6.5 % of the proteins were up-regulated in NRP-1, while 20.4 % of the proteins were up-regulated in NRP-2. The analysis of the proteomic profile showed that the NRP-1 state displayed a significant increase in proteins involved in small molecule degradation and the NRP-2 state a significant increase in energy metabolism, altogether suggesting an adaptive mechanism of *M. tuberculosis* to enter into the anaerobic environment.

M. tuberculosis pathogenicity is directly associated with its ability to establish invasion and division inside the host's macrophages, despite the antimicrobial properties of these cells. The study of the *M. tuberculosis* proteome in this physiological environment is a crucial step towards understanding the mechanisms involved in its pathogenicity. In spite of the enormous advances in biochemical analytical techniques, the purification and identification of proteins is not always an easy task. In a recent paper, Mattow *et al*. (Mattow 2006) applied subcellular fractionation of infected murine bone marrow-derived macrophages in combination with high-resolution 2-DE and MS/MS to analyze the proteome of *M. tuberculosis* inside the phagosome. The proteome was compared with that derived from broth cultures. Using this approach, 121 unique spots were detected in intra-phagosomal mycobacteria. Only 11 of them were identified as *M. tuberculosis* proteins, the remaining 110 were identified as murine proteins. The 11 identified proteins were: *Rv1240* (Malate dehydrogenase, MdH), *Rv1077* (Cystathione (beta)-synthase, CysM2), *Rv3396c* (GMP synthase, GuaA), *Rv0489* (Phosphoglycerate mutase I, Gpm), *Rv2773c* (Dihydrodipicolinate reductase, DapB), *Rv0009* (Peptidyl-prolyl cis-trans isomerase, PpiA), *Rv1627c* (lipid carrier protein), *Rv2961* (putative potassium uptake protein, TrkA), and hypothetical protein *Rv1130*, which was detected in two separates spots, *Rv0428c*, and *Rv1191*. Some of these proteins (CysM2, DapB, GuaA, MdH and PpiA) had previously been detected using 2-DE patterns of whole cell lysates and/or culture supernatants of *M. tuberculosis* H37Rv, indicating that they are not exclusive of the phagosomal millieu.

Proteomic studies seem to be a successful way to discover new virulence factors, drug target molecules and proteins involved in pathogenic mechanisms. Thousands of proteins have now been identified and many more await identification.

4.5. An insight into *M. tuberculosis* metabolomics

4.5.1. Metabolomics state-of-the-art

The term **metabolomics** was first coined in 1998 (Oliver 1998) to describe the "*change in the relative concentrations of metabolites as the result of deletion or over-expression of a gene*". At the same time, the term **metabolome analysis** referred to the analysis of metabolites in the phenotypic profile of *E. coli* (Tweeddale 1998). Later on, metabolomics was considered the detection and measurement, under defined conditions, of cellular metabolites such as low molecular weight molecules present in an organism or biological sample. The field also includes information on the level of metabolite activities in the cell. Metabolites are in general defined as those small molecules, usually intermediate and final products of metabolism, but the definition also applies to high molecular weight molecules such as lipids, peptides and carbohydrates (sometimes referred to as "lipidomics", etc).

Metabolomic approaches are now feasible due to the rapid improvements that have taken place during the last decade in two areas: analytical chemistry and bioinformatics. Metabolomic methodologies include the combination of classical technologies, such as gas chromatography-mass spectrometry (GC-MS), or nuclear magnetic resonance (NMR), with new developments to achieve improved sensitivity and discriminative power. Sophisticated informatic analysis and data mining are an important part of the methodology. The complete analysis involves *in silico* models on metabolite-protein interactions. This analysis can be qualitative or quantitative. In the latter case, all the conditions required for an accurate quantification should be considered, such as the use of appropriate data standards, etc (Nielsen 2005). Metabolomics can also help to validate *in silico* pathways prepared on the basis of available genome sequences and established databases (Park 2005).

Metabolomic analysis has mainly been used in studies on plants and human pathology; in this latter case, the attention was focused on searching for metabolites associated with disease, in other words, "*metabolites as biomarkers of disease*" (Weckwerth 2005). Microbial metabolomics has initially been devoted to explore bacterial or fungal strains carrying improved phenotypes with a certain biotechnology usefulness value (Wang 2006). Metabolomic approaches have also been directed to the development of new drugs addressed against novel microbial targets. A review on the basics and applications of microbial metabolomics can be read in van der Werf *et al*. (Werf 2005).

4.5.2. Has the metabolomic analysis of tuberculosis actually started?

From the beginning, a unique property of mycobacterial cells called the attention of scientists: the remarkably high lipid content of the cell envelope, which accounts for the most conspicuous mycobacterial features, including physiology and pathogenicity (Asselineau 1998, Barry 2001) (see Chapter 3). Many studies have been published on identification, characterization, and even practical applications (e.g. diagnostics) of several mycobacterial lipids. Almost all books on TB or mycobacteria have at least one chapter dedicated to lipids. Older books refer in more depth to the structural and chemical characterization of the envelope, as well as to the biosynthesis of lipids (Ratledge 1982, Kubica 1984); more recent books lay stress on the genetics and genes related to lipid metabolism (Cole 2005). Thus, the analysis of the lipid metabolic profiling cannot be regarded as a new field in mycobacteria, at least when considering all lipids as metabolites (Ortalo-Magne 1996). The main objection to doing so is the size of mycobacterial lipids. Indeed, mycobacterial lipids are rather big and complex. Most of them have a fatty acid backbone covalently linked to other kind of molecules, most frequently several types of saccharides (Asselineau 1998). Many lipids also belong to the molecular structure of bacterial lipoproteins (Sutclife 2004).

A detailed revision of mycobacterial lipids has been published relatively recently (Kremer 2005). The most representative lipids in mycobacteria are the mycolic acids. These molecules are larger in mycobacteria, compared to those of other related bacteria, such as *Corynebacterium* or *Nocardia.* Mycolic acids are the lipid component in the structure of complex glycolipids, including Mycolyl-ArabinoGalactan (mAG) and Trehalose-6-6'-Dimycolate (TDM). Another important group of lipids also contains trehalose as the glycosyl radical molecules and their fatty acids chains are multi-methylated. This group includes Di- Tri- and Pentaacyl Trehalose (DAT, TAT and PAT) and Sulfolipids (SL); the Phthiocerol Dimycocerosates (PDIMs) are also very important. These compounds and the closely related Phenolic Glycolipids (PGL) participate in the integrity of the cell envelope of *M. tuberculosis*. A last group of glycolipids contain D-mannan and D-arabinan in their molecules and have been considered of relevance in bacterial pathogenicity for a long time: Lipomannans (LM) and Lipoarabinomannans (LAM) (see Chapter 3).

Many lipids are unique to mycobacteria and therefore their metabolic analysis cannot be addressed by comparative lipidomic studies with other bacteria. Such specific metabolic pathways are viewed, in turn, as excellent targets for the design of new specific drugs (Draper 2000). Renewed efforts have been applied to the detection of metabolic routes and genes that participate in the biosynthesis and

degradation of complex lipids (Brennan 2003, Reed 2004, Portevin 2004, Veyron-Churlet 2004, Trivedi 2005, Kaur 2006), as well as genes involved in lipid transportation (Domenech 2004, Jain 2005). A review has recently been published on the biosynthesis, regulation and transport of long-chain multiple methyl-branched fatty acids, such as PDIM, PGL, and SL (Jackson 2006). This paper updates the knowledge on these complex topics, indicating that mycobacterial lipids share mechanisms in their metabolic routes, and that changes in a pathway could influence another pathway; in fact, some small molecules, namely metabolites, could be precursors of the more complex synthesis of lipids, and also be synthesized themselves during the lipids' metabolic pathway, thus being by-products or secondary products of the lipid's metabolism.

Few studies deal with the small metabolites from mycobacterial. A recent study on *Rv2221*, coding for a highly efficient *M. tuberculosis* adenyl-cyclase, indicates that its catalytic activity is regulated by fatty acids. Thus, *M. tuberculosis* lipids seem to be involved in signal transduction through the main metabolite cAMP (Abdel-Motaal 2006). In fact, small metabolites are often involved in signaling transmission in many bacteria. The relevant PhoP/PhoR two-component system was demonstrated to be related to lipid metabolism in *M. tuberculosis* (Gonzalo Asensio 2006). Although the specific signal sensed by PhoR is still unknown (Jackson 2006), some small molecules (metabolites) might behave as its signaling effectors. In fact, the homologous two-component system (PhoP/PhoQ) is sensored by magnesium in other bacteria (Martin-Orozco 2006).

The association of mycobacterial lipids to *M. tuberculosis* pathogenicity is a matter of renewed interest (Riley 2006). A role in the establishment and progress of the pathology caused by the tubercle bacilli has been classically assigned for years to many of those lipids (Bloom 1994). However, most of the studies were conducted using lipids as isolated molecules, overlooking the interactions with other molecules within the bacterial cell and the environment. In fact, the lipid contents of the bacillus change according to the environmental conditions. Garton *et al*. described an increase of lipophilic inclusions according to the lipid content in the *in vitro* culture medium where bacteria were grown (Garton 2002). Lipid availability is probably not low inside man, the natural host, and *in vivo* bacilli could be lipolitic rather than lipogenic (Wheeler 1994). Trafficking of mycobacterial lipids from bacterial vacuoles to the endosomes of macrophages was demonstrated in *M. tuberculosis* infected macrophages, and mycobacterial lipids were detected even in uninfected cells. These findings indicate that through its own lipids, *M. tuberculosis* exerts a wide influence on its environment that extends beyond truly infected cells (Beatty 2000). Altogether, these data underline the great importance of the

metabolomic analysis for the interpretation of the biology of the tubercle bacillus and its relation with the host.

4.6. Concluding remarks

The availability of the first *M. tuberculosis* genome sequences triggered an overwhelming amount of knowledge on the genetics and the biology of *M. tuberculosis*. The way was opened for comparative and functional genomics. Scientists and medical doctors started to appreciate the potential coding capacity of this extraordinary organism. A broad picture of the *M. tuberculosis* gene content and coding capacity has been revealed. Sequencing and comparison with other genomes have shown the close relations that exist among the members of the *M. tuberculosis* complex, and have allowed the identification of a core mycobacterial genome, a minimal set of genes conserved in the different mycobacterial species. These advances were generated in a little more than seven years by no more than five publicly available genomes. Now, with the advent of new technologies and 21 genome projects in process, the study of mycobacteria and comparative genomics seems not only promising but very exciting. The application of new technologies, such as DNA microarray for the comparison of *M. tuberculosis* wild isolates, is a promising approach towards understanding its natural biology and adaptative evolution in the human population.

As research on the biology of TB expands, new and more accurate information is generated. In the coming years, knowledge about the real coding capacity of the tubercle bacillus will increase exponentially, and genome sequences will feed back from transcriptome and proteome analysis, filling old gaps and opening new ones in the understanding of *M. tuberculosis* biology. Functional genomics has become a key tool in the understanding of the biology of *M. tuberculosis*. By providing information about the pathogenesis of the disease, it is expected to promote the discovery of vaccine candidates and the investigation of novel drug targets. Investigations on complex biological systems can be now envisaged under a metabolomic perspective (Forst 2006). Metabolomics is a newborn methodology in microbiology and is even younger in mycobacteriology, therefore, almost everything remains to be learned in TB concerning that discipline.

It is clear that a long way still remains to be walked to understand how the tubercle bacillus behaves inside the host, its unique known environment. A more comprehensive integration of the knowledge generated by genomics, transcriptomics, proteomics and various molecular tools will surely provide a clearer picture of the amazing pathogen *M. tuberculosis* and the illness that it causes.

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Chapter 5: Immunology, Pathogenesis, Virulence

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5.1. Immune response against *Mycobacterium tuberculosis*

The immune response against tuberculosis (TB) plays a fundamental role in the outcome of *M. tuberculosis* infection. It is clear that the immune system reacts efficiently in the vast majority of infections. This is particularly evident in the case of TB, where most people infected by the tubercle bacillus (~ 90 %) do not develop the disease throughout their lifetimes. Nevertheless, the risk of developing the disease increases considerably when TB infection co-exists with an alteration in the immune system, such as co-infection with human immunodeficiency virus (HIV).

Also, it is well known that bacille Calmette-Guérin (BCG) vaccination has not been completely efficient in the prevention of pulmonary TB. Thus, the design of vaccines against TB is a field in which much effort has been invested with the aim of fighting this disease. Recently, it has become clear that, in order to develop a more efficient vaccine, a better understanding of the relation between the immune response of the host and the tubercle bacillus is needed.

In view of this, the present chapter provides an updated overview of the cellular and molecular immune mechanisms involved in the development of the disease.

5.1.1. Innate immune response

5.1.1.1. Neutrophil leukocytes

Even though macrophages are considered the main targets for infection by *Mycobacterium tuberculosis*, it has been recently proposed that other cell populations can also be infected by mycobacteria and therefore may be important in the development of the disease. Neutrophils are found within this group of cells (Figure 5-1). Characteristically, they are among the earliest cells recruited into sites where any noxious agent enters into the body and/or inflammatory signals are triggered. They also have well-characterized microbicidal mechanisms such as those dependent on oxygen and the formation of neutrophil extracellular traps (Urban 2006).

Using the murine experimental model, the role played by neutrophils in TB is controversial. These cells have been detected at the beginning of infection as well as several days after infection (Pedrosa 2000, Fulton 2002) and were thought to have

an important role in the control of mycobacterial growth. Indeed, if neutrophils are eliminated before infection, mycobacterial growth increases in the lungs of experimentally infected animals; and conversely, if mice are treated with an agent that increases neutrophils, the bacillary growth rate decreases (Appelberg 1995, Fulton 2002). However, when the microbicidal ability of neutrophils against mycobacteria was analyzed, controversial results were obtained. There are reports of neutrophils being able to kill mycobacteria (Jones 1990) and other reports where this phenomenon was not observed (Denis 1991). Nevertheless, it is believed that the function of neutrophils goes beyond their microbicidal ability. Therefore, these cells are thought to contribute to the control of infection through the production of chemokines (Riedel 1997), the induction of granuloma formation (Ehlers 2003) and the transference of their own microbicidal molecules to infected macrophages (Tan 2006).

Figure 5-1: Neutrophils ingest *Mycobacterium tuberculosis*. Human purified neutrophils were incubated with *Mycobacterium tuberculosis* H37Rv and DNA was stained with SYTOX™ Green. Fluorescent rods on the left are intracellular bacilli.

On the other hand, neutrophils have recently been ascribed a role in the development of the pathology, rather than the protection of the host. TB susceptible animals were found to have a larger and longer accumulation of neutrophils in TB lesions compared to TB resistant animals (Eruslanov 2005). This event seems to be influenced by the differential expression of molecules which are chemoattractant to

neutrophils (Keller 2006). The different susceptibility of the hosts may explain the discrepancies in the results of these recent studies and those of earlier ones, which suggested a protective role of neutrophils in the control of TB infection. While those early studies showing protection were conducted in mouse strains that were naturally resistant to TB, the later studies mainly focused on the role of these cells in TB susceptible mouse strains. Evidently, a more precise definition of the role played by neutrophils during infection will depend on an evaluation of the kinetics and magnitude of the response that these cells have in the early stages of the disease.

5.1.1.2. Mast cells

Mast cells are effector cells with a relevant role in allergic reactions (Woodbury 1984, Miller 1996, Galli 1999, Williams 2000); and are also critical for the development of a T helper 2 (Th2) response (Galli 1999, Metcalfe 1997). They are found in the mucosa of the respiratory, gastrointestinal, and urinary tracts and can also be observed in the vicinity of blood and lymph vessels.

These cells express a receptor with high affinity for IgE (FcεRI) and therefore this immunoglobulin is bound to their membrane. Upon the union of the antigen to the active sites of FcεRI-bound IgE, mast cells liberate several molecules, including preformed mediators and mediators synthesized *de novo* (Metzger 1992, Turner 1999, Williams 2000). Among the preformed mediators contained in mast cell granules are histamine, tryptase, chymase, carboxypeptidase, and heparin, while mediators synthesized *de novo* include leukotriene C4, prostaglandin D2, platelet-activating factor (PAF), tumor necrosis factor alpha (TNF-α), transforming growth factor (TGF-β), fibroblast growth factor 2 (FGF-2), vascular endothelial growth factor (VEGF), and interleukins IL-4, IL-5 and IL-8 (William 2000, Turner 1999, Sayama 2002).

Besides this interaction between IgE and the antigen, other agents are able to induce the activation of mast cells and the liberation of cytokines and other mediators. For instance, microbial products (Di Nardo 2003, Feger 2002) stimulate mast cells via two members of the toll-like receptor (TLR) family, TLR-2 and TLR-4 (Supajatura 2002, Sabroe 2002, McCurdy 2003).

The locations where mast cells are usually found are common gateways for infectious agents and there is evidence of these cells being excellent mediators of the inflammatory response (Williams 2000, Metcalfe 1997). At least in bacterial infections by *Klebsiella pneumoniae* and *Escherichia coli,* mast cells are required for the triggering of innate immunity (Malaviya 1996, Malaviya 2001). In addition, due to their strategic distribution within the lung, mast cells have a fundamental role in the

defense of the host against mycobacteria. An early study showed an increased number of mast cells and their degranulation in the lungs of animals experimentally infected with *M. tuberculosis* (Ratnam 1977). The presence of mast cells has also been described in the duodenum and the ileum of cows infected with *Mycobacterium paratuberculosis,* a microorganism that causes granulomatous enteropathic lesions (Lepper 1988). Muñoz *et al* (2003) demonstrated that there is an interaction between mast cells and *M. tuberculosis* through the CD48 molecule. This interaction triggers the release of preformed mediators, such as histamine and β-hexosamidase, and the liberation of *de novo* synthesized cytokines, such as IL-6 and TNF-α, which are involved respectively in the activation of neutrophils and the maintenance of the integrity of the granuloma (Muñoz 2003, Law 1996, Adams 1995). The secretory proteins *Mycobacterium tuberculosis* secreted antigen (MTSA-10) and 6-kiloDalton (kDa) early secretory antigenic target (ESAT-6) contribute to the activation not only of macrophages and dendritic cells but also of mast cells for the liberation of their pro-inflammatory mediators (Muñoz 2003, Trajkovic 2004).

5.1.1.3. Macrophages

The macrophage is the paradigmatic cell with regard to *M. tuberculosis* infection. Indeed, alveolar macrophages have been shown to play an essential role in the elimination of particles that enter the organism through the airways; and have long been considered the first cell population to interact with the tubercle bacillus. More macrophages are recruited afterwards from the bloodstream, and are in charge of maintaining the infection in the host (Dannenberg 1991, Dannenberg 1994).

The initial interactions of the bacilli with the macrophage take place through cellular receptors, such as receptors for Fc, complement (Schlesinger 1990), mannose (Schlesinger 1993), surfactant protein (Zimmerli 1996), CD14 (Peterson 1995), and CD43 (Randhawa 2005). Though it is unknown if the bacteria interact with one or more of these receptors during *in vivo* infection, the results of *in vitro* experiments suggest that the macrophage response depends on the type of receptor with which the bacteria interact. Their interaction with Fc receptors increases the production of reactive oxygen intermediates and allows the fusion of the bacteria-containing phagosomes with lysosomes (Armstrong 1975). On the other hand, interaction of the bacteria with the complement receptor 3 (CR3) prevents the respiratory burst (LeCabec 2000) and blocks the maturation of phagosomes harboring the bacteria, thus preventing fusion with lysosomes (Sturgill-Koszycki 1996).

The interactions of mycobacteria with members of the Toll-like receptor family have been studied for some years. TLR-2 (Brightbill 1999) and TLR-4 (Jeans

1999) are activated by several *M. tuberculosis* components. Among others, the 19-kDa lipoprotein and lipoarabinomanann (LAM) activate macrophages through TLR-2, promoting the production of IL-12 and inducible nitric oxide synthase (iNOS) (Brightbill 1999).

Regardless of the receptor with which the bacteria interact, it has been observed that the cellular cholesterol present in the macrophage cell membrane is an essential molecule for the internalization of the bacteria (Gatfield 2000). It is believed that cellular cholesterol works as a direct anchorage point for the bacterium and stabilizes its interaction with the macrophage membrane. Afterwards, the bacterium is efficiently internalized (Pieters 2001).

Once the bacteria enter the macrophage, they generally locate themselves in the mycobacterial phagosome (Armstrong 1971, Armstrong 1975). This structure derives from the plasma membrane and presents some cell surface receptors (Russell 1996, Hasan 1997). In contrast to normal phagocytosis, during which the phagosomal content is degraded upon fusion with lysosomes, the mycobacteria block this process (Armstrong 1971, Armstrong 1975).

This inhibition depends on an active process induced by viable mycobacteria, since dead bacilli can be easily found in lysosomal compartments (Armstrong 1971, Armstrong 1975). Besides having a different morphology, the vacuoles in which the bacteria reside present "early" endosomal compartment markers instead of the characteristic "late" endosomes (Hasan 1997, Clemens 1996, Baker 1997). In addition, these mycobacterial phagosomes retain "early" markers, such as Rab5 and Rab14 GTPases, and do not acquire the "late" Rab7 molecule; a finding which is also consistent with a blockage of the maturation process from early to late endosome (Via 1997, Kyei 2006).

Another characteristic of the mycobacterial phagosome is its limited acidification (Crowle 1991). Normally, material transported through an endosomal route finds an acidic medium due to the action of the vesicular proton-pump adenosine triphosphatase (V-ATPase) in the late endosome. It is suggested that such reduced acidification is the result of a low or zero concentration of V-ATPase in the mycobacterial phagosome (Sturgill-Koszycki 1994). A more recently described property is that this mycobacterial phagosome can not physically associate with iNOS (Miller 2004).

The inability of the mycobacterial phagosome to mature has been attributed to the active retention of a protein present in phagosomes, known as tryptophan aspartate coat protein (TACO), which was elegantly demonstrated by Ferrari *et al.* When these authors infected TACO-deficient cells, the maturation of mycobacterial

phagosomes was not arrested and therefore these cells were able to eliminate bacilli by fusion of phagosomes with lysosomes (Ferrari 1999). It is also worth noting that TACO binds itself to the plasmatic membrane of macrophages through cholesterol, which also plays an essential role in mycobacterial uptake by macrophages. These events show both molecules to be importantly associated in the mycobacterial mechanisms for survival (Gatfield 2000).

The inhibition of phagosome maturation by mycobacteria may be reverted by cytokines, such as interferon-gamma (IFN-γ) and TNF-α, which also stimulate microbicidal mechanisms, including the production of reactive oxygen and nitrogen intermediates (Flesch 1990, Chan 1992). The protective role of nitrogen intermediates has been demonstrated in different murine models (MacMicking 1997, Flynn 1998), and a similar function has been suggested for these molecules in human TB (Nicholson 1996). In contrast, the role played by the reactive oxygen intermediates during infection has not been completely explained, though it is known that hydrogen peroxide produced by macrophages activated by cytokines has a mycobactericidal activity (Walter 1981). Also, it has been found that the tubercle bacillus presents molecules, such as LAM and phenolic glycolipid I, which work as oxygen radical scavenger molecules (Chan 1989, Chan 1991).

5.1.1.4. Dendritic cells

Dendritic cells are clearly involved in the protective immune response to *M. tuberculosis* infection. As explained above, when *M. tuberculosis* bacilli are inhaled and phagocytosed by the pulmonary macrophages, they remain, and even replicate, within the cell phagosome. Dendritic cells recruited from blood, and probably also from lung tissues, may play a role in protective immunity since they are found in increased numbers in TB lesions (Sturgill-Koszycki 1994, Pedroza-González 2004, García-Romo 2004).

Dendritic cells recognize, capture and process antigens, thus being able to present them in the context of major histocompatibility complex (MHC) molecules, as well as through CD1 (Banchereau 1998, Gumperz 2001). Dendritic cells bind antigens via C-type lectin receptors and Fcγ/Fcε receptors, and internalize them by endocytosis (Engering 1997, Fanger 1996, Jiang 1995). *M. tuberculosis* endocytosis is carried out through known C-type lectin receptors, such as dendritic cell-specific intercellular-adhesion-molecule-grabbing non-integrin (DC-SIGN) (Geijtenbeek 2003, Tailleux 2003). This molecule interacts with mannose capped-LAM, a component of the mycobacterial cell wall (Geijtenbeek 2003, Figdor 2002). In addition, peripheral blood dendritic cells and immature dendritic cells derived from monocytes express TLR-2 and TLR-4 (Jarrossay 2001, Kadowaki 2001), two Toll-like

receptors with which mycobacteria seem to interact. Thus, it can be assumed that a protective host response may be induced through these signals. Additional signals generated by the association of mannose capped-LAM to DC-SIGN induce IL-10 production (Geijtenbeek 2003), while the union of a 19 kDa *M. tuberculosis* lipoprotein to TLR-2 induces production of IL-12, TNF-α, and IL-6 (Means 2001, Means 1999, Underhill 1999).

Once the antigens have been captured and internalized, dendritic cells become mature (indicated by phenotypical and functional changes) and efficiently migrate to peripheral lymph nodes. There is evidence of *in vivo M. tuberculosis* and BCG transport from lung tissues to the lymph nodes inside infected dendritic cells (Dieu 1998). This migration of infected dendritic cells requires the expression of the chemokine receptor 7 (CCR7) on their surface, which makes them sensitive to chemokines (CC) CCL19 and CCL21 (Dieu 1998, Gunn 1998, Kriehuber 2001, Bhatt 2004). It is important to mention that maturation of dendritic cells is not only accompanied by an increased synthesis of MHC class I and II, but also by the expression of co-stimulating molecules, such as CD80 and CD86 (Turley 2000), and the production of IL-12 (Steinmann 2001).

The internalization of *M. tuberculosis* into human and murine dendritic cells has been observed in several *in vitro* (Bodnar 2001, Fortsh 2000, Giacomini 2001, Hanekom 2002, Henderson 1997, Inaba 1993) and *in vivo* (Jiao 2002, Pedroza-González 2004, García-Romo 2004) studies. Reportedly, when dendritic cells derived from monocytes are infected with *M. tuberculosis,* their ability to present lipidic antigens is impaired and thus the expression of CD1 decreases (Stenger 1998). Components of the mycobacterial cell wall were also shown to inhibit the phenotypical maturation of dendritic cells induced by lipopolysaccharides. Different lineages of *M. tuberculosis* may vary in the degree by which they affect the dendritic cells. In particular, the enhanced virulence ascribed to Beijing strains might well be related to their inability to stimulate dendritic cell maturation (Lopez 2003, Ebner 2001).

In a protective immune response, dendritic cells induce maturation of T cells towards a T helper 1 (Th1) profile by secreting cytokines, such as IL-12, IL-18, IL-23, and probably IFN-α and β, but not IFN-γ (Wozniak 2006, Kadowaki 2001, Kalinski 1999, Thurnher 1997). Th1 cells expand in response to the BCG antigens presented by the dendritic cells in the lymphoid nodules and migrate toward infection sites, such as the lung tissue, where they liberate IFN-γ, thus activating local macrophages that control bacilli replication (Humphreys 2006).

5.1.1.5. Natural killer cells

Natural killer cells play a very important role in the development of the innate immune response. Their main function has been associated with the development of cytotoxicity to target cells and they are among the first cell populations to produce IFN-γ during the immune response. For a long time, the study of this cell population was focused on their role in viral and tumoral diseases. More recently, however, increasing interest has arisen in their eventual function in several bacterial infections.

The number of natural killer cells was shown to increase in the lungs of C57BL/6 mice during the first 21 days after aerosol infection with *M. tuberculosis* complex strains. This cell expansion was associated with an increased expression of activation and maturation markers, and IFN-γ production. However, the depletion of natural killer cells had no influence on the lung's bacterial load, indicating that although these cells become activated during the early response in pulmonary TB, they are not essential for host resistance (Junqueira-Kipnis 2003). Natural killer cells also play an important role in human TB by regulating different aspects of the immune response. Human natural killer cells have been shown to have an enhanced cytotoxicity for macrophages infected with *M. tuberculosis.* They also optimize the ability of CD8+ T lymphocytes to produce IFN-γ and to lyse *M. tuberculosis* infected cells, thus joining innate to adaptive immune responses (Vankayalapati 2002, Vankayalapati 2004).

5.1.1.6. CD1d-restricted natural killer T cells

These are a unique subset of human natural killer T cells characterized by the expression of an invariant V alpha 24 T cell receptor that recognizes the nonclassical antigen-presenting molecule CD1d. The activity of CD1d-restricted killer cells is notably enhanced by the marine glycolipid alpha-galactosylceramide derived from sponges. Once activated by alpha-galactosylceramide, CD1d-restricted natural killer T cells contribute to human host defense against *M. tuberculosis* infection. Human monocyte-derived macrophages expressing CD1d can induce effector functions of natural killer T cells against cells infected with *M. tuberculosis* when activated with alpha-galactosylceramide. These functions include IFN-γ secretion, proliferation, lytic activity, and anti-mycobacterial activity; this latter via the antimicrobial peptide granulysin, which damages the mycobacterial surface. There is further support of the potential interaction of natural killer T cells with CD1d-expressing cells at the site of disease, since CD1d can be readily detected in granulomas of TB patients (Gansert 2003). Such a role has not been proved in *M. tuberculosis* infected mice. Rather, natural killer T cells have been shown to play a

detrimental role, at least in the late phase of mouse experimental infection (Sugawara 2002).

5.1.1.7. Epithelial cells

Alveolar macrophages have been considered for a long time to be the first cell population to interact with *M. tuberculosis*. However, the number of epithelial cells in the alveoli is 30 times higher than the number of macrophages and thus, the likelihood that they are the first cells exposed to the infecting bacilli is similarly higher. The first indication of the involvement of epithelial cells in *M. tuberculosis* infection was derived from a study where the presence of mycobacterial DNA was detected in necropsy specimens from people who had died from diseases other than TB. In that study, *M. tuberculosis* DNA was detected in macrophages, type II pneumocytes, fibroblasts, and endothelial cells (Hernandez-Pando 2000). In addition, several *in vitro* studies have characterized the interaction between epithelial cells and *M. tuberculosis*. These cells can host *M. tuberculosis* bacilli and allow their replication (Bermudez 1996). Moreover, epithelial cells are able to establish an initial pro-inflammatory environment by secreting IL-8 (Wickremashinge 1999) and inducing the production of nitric oxide (NO) (Roy 2004). Obviously, *in vivo* experiments are necessary to better understand the role played by alveolar epithelial cells in *M. tuberculosis* infection.

5.1.1.8. Defensins

A conspicuous element of the innate immune response against microorganisms is a group of small endogenous antimicrobial peptides known as defensins (Diamond 1998). These cationic peptides, consisting of approximately 30 to 50 amino acids, are present in myeloid and epithelial cells of all animal species. They were shown to display antibacterial (Gabay 1989, Ganz 1985, Selsted 1987), antifungal (Selsted 1985), and antiviral (Daher 1986) activities. These molecules are classified as alpha, beta, and theta defensins based on the position of cysteine residues and the number of disulfur bonds (Bals 2000, Hoover 2000, Lehrer 1993). In phagocytic cells, defensins represent the main microorganism destruction components independent of oxygen metabolism (Miyakawa 1996, Ogata 1992). Allegedly, these peptides break the membrane of several microorganisms and some of them are even able to pass through the cytoplasmic membrane and enter the infected cell (Ganz 2003, Rivas-Santiago 2006).

Defensins were first described in guinea pig and rabbit neutrophils (Zeya 1963, Zeya 1966). There is no report of human monocytes and macrophages having defensins, although neutrophils have been reported to have four known human neutrophil defensins peptides (Ganz 1990), of which three (HNP-1, HNP-2 and HNP-3) were found to be active against *Mycobacterium avium-intracellulare* and *M. tuberculosis* (Ogata 1992, Miyakawa 1996).

In vitro, the human alpha defensins present in human neutrophils directly attracts CD4+/CD45RA+ T cells, CD8+ cells, and dendritic cells. The expression of human beta-defensin 1 is constitutive in epithelial cells but the expression of human beta-defensins 2 and 3 is inducible by IL-1, TNF-α, and by Toll-like receptor recognition of bacteria and fungi (Kaiser 2000, Lehrer 1993, Stolzenberg 1997). Human beta-defensins are also chemoattractants for T CD4+/CD45RO+ cells through receptor CCR6 (Chertov 2000).

Mice infected with *M. tuberculosis* express murine beta defensins mBD-3 and mBD-4 n. In the first stages of infection, the epithelial cells of the respiratory tract express both defensins, which correlates to the early control of bacterial proliferation. However, their expression decreases as the disease progresses. In the latent infection model, mBD-3 and MBD-4 are continuously expressed, but their expression is suppressed if the infection is reactivated (Rivas-Santiago 2006).

Genetic expression of human beta-defensin 2 (HBD-2) has been identified in epithelial cells of the skin, lung, trachea, and urogenital system (Bals 1998, Kaiser 2000, Lehrer 1993, Linzmeier 1999, Singh 1998, Stolzenberg 1997). This defensin was also detected in bronchial lavage cells from patients infected with *M. avium-intracellulare* (Ashitani 2001). Peripheral blood monocytes transfected with human beta-defensin HBD-2 have a better control of *M. tuberculosis* growth than non-transfected monocytes (Kishik 2001). Human alveolar epithelial cells infected with *M. tuberculosis* were also found to express human beta-defensin HBD-2 (Rivas-Santiago 2005).

M. tuberculosis infected mice that have been treated with the defensin peptide HNP-1 show a reduction of bacterial load in the lungs, liver, and spleen (Sharma 2001). This observation suggests that defensins could represent important components of the innate response mechanisms against *M. tuberculosis* and could be used as new therapeutic tools.

5.1.2. Acquired immune response against *M. tuberculosis*

In contrast to innate mechanisms, the specific or adaptive immune response requires the specific recognition of foreign antigens. The innate immune system has a profound influence on the type of acquired immune mechanisms generated, and *vice versa*, the specific immune response executes several of its effector functions via the activation of components of the innate immunity. Specific immune responses can be divided into cell-mediated mechanisms, which include T-cell activation and effector mechanisms, and the humoral immune response, consisting of B-cell maturation and antibody production. Both mechanisms are not mutually exclusive, and T helper cells are required for antibody maturation, isotype switching and memory. B cells also function as antigen presenting cells by activating T cells in a specifically driven manner. In the following pages we will focus on the generation of both humoral and cellular immune responses against *M. tuberculosis*.

M. tuberculosis is the most conspicuous example of an intracellular bacterium that persists for long periods within the host, causing a latent infection, namely a chronic asymptomatic infection without tissue damage. This is best illustrated by the fact that two billion people worldwide are infected with *M. tuberculosis*, but more than 90 % of them remain healthy and free of clinical disease and the tubercle bacilli remain within them in a state of dormancy. Therefore, although the host cell-mediated immunity is enough to control the progression of disease, it fails to exert sterile eradication and hence, those two billion infected persons suffer the latent form of TB (Collins 2002).

As for other intracellular infections, the primary protective immune response is cell mediated rather than antibody mediated. *M. tuberculosis* resides inside the macrophage and is relatively resistant to microbicidal mechanisms that efficiently eliminate other phagocytosed bacteria. This is due in part to the ability of the tubercle bacilli to hinder macrophage activation by IFN-γ and IL-12. Several studies have confirmed the critical importance of these cytokines in both human and mice *M. tuberculosis* infection. In addition, deficiencies in IL-12 or IFN-γ, or their receptors, render the individual more susceptible to mycobacterial infections (Jouanguy 1999, Alcais 2005). For the last 20 years, it has been assumed that the induction of a Th1-type immune response affords the host the greatest protective capacity.

Despite the fact that there are hundreds of studies published on TB immunity, still there is a lack of information regarding important issues, such as the role of lung antigen presenting cells *in vivo* during pulmonary TB (Pedroza-González 2004). This type of information would allow a better understanding of the induction of

specific immune responses against *M. tuberculosis*, and therefore the development of tools that could control the disease more effectively.

5.1.2.1. Humoral immune response

Because of their intracellular location, it is frequently assumed that tubercle bacilli are not exposed to antibody and therefore this type of immune response is considered to be non-protective. However, during the initial steps of infection, antibodies alone or in conjunction with the proper cytokines may provide important functions, such as prevention of entry of bacteria at mucosal surfaces. Even though the issue remains controversial, the role of antibodies in intracellular bacterial infections has gained renewed attention. Lately, their participation in the control of acute infections, such as chlamydial respiratory infection (Skelding 2006), and chronic infections produced by Actinomycetes, including *M. tuberculosis* (Salinas-Carmona 2004, Williams 2004, Reljic 2006), was explored.

Antibodies can be exploited in two ways in the clinical management and control of TB: as serological diagnostic tools; and as active participants in protection. Serological methods have been regarded for a long time as attractive tools for the rapid diagnosis of TB due to their simplicity, rapidity, and low cost. As early as 1898, Arlöing showed that sera from TB patients could agglutinate tubercle bacilli (cited in Daniel 1987). With the introduction of the enzyme-linked immunosorbent assay in the '70s, interest was renewed and several groups of investigators committed themselves to finding an optimum antigen for TB serodiagnosis. At that time, complex antigens were used in most cases, such as whole bacteria, culture filtrates, bacterial extracts, tuberculins and their purified derivates (PPD). More recently, individual purified antigens have also been assayed, including proteins, lipopolysaccharides and glycolipids, *i.e.,* Ag 85, 38-kDa protein, LAM or diacylthrehaloses. To date, however, no test has shown sufficiently high sensitivity and specificity values for diagnostic purposes (Al Zahrani 2000, Bothamley 1995, Singh 2003, Raqib 2003, Julián 2004, Lopez-Marin 2003, see also chapter 13).

As for their use in protection against TB, antibodies could enhance immunity through many mechanisms including neutralization of toxins, opsonization, complement activation, promotion of cytokine release, antibody-dependent cytotoxicity, and enhanced antigen presentation. In this sense, data from several laboratories indicate that anti-mycobacterial antibodies play an important role in various stages of the host response to TB infection (Costello 1992, Hoft 1999, Hoft 2002, Teitelbaum 1998, Williams 2004, De Valliére 2005). In particular, De Vallière *et al.* showed that specific antibodies increased the internalization and killing of BCG by neutrophils and monocytes/macrophages. Moreover, antibody-coated BCG bacilli

were more effectively processed and presented by dendritic cells for stimulation of CD4+ and CD8+ T-cell responses.

This enhanced anti-mycobacterial activity of phagocytes by antibody-coated bacilli is extremely important in the context of mucosal immunity. IgG and IgA antibody classes have been shown to be present in the mucosal secretions of the human lower respiratory tract (Boyton 2002). The specific mycobacterial targets for antibody-mediated enhanced interiorization and/or killing are not known, but surface antigens such as LAM or proteins expressed under stress conditions, such as alpha crystallin protein, may be relevant. In an experiment where 17 recombinant mycobacterial protein antigens, native Ag85 complex, LAM, and *M. tuberculosis* lysate were used to detect antibody responses induced by BCG vaccination, only LAM-reactive serum IgG responses were significantly increased in both BCG vaccinated individuals and active TB patients. As expected, oral BCG vaccination leads to a significant increase in LAM-reactive secretory IgA (Brown 2003).

A new approach toward protection against TB, using passive inoculation with IgA antibodies, was tested in an experimental mouse model of TB lung infection (Williams 2004). Intranasal inoculation of mice with an IgA monoclonal antibody against alpha crystallin protein reduced the *M. tuberculosis* colony up to 10-fold, forming units (cfu) in the lungs nine days after either aerosol or intra-nasal challenge. Both monomeric and polymeric IgA reduced cfu to the same extent, suggesting that the antibody may target the Fc alpha receptor (Fc-αR) rather than polymeric immunoglobulin receptor (poly-IgR) in infected lung macrophages. As expected, protection was of short duration, probably due to the rapid degradation of the intranasally-applied IgA.

More recently, in a follow-up of this study (Reljic 2006), the duration of protection was extended by inoculation of IFN-γ three days before infection, and further co-inoculation with IgA at different time points (2 h, 2 and 7 days) after aerosol infection with *M. tuberculosis* H37Rv. Instead of a 10-fold reduction in cfu, a 17-fold reduction was observed, as well as lower granulomatous infiltration of the lungs. Thus, the combined administration of IFN-γ and IgA shows promise as a prophylactic treatment of immunodeficient patients or as an adjunct to chemotherapy.

Taken all together, these findings suggest an urgent need to reassess the role of antibody responses in TB. In particular, the mechanism involved in antibody-mediated enhancement of innate and cell-mediated immunity should be addressed, in order to analyze whether these mechanisms could be exploited to develop better TB vaccines or to design alternative immunotherapeutic tools.

5.1.2.2. Cellular immune response

Since the tubercle bacilli reside inside a compartment within the macrophage, their antigens are presented by MHC class II molecules to CD4+ T lymphocytes. These cells play an important role in the protective response against *M. tuberculosis* and, when they are absent, growth of the bacilli cannot be controlled (Caruso 1999, Muller 1987). This is the case in patients with an immunodeficiency, such as that caused by HIV infection.

The main function of CD4+ T cells is the production of cytokines including IFN-γ, which activates macrophages and promotes bacilli destruction. Recently, another function has been ascribed to these cells, i.e., helping to develop the CD8+ T cell-mediated response (Scanga 2000, Serbina 2001). In the same way, CD4+ T cells may participate in the induction of apoptosis of infected cells and the subsequent reduction of bacterial viability through the CD95 Fas ligand system (Oddo 1998).

The participation of CD8+ T cells in the control of the infection is well recognized. Mice deficient in molecules such as CD8α, transporter associated with antigen processing (TAP), and perforin, were shown to be more susceptible to *M. tuberculosis* infection than animals which produced these molecules (Flynn 1992, Behar 1999). The mechanisms used by these cells for the control of TB seem to be mainly cytokine production and bacterial lysis.

In the lungs of infected mice, CD8+ T cells showed to be able to secrete IFN-γ through activation of the T-cell receptor or by interaction with infected dendritic cells (Serbina 1999). Once again, the function performed by this IFN-γ is the activation of the macrophage and promotion of bacterial destruction.

In addition, CD8+ T cells proved to be efficient in lysing infected cells and in reducing the number of intracellular bacteria (Stenger 1997, Cho 2000). The mechanisms of control of the bacterial load seem to be associated with granular exocytosis involving perforin and granzymes. Still, granulysin, which is found in CD8+ T granules, is the molecule responsible for killing the bacterium (Stenger 1998).

5.1.2.3. Gamma/delta T cells

Previously, the CD4- CD8- T cells, known as γδ T cells, were considered to play a low-profile role in the immune response. They were believed to proliferate only in response to non-peptidic antigens (Schoel 1994). As they were found in early lesions, they were thought to react to infected macrophages only through the production of IFN-γ (Ferrick 1995). In the last few years, however, γδ T cells proved to be relevant for the regulation of the immune response.

High levels of γδ T cells are usually found in the peripheral blood of TB patients (Ito 1992). *Ex vivo,* γδ T cells from human TB patients display a lytic activity that is independent of the MHC. While the lytic activity of CD4+ and CD8+ T cells decreases gradually as the disease becomes more severe, γδ T cells increase their activity, lysing target cells infected with *M. tuberculosis* through the Fas-FasL mechanism and the perforin pathway (De la Barrera 2003).

In a murine model of TB infection, γδ T cells were shown to contribute to the elimination of *M. tuberculosis* and to have an anti-inflammatory effect. Indeed, when these cells are eliminated by genetic manipulation or by using a specific monoclonal antibody, inflammatory damage is accelerated in the lungs of mice infected with *M. tuberculosis* (D'Souza 1997, Ladel 1995). In addition, γδ T cells produce IL-17 during early infection, which probably promotes the flow of cells towards infection sites. This IL-17 secretion may be produced in response to IL-23 secretion by dendritic cells infected with *M. tuberculosis* (Lockhart 2006).

The role of γδ T cells in protective immunity is not limited to cytokine secretion (such as IL-17 and IFN-γ) and cytotoxic activity. These cells also behave as antigen-presenting cells. Like dendritic cells, they can process and efficiently present antigens and give the co-stimulating signals needed to induce proliferation of αβ T cells (Brandes 2005). As noted, γδ T cells act as a link between the innate immune response and the adaptive immune response, although other roles played by these cells still remain unknown.

5.2. Tuberculosis pathogenesis and pathology related to the immune response

An important reason for the current failure to control TB is that, even when applying the best available chemotherapy, treatment must be continued for at least six months. This treatment regimen is not a realistic option in limited-resource countries, or even in large cities of developed countries, because after a few weeks of treatment the patients start to feel well again and stop taking the drugs.

There are two main reasons for prescribing such long-term treatments. The first is that the antibiotics kill the vast majority of the bacilli within a few days, but persisting bacteria are not killed by the drugs. These persisting bacilli may be in a true stationary phase with very low metabolism, and may be non-replicating or replicating very slowly (latent infection). The other reason is the necrotizing tissue response that is analogous to the Koch phenomenon (Koch 1891). Robert Koch

demonstrated that the intradermal challenge of guinea pigs with whole organisms or culture filtrate, four to six weeks after the establishment of infection, resulted in necrosis at both the inoculation site and the original tuberculous lesion site. A similar phenomenon occurs in persons with active TB, in whom the PPD test site may become necrotic. Koch tried to exploit this phenomenon for the treatment of TB and found that subcutaneous injections of large quantities of culture filtrate (old tuberculin) into TB patients evoked necrosis in their tuberculous lesions. In fact, this treatment was shown to have extremely severe consequences associated with extensive tissue necrosis and was discontinued (Anderson 1891). Still today, the task for the researchers working in this field is to understand the differences between protective immunity and progressive disease, including the Koch phenomenon (Rook 1996). The final aim is to learn how to replace the pathological immune response by the protective one, and with such knowledge, to design short-course chemotherapy schemes supplemented with immunotherapy, which would enable TB control worldwide. These important topics on immunopathology and immunotherapy are revised in the following sections.

The role of individual immune cells in the protection against *M. tuberculosis* has been described in previous sections of this chapter. The next paragraphs of the 5.2 section describe patterns of immune response to infection and disease.

5.2.1. Mechanism of protective immunity

In the experimental murine TB model, the protective response has a distinct Th1 type of cytokine pattern, as demonstrated by manipulation of the immune system through genetic knockout or the administration of specific monoclonal antibodies (Cooper 1995, Dalton 1993, Flynn 1993). The same cytokine pattern seems to be protective in humans because children with defective receptors for IFN-γ or IL-12 are susceptible to mycobacterial disease (Jouanguy 1999, Alcaïs 2005).

In addition to these Th1-type cytokines, TNF-α is also essential for immunity to *M. tuberculosis* in mice (Kindler 1989) as well as in humans (Keane J 2001). These observations further suggest a role for NO, because TNF-α triggers the release of NO from IFN-γ-activated cells. In fact, iNOS expression is essential to control infection in mice (Chan 1992) and is also highly expressed in human tuberculous lesions (Schon 2004).

5.2.2. The immune response related to progressive disease: mechanisms of immunopathology

In the early '90s, some researchers reported an increased expression of Th2 cytokines, especially IL-4, in patients with advanced pulmonary TB (Schauf 1993, Sanchez 1994). Later, other authors failed to detect IL-4 and the issue remained controversial (Lin 1996). More recent data indicate that these failures could be attributed to technical problems (Rook 2004), and now there is substantial evidence that demonstrates a Th2 response in human TB (Seah 2000, Van Crevel 2000, Marchant 2001, Leinhardt 2002). Interestingly, IL-4 levels tend to be higher in patients living close to the Equator, possibly as a consequence of simultaneous infection with helminths and/or higher mycobacterium inoculum (Malhotra 1999). Indeed, TB patients have several IL-4-dependent manifestations, including high IgE antimycobacterial antibodies (Yong 1989), antibodies against cardiolipin (Fisher 2002), and high expression of DC-SIGN in dendritic cells (Relloso 2002). IL-4 has been detected in human TB lung lesions by in-situ hybridization (Fenhalls 2000). Also, IL-4 messenger ribonucleic acid (mRNA) and T-cells containing IL-4 are increased in human pulmonary TB. This high IL-4 expression correlates significantly with serum IgE, serum soluble CD-30, and also with the extent of cavitation (Seha 2000, van Crevel 2000, Lienhardt 2002). It has been demonstrated that CD8+ cells are another source of IL-4, and this correlates with cavitation (van Crevel 2000). The presence of IL-4 at late stages of the disease has a direct pathogenic role because it downregulates the protective Th1 responses (Biedermann 2001).

Similar abnormalities are also observed in the lungs of Balb/c mice, which have been experimentally infected via the trachea with a high dose of *M. tuberculosis* H37Rv (Hernandez- Pando 1996, Hernandez-Pando 1998). In this model, there is an initial phase of partial resistance dominated by Th1 cytokines plus activated macrophages that produce TNF-α and express iNOS. The phase of progressive disease starts after one month of infection. This late phase is characterized by a drop in the number of cells expressing INF-γ, IL-2, TNF-α, and iNOS, progressive pneumonia, extensive interstitial fibrosis, high bacillary counts and very high levels of IL-4 and TGF-β, produced by a distinctive type of macrophage with numerous cytoplasmic vacuoles (foamy macrophages) (Figures 5-2 and 5-3). It is important to point out that this animal model resembles the disease in developing countries, where the infecting dose is usually high and the progressive disease tends to show a high IL-4 response (Rook 2004). This Balb/c model can be considered a suitable model for human TB because it mimics the well-characterized response observed in the progressive human disease that consists of an increased expression of Th2

cytokines and TGF-β. This switch to Th2 cytokine production seen in Balb/c mice and, albeit to a lesser extent, in humans, is absent in the experimental TB model developed in C57Bl mice, which is characterized instead by progressive lung granulomas and extensive lung consolidation with a strong and sustained Th1 response (Flynn 2006).

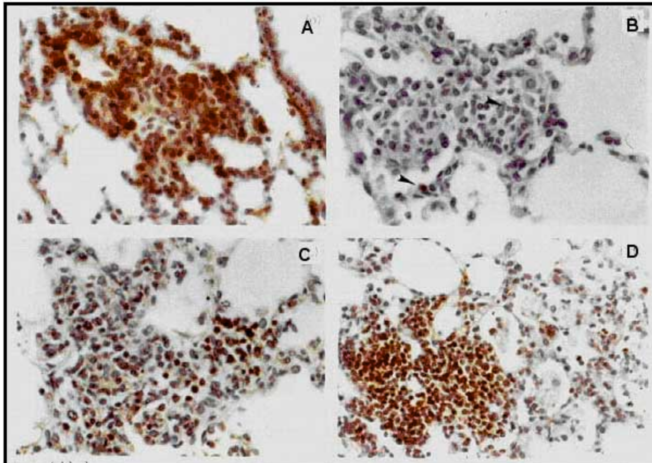

Figure 5-2: Immunohistochemical detection of IFN-γ (Th1 cells) and IL-4 (Th2 cells) in lung granulomas during the course of experimental TB in Balb/c mice infected by the intratracheal route. (A) Numerous IFN-γ immunostained cells (brown staining) exist in early granulomas, after 2 weeks of infection. (B) In contrast, few IL-4 positive cells (arrows) are seen in a serial section from the same granuloma exhibited in A. (C) After 4 months of infection, during active and advanced disease, there are some IFN-γ immunostained cells in granulomas. (D) Numerous IL-4 immunostained cells are seen in the same late granuloma exhibited in C. Thus, during early infection (first month) there is a predominance of Th1 cells, while during progressive disease a mixed Th1/Th2 pattern exist in this animal model.

In the Balb/c model of progressive pulmonary TB described above, the mixed Th1/Th2 cytokine pattern is associated with the pathology and reduced protection (Rook 1996). When pre-sensitized with 10^7 cfu of *Mycobacterium vaccae,* a saprophytic, highly immunogenic mycobacteria, mice infected with *M. tuberculosis* mount a strong Th1 response and are partially protected. In sharp contrast, when pre-immunized with a higher dose of the same mycobacterial preparation (10^9 cfu), mice develop a response with a mixed Th1/Th2 pattern that leads to increased severity of infection with the disease, and death (Hernandez-Pando 1994, Hernandez-Pando 1997). Thus, pre-exposure to saprophytic mycobacteria can determine either

resistance or susceptibility to *M. tuberculosis* infection, and the effect seems to be dose dependent. The nature, route, and dose of mycobacterial exposure depend on where and how an individual lives, because mycobacteria are not part of the usual commensal flora of human beings. This variable priming of antimycobacterial responses by saprophytes can either protect or predispose to infection, and might well be responsible for the uneven efficacy of BCG vaccination in different parts of the world (Rook 2005).

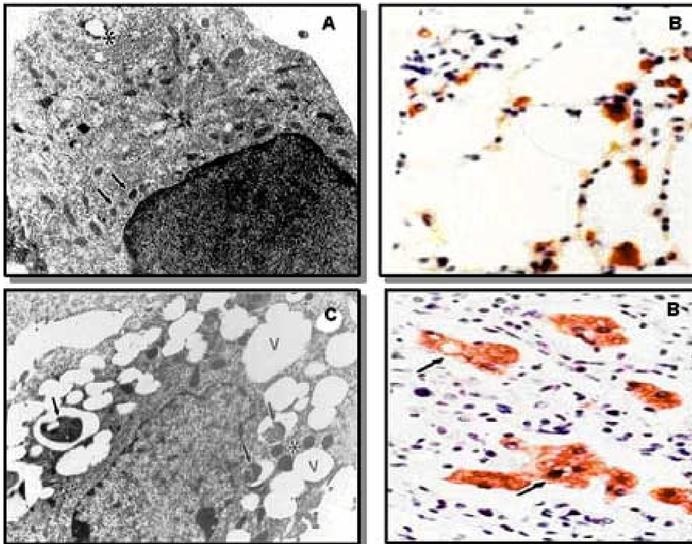

Figure 5-3: Representative immunohistochemical and electron microscopic features of lung macrophages during experimental pulmonary TB in Balb/c mice. (A) Subcellular structure of activated macrophage during early infection, numerous primary lysosomes (arrows) and occasional bacteria (asterisk) are distinctive elements in the abundant cytoplasm of this cell type. (B) These activated macrophages show strong TNF-α immunostaining. (C) During progressive disease, vacuolated or foamy macrophages containing numerous cytoplasmic vacuoles (V) and bacilli (arrows) are the predominant cells in the areas of pneumonia (E) These foamy macrophages show strong TGF-β immunostaining. Thus, activated macrophages are efficient producers of pro-inflammatory cytokines, such as TNF-α, and contribute to the control of the infection, while vacuolated macrophages are severely infected cells and efficient producers of anti-inflammatory cytokines that enable bacilli growth, such as TGF-β.

Various symptoms of TB, such as fever, weight loss and tissue damage, resemble the pathological effects of TNF-α. Evidence that such symptoms may be produced by this cytokine has come from experiments with thalidomide, a compound that

decreases the half-life of TNF-α mRNA (Moreira 1993). TB patients treated with this drug show rapid symptomatic relief and weight gain (Kaplan 1994). Thus, TNF-α has a paradoxical participation in the immunopathology of TB: it plays an essential role in protection but may also be a significant factor in its pathology. This ambiguous activity of TNF-α might be defined on site in the light of the predominant cytokine pattern, Th1 or Th2. In fact, the sensitivity of a given inflammatory site to TNF-α is dependent on the cytokine profile of the prevailing CD4+ T cells.

This ambiguous effect of TNF-α was also observed in Balb/c mice immunized with different doses of *M. vaccae* two months before challenge with fully pathogenic *M. tuberculosis.* Only a Th1 cytokine response was elicited in response to 10^7 cfu of *M. vaccae,* as demonstrated by high INF-γ production by splenocytes and high cutaneous delayed type hypersensitivity (DTH) against mycobacterial antigens. If 1 µg of TNF-α was injected into the site of the DTH response (right footpad) that had been elicited 24 h earlier in such animals, no necrosis was caused. However, in mice that had been sensitized with a 100-fold higher dose (10^9) of *M. vaccae*, a mixed Th1/Th2 cytokine pattern was evoked, with very high IL-4 production that elicited much lower DTH reactions. In these animals, the injection of TNF-α at the site of DTH response elicited massive inflammation and local necrosis (Hernandez-Pando 1997). Therefore, a possible explanation of the TNF-α paradox can be proposed: it seems that the TNF-α was released into a relatively pure Th1-mediated inflammatory site, where it may act merely as a supplementary macrophage-activating cytokine. But, when released into a mixed Th1/Th2 site with high IL-4 concentration, it causes damage. These observations were confirmed in a further study using the Balb/c model of progressive pulmonary TB (Hernandez-Pando 2004). In the early stage of infection (21 days after infection), while the Th1 cytokine response predominated and controlled the growth of bacilli, the DTH response was the highest and DTH sites were not vulnerable to necrosis by TNF-α. In contrast, during the progressive phase of the disease (50 days after infection) extensive tissue damage and high IL-4 production are manifested, the DTH response was very low, and TNF-α administration in the DTH sites provoked extensive inflammation with necrosis. Moreover, mice that have been preimmunized with a high dose (10^9) of killed *M. vaccae*, which induces mixed Th1/Th2 cytokine responses with high IL-4 production, developed higher and more rapid TNF-α-sensitive DTH response and became more susceptible to intratracheal *M. tuberculosis* challenge than unimmunized control animals (Hernandez-Pando 1997). Thus, this immunopathological response is a clear reminder of the Koch phenomenon. Another experimental confirmation of the TNF-α-mediated immunopathology associated with IL-4 comes from IL-4 gene knockout of tuberculous Balb/c mice that exhib-

ited not only diminished bacterial proliferation, but also complete absence of TNF-α-mediated toxicity following a TNF-α challenge in the DTH sites (Hernandez-Pando 2004).

We have explained above that an inappropriate Th2 component is present in both murine and human TB. Its effect becomes more striking when the disease becomes more severe. What then are the likely causes of this shift in cytokine profile, and what is the participation of other factors that deregulate the protective immunity against TB? These questions will be addressed in the next section, but it is certain that there are many significant participant factors that we do not yet know about, and their characterization will contribute significantly to the knowledge of the immunopathology and control of this significant infectious disease.

5.2.3. Factors that deregulate the protective type 1 response

Figure 5-4 graphically summarizes the participating factors in protection and progression of pulmonary TB. An increase in antigen load is clearly a participating factor, as shown by the striking linkage of the Th1/Th2 balance to the dose after immunization with particulate antigens such as mycobacteria (Hernandez-Pando 1994) or *Leshmania* (Bretscher 1992). Thus, low antigen loads, such as the low dose of *M. vaccae* (10^7 cfu) used to presensitize mice in the above-mentioned experiments, or the relatively low bacterial lung burden during early infection in the Balb/c model of progressive pulmonary TB, prime the Th1 response. In contrast, high antigen loads, for example the 10^9 cfu of *M. vaccae* or the high bacillary loads produced in the lungs during the progressive phase of the Balb/c model, efficiently induce the Th2 response.

Another mechanism that participates in the declination of the Th1 cytokine pattern during progressive disease in the Balb/c model is the selective apoptosis of CD4+/Th1 cells (Rios Barrera 2006). Indeed, Th1 cell apoptosis can partly be induced by foamy macrophages through a Fas/Fas ligand mechanism. Foamy macrophages predominate in advanced TB, they contain numerous bacilli, and their cytoplasmic vacuoles display strong immunostaining for mycobacterial lipids such as LAM. Vacuolated macrophages show little immunostaining for TNF-α or iNOS, but strong TGF-β immunoreactivity (Hernandez-Pando 1997, Hernandez-Pando 2001), and also express high levels of the anti-apoptotic Bcl2 molecule. Due to these properties, foamy macrophages are long-lived cells that harbor mycobacteria for long periods, and at the same time are a significant source of immunosuppressing cytokines that facilitate bacilli proliferation.

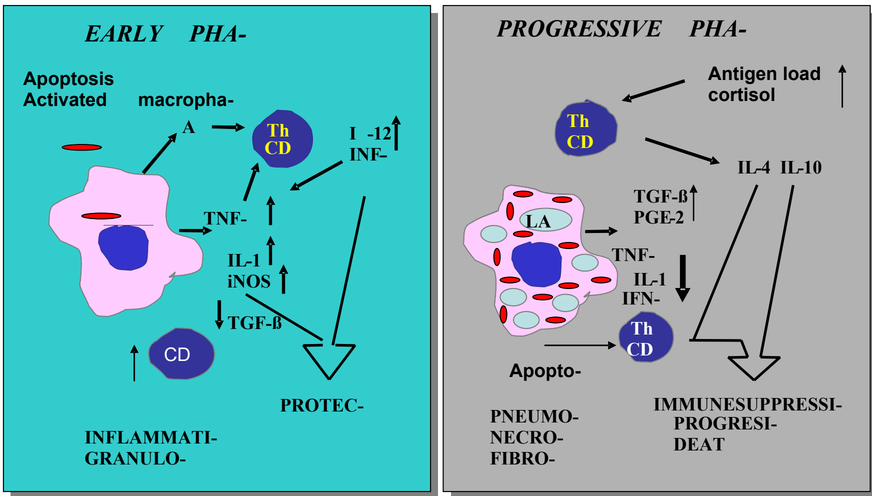

Figure 5-4: Left: Relevant immunopathological events during experimental pulmonary TB in Balb/c mice. During early infection, bacilli are efficiently phagocytosed by lung macrophages, which also secrete proinflammatory cytokines such as TNF-α and IL-1, and present bacillary antigens to Th1 cells. Th1 cells thus activated produce IL-12. Right: During progressive disease, Th2 cells emerge to deactivate Th1 cells, which together with overproduction of anti-inflammatory and immunosuppressive molecules such as cortisol, prostaglandin E (PGE) and TGF-β, deactivate macrophages, enabling bacilli growth and progressive tissue damage that causes death.

Another factor that deregulates the protective immune response against TB is prostaglandin production. Prostaglandins, in particular PGE-2, are potent mediators of intercellular communication. Indeed, at high concentrations PGE-2 is immunosuppressive for T-cell-mediated immunity (Phipps 1991). In the Balb/c model of *M. tuberculosis* infection, pulmonary PGE-2 concentrations remain relatively low and stable during the early phase, and if mice are promptly treated with niflumic acid, a potent and specific blocker of cyclo-oxygenase 2 (the rate limiting enzyme of prostaglandin production), infected lungs show a higher degree of inflammation and expression of TNF-α, IL-1α and IFN-γ, but almost complete disappearance of iNOS expression, which coexists with a significant increment of the bacterial load. Interestingly, during the late progressive phase in this experimental model, foamy macrophages from the pneumonic areas exhibit strong PGE-2 immunostaining, and PGE-2 concentrations four fold higher than those of the early phase. When prostaglandin production was suppressed in animals suffering from advanced disease, a significant reduction of pneumonia and bacillary load, with a striking increment in

the size of the granuloma was seen, and the expression of IFN-γ, TNF-α and iNOS was also improved. Therefore, PGE-2 is a significant factor participating in the pathogenesis of pulmonary TB and has contrasting functions depending on its concentrations. During the early phase of the infection, the low PGE-2 concentrations contribute to iNOS expression, permitting the temporal control of bacilli growth; while the high PGE-2 concentrations during the late phase contribute to the downregulation of cell-mediated immunity, allowing disease progression (Rangel 2002).

Excessive release of TGF-β has been implicated in the pathogenesis of human and murine TB. Blood mononuclear cells from TB patients were found to release increased levels of TGF-β, which was found in abundance in tuberculous lung lesions (Tossi 1995). Serum levels of TGF-β were strikingly raised in patients with advanced disease (Fiorenza 2005). The mannose-capped lipoarabinomannan from the cell wall of *M. tuberculosis* is a potent inducer of TGF-β (Dahl 1996). Interestingly, human blood monocytes and alveolar macrophages produced bioactive TGF-β upon stimulation with *M. tuberculosis*, so a signal from the organism was causing activation of TGF-β, as well as its secretion (Aung 2005). Several reports indicate that TGF-β also suppresses protective immunity to TB *in vivo*. In a mouse model, latency-associated peptide, a natural modulator of TGF-β function, decreased BCG growth in the lung and enhanced expression of IFN-γ (Wilkinson 2000). When mycobacterial infected guinea pigs were given intraperitoneal injections of recombinant human TGF-β, mycobacterial loads increased significantly (Dai 1999). In the Balb/c mouse model, high expression of TGF-β during the progressive phase of the infection was seen, and when TGF-β was blocked by recombinant β-glycan (type III TGF-β receptor) expression of IFN-γ and IL-2 increased with strong downregulation of IL-4, in co-existence with a significant reduction in bacterial counts in the lungs (Hernandez-Pando 2006), but with more lung consolidation by pneumonia than in non-treated control animals. Thus, TGF-β is a downregulator of cell-mediated immunity and a suppressor of excessive inflammation preventing tissue damage. This implies that the participation of classical regulatory cytokines, such as IL-10 or TGF-β, is necessary in order to avoid excessive inflammation, and to preserve the architecture and function of the lungs. Therefore, the fine balance between proinflammatory and anti-inflammatory cytokines seems to be a key factor in the immunopathogenesis of TB.

Fibrosis is a major cause of permanent respiratory dysfunction in TB. In human TB, fibrosis might be related to a high production of the potent fibrogenic cytokine TGF-β and to the presence of the Th2 response. Interestingly, pulmonary fibrosis in systemic sclerosis is associated with CD8+ cells secreting IL-4 (Atamas 1999).

Also, when IL-4 genes are knocked-out in Balb/c mice, the subsequent absence of IL-4 is associated with very low TGF-β production during the progressive phase of the disease, with lesser fibrosis and diminished bacterial growth (Hernandez-Pando 2004), confirming that Th2 cytokines are directly involved in the development of fibrosis, probably by inducing TGF-β production (Lee 2001).

Adrenal steroids may also contribute to the dysfunction of Th1 responses in TB. Reactivation or progression of infection is sensitive to activation of the hypothalamic-pituitary adrenal axis. The exposure of humans to the stress of war or poverty (Spence 1993), or cattle to the stress of transportation, is efficient in causing reactivation of latent infection. In mice, it has been demonstrated that this is due to glucocorticoid release (corticosterone in mice) (Brown 1995, Tobach 1956), which reduces macrophage activation and Th1-cell activity (Daynes 1991), while synergizing with some Th2 functions (Rook 1994). Tuberculous patients lose the circadian glucocorticoid rhythm, provoking constant exposure of peripheral lymphocytes to cortisol (Sarma 1990). In addition, the total output of cortisol derivatives and of androgens is frequently reduced (Rook 1996). Cortisol undergoes reduced conversion to inactive cortisone, producing normal serum cortisol concentrations in TB patients (Post 1994). The lung enzyme 11-beta-hydroxysteroid dehydrogenase converts inactive cortisone to active cortisol, producing higher concentrations of cortisol in the tuberculous lung (Rook 2000). In the Balb/c model the high production of TNF-α by activated macrophages in mature granulomas during the early phase of the infection (day 21), activates para-ventricular neurons in the hypothalamus, inducing higher expression of corticotrophin-releasing factor (Hernandez-Pando 1998). This factor induces adrenocorticotropic hormone production in the pituitary and in turn, this hormone stimulates the adrenals to produce glucocorticoid. The stimulus is so strong that both adrenals duplicate their weight due to nodular and diffuse hyperplasia (Hernandez-Pando 1995). In consequence, high concentrations of corticosterone are produced, contributing to the activation of Th2 cells and bacilli cell growth. Perhaps this immuno-endocrine response is another mechanism to avoid excess lung inflammation due to the well-known anti-inflammatory activity of glucocorticoids, but at the same time, this response contributes to deregulation of the protective immunity and bacilli growth. Interestingly, during experimental late progressive disease, a striking adrenal atrophy is produced (Hernandez-Pando 1995). This situation is similar to the reduced adrenal reserve observed in patients with severe TB, that die suddenly and without obvious cause during treatment (Onwubalili 1986, Scott 1990). Occasionally, the adrenals are destroyed by TB, but there are patients whose adrenals are found on postmortem examination to be small and without evidence of direct infection, as in tuberculous mice. Interestingly, TNF-α and IL-1 are much more toxic in adrenalectomized than

in control animals (Zuckerman 1989, Bertini 1998). Thus, a reduced adrenal reserve could play a role in the above-described toxicity of TNF-α, and in the toxicity of TNF-α in tuberculous mice once they have entered the phase of adrenal atrophy in the late progressive stage of the disease (Hernandez-Pando 1995). It is also important to consider that the function of cortisol within lymphoid tissue is regulated by local production of the metabolites of dehydroepiandrosterone sulfate, an androgenic adrenal steroid that has "anti-glucocorticoid effects", inducing strong activation of Th1 cells (Hernandez-Pando 1998). Administration of dehydroepiandrosterone or its derivative 3,17-androstenediol causes a Th1 bias, so this could be an efficient form of immunotherapy, as discussed below.

5.2.4. Susceptibility to tuberculosis: the importance of the pathogen

From the first exposure to *M. tuberculosis*, the host immune system triggers a series of responses which define the course of infection. However, this defense is not uniform in exposed persons. As mentioned above, the vast majority never develop active disease (Bloom 1992), but in those persons that become sick, a wide spectrum of possible clinical manifestations may occur, and the immune response, as seen for example in *in vitro* T- and B-cell reactivity against mycobacterial antigens, differs significantly from person to person. Thus, the clinical course of the infection and its epidemiological consequences depend on a complex interplay of host, environmental and bacterial factors (Nardell 1993, Hill 1998, Bellamy 1998, Stead 1992, Kramnik 2000).

As mentioned above, environmental factors that determine TB susceptibility include poverty, malnutrition, stress, overcrowding, and exposure to mycobacterial saprophytes. In the host, there is evidence of multifactorial genetic factors that influence susceptibility to *M. tuberculosis* (see Chapter 6). Mouse genes that participate in the control of early mycobacterial multiplication or TB progression in the lungs have been distinguished (Kramnik 2000). However, it seems that the independent participation of these genes is not sufficient to confer full protection against virulent *M. tuberculosis.*

As illustrated in this chapter, the host immune response against mycobacterial infection is the most investigated factor; but recent studies indicate that the genetic variability of *M. tuberculosis* has a significant role in the outcome of the infection. For many years, *M. tuberculosis* was considered to be highly conserved with a high degree of sequence homology and lack of antigenic diversity (Kapur 1994, Kremer 1999). Therefore, most of the immunological research has been done with a limited number of laboratory strains, including H37Rv or Erdman. However, DNA finger-

printing techniques have demonstrated a high degree of DNA polymorphism in the genome of *M. tuberculosis,* associated with repetitive DNA sequences and insertion elements (van Embden 1993). This genetic variability is related to recent epidemiological data indicating striking differences in virulence and transmissibility (Valway 1998, Caminero 2001). Particular outbreak strains were found to elicit distinct immune paths and mortality rates in the course of experimental infection. The investigation of an outbreak produced by a newly identified, genetically distinct *M. tuberculosis* strain named CDC1551 revealed that this strain produced a high rate of transmission in humans; in mice, it induced higher levels of TNF-α, IL-10, IL-6 and IFN-γ (Valway 1998). The genetic comparison between *M. tuberculosis* H37Rv and CDC1551 demonstrated single nucleotide polymorphism in many different genes. The clinical and epidemiological differences in this strain have therefore now been linked with immunological and genetic differences (McShane 2003). In another study, the clinical isolate HN878 was found to be hypervirulent and mice infected with this strain failed to induce a Th1 response, with lower levels of IFN-γ and TNF-α in the infected lungs (Manca 1999).

Using the Balb/c mouse model of progressive pulmonary TB, 12 distinct strains of *M. tuberculosis,* defined on the basis of IS*6110* RFLP patterns, and representing four major genotype families found throughout the world, were studied (Lopez 2003). This study demonstrated marked differences in virulence, cytokine induction and immunopathology among the different strains. In comparison, with animals infected with the laboratory strain H37Rv used as a control, mice infected with Beijing strains induced significantly high mortality, high bacillary load and a non-protective pattern of cytokine production (low IFN-γ expression with high but ephemeral TNF-α and iNOS production). "*M. canettii*" strains induced long survival with low bacillary load and significantly fewer areas of pneumonia in coexistence with constant and stable expression of IFN-γ, TNF-α and iNOS. The differences among other strains were less marked and showed intermediate rates of survival. Interestingly, the protective efficacy of BCG against the different strains of *M. tuberculosis* was found to vary and BCG was least protective against the hypervirulent Beijing strain 9501000 (Lopez 2003). This is important, considering that the Beijing genotype is the predominant strain in several distinct geographical areas, presumably due to a selective advantage over other strains (van Soolingen 1995).

The use of microarrays would help to characterize the genetic differences between these strains. Perhaps specific SNPs could be identified facilitating the identification of virulence genes, which will allow the development of attenuated strains and potential vaccine candidates (Hernandez-Pando 2006). Thus, further animal studies

using different clinical isolates and mutant strains are necessary to evaluate how the genetic differences translate into functional differences.

5.3. Latency and maintenance of the immune response

M. tuberculosis is a pathogen capable of producing both progressive disease and latent infection (Parrish 1998). The initial infection usually occurs in the lungs and in most cases is controlled by the immune system. Only 10 % of these infections lead to progressive disease (Sudre 1992, Parrish 1998). Even after successful control of the primary TB infection, some bacilli remain in a non-replicating or slowly replicating dormant state for the rest of the life of the individual. This infectious state, termed latent TB infection, is clinically asymptomatic, and most active TB cases arise as a result of reactivation of dormant bacilli (Parrish 1998, Dolin 1994). Up to one third of the world's population is estimated to carry latent *M. tuberculosis* infection, and hundreds of millions of TB reactivations are anticipated specifically in areas of low or moderate endemicity, where most cases of active TB result from reactivation of latent infection (Parrish 1998, Fine 1999).

It has been established that the low concentrations of oxygen and nutrients in chronic granulomas that remain after efficient control of the primary infection, as well as the local production of TNF-α and NO, are significant factors for the induction and maintenance of latent infection (Parrish 1998, Voskuil 2003, Arriaga 2002, Flynn 1998). Indeed, immunological studies in animal models of latent TB have demonstrated that cytokines such as TNF-α and IFN-γ, as well as NO contribute significantly to maintaining infection in the latent state (Arriaga 2002, Flynn 1998). These types of immune responses, of which the tuberculin skin test is a conspicuous exponent, is also crucial for protection in latently infected individuals.

It has been shown that in a well-characterized experimental latent infection model, as well as in necropsy tissues from humans with latent TB, mycobacterial DNA can be detected by *in situ* PCR in a variety of cell types in the histologically normal lung, including epithelial cells (Hernandez-Pando 2000, Arriaga 2002). This could be an efficient mechanism of the bacilli to evade elimination. As these cells are considered to be non-professional phagocytes, they can not destroy ingested bacilli or present antigens in the MHC II context. Interestingly, in the mouse model of latent infection, we occasionally observed intracytoplasmic bacilli in bronchial cells and type 2 pneumocytes, suggesting that, as happens *in vitro* (Bermudez 1996), mycobacteria can also infect the lung epithelium during experimental latent infection, but in a microenvironment that is completely different from that found in

chronic granulomas. Infected lung epithelium is directly exposed to the air, so perhaps the efficient growth control or elimination of these bacilli during latent infection could be mediated by natural antimicrobial peptides such as NO or β-defensins. It is also important to consider that epithelial cells have a short life span, thus the maintenance of latent bacilli in this cell type should be different from those located in chronic granulomas.

Apart from the lung and the lymph nodes, other organs and tissues are likely to host persistent bacilli during TB latency. Indeed, nearly 15 % of the cases of reactivated TB occur at extrapulmonary sites, without apparent pathology in the lungs (Farer 1979, Hopewell 1994). In those cases, it is likely that the growth of the bacilli resumes directly from the reactivation site rather than from pulmonary sites. In this regard, it has recently been demonstrated that adipose cells could be one of these sites. *M. tuberculosis* can infect mature adipocytes by interaction with CD36, and intracellular bacilli cannot replicate and are not accessible to antibiotics. Moreover, by in-situ PCR, it was demonstrated that adipose tissue from individuals that died from causes other than TB frequently showed mycobacterial DNA, suggesting dormant bacilli infection (Neyrolles 2006).

5.4. Immunotherapy for tuberculosis

The current six-month regimens for TB treatment are too long, causing problems in logistics and compliance.; and they often lead to the development of drug resistance, which can be extreme, as demonstrated by the currently emerging extensively drug resistant TB (XDR-TB) (CDC 2006). These are the most important reasons for seeking efficient immunotherapeutic regimens in TB treatment. Immunotherapy aims at reverting the non-protective immune response, which is usually elicited during the progressive phase of the disease, to the protective Th1 response, and thus to cure TB or act as an adjunct to shorten conventional chemotherapy. Animal work suggests that it might be possible to potentiate the immune system to destroy the organisms more efficiently and even to eliminate bacilli that persist in latently infected tissues.

Following Robert Koch's discovery of *M. tuberculosis,* attempts at immunotherapy were made by Koch himself using subcutaneous injections of *M. tuberculosis* culture filtrate, and by other researchers, such as Macassey and Jousset in 1934, who used antisera raised in animals. As mentioned before, the injection of culture filtrate led to necrotic reactions, both at the site of injection and in distant lesions, and was abandoned (Anderson 1891). However, during the last decade, several studies have demonstrated striking therapeutic effects in experimental animal models by

manipulating the immune response. In general, two approaches have been used: the first one consists of direct stimulation of the Th1 response; and the second aims at the inactivation of factors that suppress the cellular immune response, which are also, usually natural anti-inflammatory factors.

5.4.1. Immunotherapy induced by direct stimulation of the protective Th1 response

The adrenal steroid dehydroepiandrosterone and its derivative, 3β,17β androstenediol, are efficient inducers of Th1 cell activation, favoring the production of the cytokines IFN-γ and TNF-α; both essential for protection against TB. Administration of either hormone has previously been shown to improve the course of pulmonary TB in Balb/c mice (Hernandez-Pando 1998). Both compounds were protective, particularly 3β,17β androstenediol, which caused reduction in bacterial counts and prolonged survival. The effects correlated with reduced expression of IL-4, and increased expression of IL-2 and TNF-α. However, these hormones have androgenic activities precluding their use in human TB. More recently, the efficacy of dehydroepiandrosterone synthetic analogs, such as 16α-bromo-5α-androstan-3β-ol-17-one (HE2000), were tested in the same model. HE2000 is a dehydroepiandrosterone derivative that does not enter sex steroid pathways, and therefore is more suitable for prolonged administration. When tuberculous Balb/c mice suffering from extensive disease were treated with HE2000, a significant inhibition of bacterial proliferation, as well as an increased expression of TNF-α, IFN-γ, and iNOS were observed, while expression of IL-4 was decreased. Moreover, when given as an adjunct to conventional chemotherapy, HE2000 further enhanced bacterial clearance (Hernandez-Pando 2004). The immunological mechanisms underlying the effects of HE2000 are not understood. It is also interesting that when HE2000 is administered as monotherapy in treatment-naïve patients with HIV-1, a significant increase is observed in the number of circulating IFN-γ+ CD8+ T cells and in the CD8+ T-cell response against two distinct GAG peptide pools (Reading 2006).

Transfer factors or leukocyte dialyzates are subcellular leukocyte components that appear to be able to transmit information for specific immune responses from experienced or memory leukocytes to naïve leukocytes (Lawrence 1955). The chemical nature of transfer factors has been difficult to elucidate, because they contain many small molecular weight components (Rozzo 1992). It seems that some transfer factor peptides correspond to the amino terminal ends of encephalins (Sudhir

1988), being very efficient factors to enhance cell-mediated immune responses (Fudenberg 1993).

Since the discovery of transfer factors, 50 years ago, the most important and interesting aspect has their therapeutic applications (Fudenberg 1993). There are many clinical reports that show the usefulness of transfer factors as efficient immunotherapeutic agents in clinical conditions characterized by inappropriate or deficient cell-mediated immune response, including different infectious diseases (Bullock 1972), some neoplastic diseases, and primary immunodeficiencies (Levin 1970, Whyte 1992). When treated with transfer factors obtained from spleen cells or peripheral blood cells of tuberculous mice or humans, Balb/c mice in a late phase of progressive pulmonary TB were able to restore the expression of Th1 cytokines, TNF-α and iNOS, to inhibit bacterial proliferation, increase DTH response, and prolong survival. This beneficial effect was dose dependent and had a synergistic effect when combined with conventional chemotherapy. Indeed, in the combined treatment, murine transfer factors eliminated bacteria from the lungs significantly faster than chemotherapy alone (Fabre 2004).

Heat-shock protein 65 (Hsp65), the mycobacterial homolog of a human stress protein, heat-shock protein 60, evokes a marked immune response in infected animals, in spite of also being highly homologous to the host stress protein (Lamb 1989, Kaufmann 1991). In addition to any putative regulatory role, a major component of the response to Hsp65 is the effect of CD8+ cytotoxic T lymphocytes that are protective in animal models (Bonatto 1998). Indeed, Hsp65-responsive CD8+ cytotoxic T lymphocytes can lyse *M. tuberculosis* infected macrophages (Silva 2000). A DNA vaccine containing the *M. leprae hsp65* sequence was therapeutic in tuberculous mice (Lowry 1999). Some researchers have not reproduced all these effects in the absence of any simultaneously administered chemotherapy, but a striking synergy with chemotherapy has been demonstrated repeatedly (Silva 2005, Nuermberger 2005). Another significant effect of the vaccine was the downregulation of IL-4-secreting T cells (Lowry 1999).

Many of the properties of the *hsp65* DNA vaccine are shared by the highly immunogenic saprophytic mycobacteria *M. vaccae,* including the induction of CD8+ cytotoxic T lymphocytes, and the downregulation of IL-4. Recombinant *M. vaccae* is a Th1 adjuvant for antigens expressed within it (Hetzel 1998, Abou-Zeid 1997), and immunization with heat-killed *M. vaccae* results in the generation of CD8+ T cells which kill syngeneic macrophages infected with *M. tuberculosis* (Skinner 1997). This property was associated with Hsp65 (Skinner 2001). Most antigens of *M. vaccae* are cross-reactive with those of *M. tuberculosis,* so it is not surprising that *M. vaccae* is able to induce a cytotoxic T cell response to *M. tuberculosis.* In

fact, the therapeutic effect of heat-killed *M. vaccae* in a Balb/c model of pulmonary TB was first published in 1996 (Rook 1996). Then in a more detailed study, it was shown that when given on days 60 and 90 after intrapulmonary infection, without any chemotherapy, *M. vaccae* caused a 1-2 log fall in bacterial counts, more granuloma, less pneumonia, and a large reduction in expression of IL-4 in granulomas (Hernandez-Pando 2000). There was an increase in IL-2 and TNF-α (Hernandez-Pando 2000).

RUTI is an experimental therapeutic vaccine made of fragmented *M. tuberculosis* delivered in liposomes made of phosphatidyl choline and sodium cholate (Cardona 2005). RUTI might be given after the initial phase of chemotherapy, when the bacterial load is greatly reduced, in order to accelerate destruction of the remaining organisms. Bacillary loads were significantly reduced with the administration of RUTI to experimentally infected animals after the termination of chemotherapy. This therapeutic effect was most likely due to the induction of CD8+ IFN-γ+ T cells in the lungs of treated animals (Cardona 2006). RUTI also induced a strong antibody response. Indeed, when these antibodies were passively transferred to SCID mice that had been infected with *M. tuberculosis* H37Rv and treated with a non-sterilizing drug regimen, sera from RUTI-treated tuberculous animals showed a reduction in the growth rate of the bacilli. In the absence of chemotherapy, however, RUTI had no therapeutic effect on late progressive disease.

5.4.2. Immunotherapy induced by suppression of the immunomodulatory anti-inflammatory response

As described before, excessive Th2 cytokine production and release of prostaglandin E and TGF-β have been implicated in the pathogenesis of TB. Thus, in addition to induction of Th1 or CD8+ cytotoxic T cell lymphocytes, downregulation of IL-4 or TGF-β is emerging as a promising immunotherapeutic protocol for established disease, with or without concomitant chemotherapy.

Evidence of the role of the Th2 response in corrupting protective functions and leading to immunopathology and fibrosis has already been described and reviewed elsewhere (Rook 2005). It is important to mention that IL-4 levels are higher in developing countries where BCG fails (Rook 2004). This may be due to both genetic (Flores Villanueva 2005) and environmental reasons, for example other tropical infections such as Th2-inducing helminthiases (Malhotra 1999). The production of IL-4 has detrimental effects in TB (Rook 2004, Rook 2005), including inhibition of apoptosis of macrophages infected with mycobacteria, and increasing intracellular availability of iron (Khanert 2006). Therefore, the effect of injecting neutral-

izing antibodies against IL-4 has been tested as a therapy for TB in Balb/c mice (Lowry 2005). A striking therapeutic effect was seen (Lowry 2005), even when monoclonal anti-IL-4 antibodies were administered as late as day 110 after infection. In this regard, it has been demonstrated that another property of *M. vaccae* is that it downregulates Th2 responses (Tukenmenz 1999, Ozdemir 2003, Zuany Amorim 2002, Hopfenspirger 2001). This ability to suppress Th2-mediated pathology is also seen when killed *M. vaccae* are given after the induction of the Th2 response has taken place (Zuany Amorim 2002, Wang 1998, Hunt 2005). The mechanism by which *M. vaccae* causes Th2 suppression is through the induction of CD25+ CD45RB^{low} regulatory T cells (Zuany Amorim 2002). This saprophyte microorganism can also inhibit an ongoing Th2 response in an allergy model, and it is at least as potent by the oral route as it is by the subcutaneous route (Hunt 2005). Therefore, the oral route should be effective in the mouse TB model. As shown for *M. vaccae* given by the subcutaneous route (Hernandez-Pando 2000), when administered by the oral route, killed *M. vaccae* organisms showed a high therapeutic value when given at the start of the infection and a less pronounced, but still significant, effect when administered after day 60 of infection (Hernandez-Pando and Rook, manuscript in preparation).

TGF-β, a potent cell-mediated immune response suppressant and anti-inflammatory cytokine, has also been implicated in the pathogenesis of TB. Blood mononuclear cells from TB patients were shown to release increased levels of TGF-β (Toossi 1995, Dluogovitzky 1999); and Balb/c mice infected by the intratracheal route showed very high expression of TGF-β during the progressive phase of the infection. Treatment with recombinant β-glycan (type III TGF-β receptor) a potent inhibitor of TGF-β, caused increased expression of IFN-γ and IL-2, with strong downregulation of IL-4, and a significant reduction in lung bacterial counts to an extent similar to that achieved by conventional antimicrobial treatment (Hernandez-Pando 2006), but with more lung pneumonic consolidation than non-treated control animals. Thus, as discussed before, TGF-β is a downregulator of cell-mediated immunity and a suppressor of excessive inflammation, thus preventing tissue damage.

TGF-β exhibits a similar immunosuppressive function in the presence of high concentrations of PGE-2, and indeed, high amounts of this molecule are produced in the lungs during late phase TB, which contributes to the modulation of the cellular immune response (Rangel 2002). What is more, the combination of soluble beta glycan and the anti-inflammatory drug niflumic acid, which blocks PGE-2 synthesis, produces a significant reduction in bacillary loads and has a significant synergistic effect on TNF-α, controling inflammation. When this combination is used

together with chemotherapy, the effects are partly additive (Hernandez-Pando 2006).

5.5. Concluding remarks

Studies on the mechanisms of disease caused by infectious agents demand a broad understanding across many specialized areas, as well as much co-operation between clinicians and experimentalists. In fact, *M. tuberculosis* infection is a fascinating model to study diverse immunopathological mechanisms because it causes a chronic disease, which provokes substantial abnormalities in the function and regulation of the immune system. Moreover, investigation of TB has also contributed seminal concepts to basic immunology. In the mid '40s, Merrill Chase demonstrated that tuberculin hypersensitivity could not be transferred by serum from skin-positive to skin-negative guinea pigs, but only by means of cells, setting the basis of cellular immunology. In the late '60s, one of Merrill Chase's graduate students, Barry Bloom, who was using mycobacterial antigens, discovered cellular mediators produced by the immune cells, such as the migration inhibitory factor, the first described lymphokine. This set the basis for the study of cytokines as essential mediators in the immune response. Thus, investigation of TB has been extremely useful in the development of immunology and immunopathology, and many concepts emerging as a consequence of ongoing research of this type will eventually contribute to novel approaches for better control of this significant infectious disease.

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Chapter 6: Host Genetics and Susceptibility

Howard E. Takiff

6.1. The difficulty in proving a genetic component for human susceptibility

6.1.1. Introduction

Tuberculosis (TB), “The White Plague” was a predominant public health problem in Europe and America in the 18th, 19th, and early 20th centuries, and considerable effort was spent trying to understand it. With the advent of effective antibiotic therapy in the ’50s, the prevalence of the disease, and research on it, declined precipitously. Since the late ’80s, however, there has been a resurgence of TB in urban settings in developed countries as well as in the developing world and Eastern Europe (Bloom 1992), and concomitantly, there has been a revival of research on TB and its causative agent, *M. tuberculosis.* Many of the questions investigated in the past are now being re-addressed at the molecular level.

One of the principal questions that occupied earlier researchers was the interplay of bacterial and host factors that determines who becomes infected and who develops TB. The discussion over the causes of TB goes back at least as far as the ancient Greeks and Romans, and basically consists of three different explanations: an inherited disorder; a contagious disease; and a disease caused by poor living conditions. Hippocrates thought it was inherited, while Aristotle and Galen believed it was contagious (Smith 2003). As the disease was most common in the urban poor, crowded into the rapidly growing cities of the recently industrialized Europe, social reformers of the time believed that TB was caused by the deplorable living conditions of the working class and rejected a contagious explanation. Although this chapter will present the published evidence supporting a heritable component to TB susceptibility, really all three explanations are correct and inter-related, which makes it difficult to separate, evaluate, and define the heritable genetic component.

While the discovery of the TB bacillus by Koch in 1882 disproved the notion that the disease had a purely hereditary etiology, or was caused solely by the unhealthy living conditions of the lower classes in the early industrial age (Hass 1996), several aspects of TB epidemiology are not explained by the germ theory and suggest that there are individual differences in susceptibility: not everyone exposed to *M. tuberculosis* becomes infected; even when infection can be demonstrated with a positive tuberculin skin test (TST), only about one in ten infected individuals becomes ill; the course of the disease varies in different individuals - before antibiot-

ics some tuberculars died rapidly of "galloping consumption" while others recovered or lived a relatively long life with chronic disease; and some infected individuals develop the disease only many years after the initial infection (Rich 1951). Without treatment, TB is fatal in about half of the patients who develop the disease.

As the disease was more common in particular families and racial or ethnic groups, a heritable component to susceptibility was a plausible assumption, but one that has defied solid experimental proof, perhaps due to the difficulty in eliminating the confounding biases of environment and exposure. In 1912, the statistician Karl Pearson, attempting to demonstrate racial differences in TB susceptibility, stated the basic question, "*We have to inquire whether persons living habitually in the same environment and with practically the same risk of infection have the same chance of developing phthisis whatever be their stock*" [cited in (Puffer 1946)].

Since the mid '80s, there have been many studies that have tried to identify genes that might be associated with TB susceptibility, as well as those testing the validity of published associations. While there are several recent reviews of the subject (Bellamy 2005, Bellamy 2006, Fernando 2006, Hill 2006, Ottenhoff 2005, Remus 2003), it is hard to come to definitive conclusions on most of the genes, because the accumulated literature is often contradictory. Studies showing that a polymorphism in a plausible gene is associated with TB susceptibility are often contradicted by subsequent work in other populations that finds no association. This has led to the recent publication of meta-analyses attempting to examine the body of published work on particular genes to determine whether a convincing consensus emerges (Kettaneh 2006, Lewis 2005, Li 2006). This chapter will attempt to summarize the current, inconclusive state of investigation on genetic determinants of TB susceptibility. In addition, it will review studies performed prior to the molecular era to illustrate the history of the field, which may help to clarify why finding genetic determinants has been elusive. It will focus on human susceptibility to *M. tuberculosis*, and will not consider susceptibility to leprosy (Geluk 2006, Schurr 2006) or other mycobacteria, except in the discussion of immune deficiencies (Casanova 2002).

The basic epidemiological designs employed in studies of genetic association, in approximate decreasing order of confidence that the results obtained are free of the complicating influences of environment and exposure are:

- twin studies comparing disease concordance in monozygotic vs. dizygotic pairs
- family linkage studies that associate the occurrence of TB in family members with the inheritance of a particular genetic marker

- case-control studies showing that, compared to controls, individuals with TB are different in some particular variable, such as exposure, race, HLA type, or the presence of polymorphisms in genes encoding elements of the immune system, such as cytokines or macrophage receptors, etc.
- anecdotal reports of family or ethnic clusters of TB cases, suggesting an increased susceptibility

As will be seen, the details and rigor of experimental design and the selection of control populations greatly affect the ability to discover associations, and the validity of the results obtained.

This tour of the literature on the genetic basis of human susceptibility to TB begins with a review of older family and twin studies that provide the basis for the belief that there is a significant component of genetic susceptibility to TB, and that show the difficulties in proving it. This is followed by an examination of racial differences in TB susceptibility, and then a summary of immunological defects, both general and specific, that confer extreme susceptibility to mycobacteria. After this comes a review of studies associating specific genes with susceptibility to common TB: first those looking at different human leukocyte antigen system (HLA) alleles; then studies on other genes thought to be important in human defense mechanisms against TB. Finally, after a review of human studies of genes equivalent to those altering TB susceptibility in mice, and work employing genomic scans, is an attempt to summarize the state of the field and put it into perspective. While this tour is not exhaustive, it attempts to critically present most of the relevant published work.

TB in famous families

French Royal Bourbon Family

Louis XIII—(1601-1643) died of galloping consumption

TB affected:	His wife
	His son – Louis XIV (1638-1715)

Simon Bolivar (1783 – 1830) died of TB

TB deaths:	Father – Juan Vincente (1786)
	Mother – Maria de la Concepción (1792)

Brontës

Chronic TB	Father (died 1861)

TB deaths: His wife

His four children:

Charlotte (1816-1855) (“Jane Eyre”)

Emily (1816-1848) (“Wuthering Heights”)

Anne (1818-1848) (“Agnes Grey”)

Patrick (1817-1848)

Ralph Waldo Emerson (1803-1882) Chronic TB

(Romantic and Transcendentalist Poet)

TB deaths: Father

Two brothers

Chronic TB: One brother

In 1949, a descendent wrote that TB had claimed lives and caused illness in 10 generations.

Henry David Thoreau (1817-1862) died of TB

(“Walden” ”On Civil Disobedience”)

TB deaths: Grandfather

Father

Sister

6.1.2. Early family and twin studies

Many early studies of TB in families compared the cumulative incidence of disease in the offspring of couples where one, both, or neither had TB, also noting other family history of TB, and whether cases were sputum positive (Puffer 1946, Stocks 1928, Frost 1933). While these studies clearly demonstrated that living in a house with a tubercular person increased the chances of developing TB, most investigators accepted that their results represented a combination of the effects of exposure and hereditary predisposition. Two examples illustrate the difficulty in separating these components.

Stocks and Karn (Stocks 1928) devised a correlation coefficient based on sibling disease concurrence expected by chance. They then used family records of 4,000 Belfast TB patients to demonstrate an excess of sibling cases occurring in families with a prior history of TB, as evidence of an inheritable factor in susceptibility. Although the attempt was interesting in its design, it could not assure comparability of environment and exposure, as a tuberculous relative could have had a confounding effect, either as a source of exposure or as a marker for lower socioeconomic status.

Puffer (Puffer 1946) attempted to separate exposure from heredity by comparing the incidence of TB in the spouses of tuberculous individuals with that in their children and siblings. Although an increased incidence of TB in the spouse of sputum positive tuberculars suggested the importance of exposure, TB was more common in consorts who additionally had a family history of TB, suggesting the greater importance of familial susceptibility. To address the obvious criticism that the spouses could have been exposed in childhood from the affected relative, Puffer stated that two thirds had no known household contact, although the contact may have been forgotten or missed. Overall, due to the near impossibility of controlling for household exposure, the family studies failed to convincingly demonstrate a genetic predisposition.

Twin studies (Table 6-1) have an experimental design that should control for the effects of environment and exposure more reliably, and several have studied inheritance of TB susceptibility. Monozygotic twins are genetically identical, while dizygotic twins are only as genetically similar as other siblings. If it can be assumed that both types of twins will share the same environments and exposures, a difference in concordance rates of TB – both twins with TB or both healthy – between the two types of twins can be attributed to the genetic components, even if multiple gene causality is suspected. The concordance in monozygotic twins can also serve as a measure of penetrance – the proportion of gene carriers who express the trait (Cantor 1992). In a large study (Kallmann 1943) performed in the United States (US) nearly three-fold greater concordance was found in monozygotic twins than in dizygotic twins, whether or not there was a history of exposure (69.2 % vs. 26.3 % with known exposure; 61.5 % vs. 12.7 % without known exposure). The concordance in dizygotic twins was the same as seen with other non-twin siblings. This study would appear to be solid evidence supporting hereditary influences, but it is weakened by several sources of potential bias specific to twin studies (Cantor 1992, Fine 1981) that are worth examining in detail because they again illustrate the difficulties in isolating genetic components from differences in exposure, and the importance of experimental design.

First, to assure validity, all affected twin pairs in the base population must be obtained. Kallman and Reisner relied upon reporting of twins from the active patients in various TB treatment facilities in New York City and New York State, a procedure that could lead to reporting bias favoring "novel" concordant monozygotic pairs, especially if there is no assurance that all twin pairs were identified. The study states that 657 twin pairs were identified, but the final analysis contained only 308 cases of "reinfection" TB, without a clear explanation of the exclusion criteria. The validity of twin studies depends upon the assumption of the equiva-

lence of environmental and exposure components, but in Kallman and Reisner's study there were more monozygotic pairs with TB in their direct ancestry (36.6 % vs. 13.4 %). They also failed to report on whether twins were living together, which tends to be more common in monozygotic pairs, and would be a source of increased concordance for uniformity of exposure, or if TB was spread from one twin to the other. In addition, even though they mention that TB is more common in females in the age group 20-35 years, the percentage of females in the two groups was not reported where the twin pairs were clustered.

Table 6-1: Twin studies

	Monozygotic		Dizygotic		Monozygotic		Dizygotic	
	Total Pairs				Concordant pairs			
Reference	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**
Diehl 1936	80	39	125	61	52	65	31	25
Dehlinger 1938	12	26	34	74	7	58	2	6
Kallman 1943	78	25	230	75	52	66	53	23
Harvald 1956	37	26	106	74	14	38	20	19
Simonds 1963	55	27	150	73	18	32	21	14

The Prophit study set out to re-examine the conclusions of Kallman and Reisner's study by trying to correct all its shortcomings (Simonds 1963). It exhaustively searched for all twins among active patients in English TB clinics, determined if the twin pairs were living together at the time of onset of TB in the index cases, whether the index case was sputum positive, and reported on the sex of all subjects. Although more concordance was found in monozygotic than in dizygotic pairs (32 % vs. 14 %), the authors believed that this difference could be explained by other factors: more female monozygotic than dizygotic twins (68 % vs. 43 %), especially in the susceptible 20-30 years age group; more monozygotic twins living together (58 % vs. 50 %); more TB concordance among those living together (42.4 % vs. 18.4 % for monozygotic, 16.3 % vs. 10.3 % for dizygotic); more sputum positive index cases among concordant pairs (72 % vs. 49 % for monozygotic, no difference for dizygotic); and more TB in parents of monozygotic than dizygotic twins (57.2 % vs. 43.5 %) – even though most of these differences were not statistically significant. Comstock's re-analysis of the data (Comstock 1978), using multiple regression to control for the sex of co-twins, age at diagnosis, type of TB,

sputum positivity of index twin, TB contact of co-twin, twins living together, and years between diagnosis of the twin pairs, still found a two-fold difference in concordance between twin types (31.4 % in evidence for monozygotic vs. 14.9 % for dizygotic; $p \leq 0.05$).

Twin studies constitute the strongest evidence for a genetic component to TB susceptibility because they control for bias better than any other experimental study design, and because there is relative consistency of the findings in most studies (Table 6-1) (Dehlinger 1938, Diehl 1936, Harvald 1956). A conservative conclusion might be that some inheritable component exists, but it has a maximal penetrance of only 65 %, and the most careful study ever performed found only 31.4 % penetrance. In other words, in as few as only a third of cases, two individuals with exactly the same genes and similar exposures will either both develop or both not develop TB.

6.1.3. Racial differences

Much of the controversy about genetic susceptibility to TB in the early part of the 20th century was concerned with allegations of racial differences, or more specifically, that Asians and especially Africans and African Americans had less innate resistance than Whites. While the near fixation on this topic by authors such as Rich (Rich 1951) might be ascribed to the prevailing racism of the period, the assumption of greater susceptibility of Africans and African Americans continues to be cited in current literature, with investigators now using molecular findings to try to explain it (Liu 2006). While Rich gave equal credit to "*the marked influence of environment... in different economic strata of individual communities within a given country*" for Whites, he attributed the higher rates in Africans and African-Americans predominantly to the effects of genetic composition. Although he cited examples of higher TB rates in Africans, he really concentrated on the more severe nature of the pathology of the disease. He proposed that because of Africa's short history of exposure to TB, Africans have not developed genetic resistance to the bacillus, and therefore many Africans, even as adults, develop a systemic, overwhelming form of the disease usually seen only in White children. While Rich states that he "*has no intention of minimizing the importance of adverse economic and environmental conditions as factors that influence the TB mortality rate of the Negro,*" one cannot help but recall the work of Dr. James McCune Smith in debunking the notion that African Americans were genetically predisposed to rickets by showing that whites of the same low socioeconomic status were similarly predisposed (Krieger 1992).

Stead and Bates (Bates 1993, Stead 1992, Stead 1997) cite several examples to support their argument that Africans and Native Americans have less resistance to TB: the greater TB mortality of the Sudanese conscripted into the Egyptian army compared to the Egyptian soldiers; the similar fate of Senegalese soldiers sent to France in the first world war; and the decimation by TB of the American Indians forced to live on US military bases. It's interesting that these three commonly cited examples all involve foreign conscripts or internees on a colonizer's military base, and rely on the dubious assumption that their physical and emotional environments were the same as those of the host soldiers.

Stead and Bates expound on the often-cited theory for the existence of racial differences in susceptibility - the duration of the exposure to endemic TB in Africa and Asia has not been long enough to select for a resistant gene pool. They postulate that a TB epidemic has a 300-year cycle, in which the more resistant survivors reproduce and increase the proportion of naturally resistant individuals in the population, so that after 50-100 years, the mortality, and subsequently morbidity, reach a peak and then progressively decline. The White populations in Western Europe and the US, where the epidemic peaked in the late 1700's and early 1800's, are now composed of individuals with a relatively resistant genetic make-up. The Africans, Eskimos and other Native Americans, however, were only exposed to TB much later, so their gene pool has yet to complete the selection for resistant individuals (Stead 1992). This theory, though still cited in current literature (Fernando 2006), is completely unproven and will likely remain so. Indeed, how could it be proven that the progressive lowering of rates for TB and other infectious diseases in Western Europe and the US, prior to the introduction of antibiotics, was the result of a changing gene pool and not of improvements in nutrition, housing, and working conditions, whose influences could outweigh any putative inheritable component (McKeown 1978)? Nonetheless, the abundance of literature describing increased susceptibility and a more progressive disease course in Africans and Native Americans suggests that some racial difference may, in fact, exist. Putting aside the theory for the origin of racial differences, are there any studies that have sufficiently controlled for environment and exposure, in order to credibly document a difference?

Kushigemachi et al. critically reviewed the epidemiological studies that relate to this question (Kushigemachi 1984). They reasoned that the only studies that could provide usable information are those that follow TST-positive groups of people for the development of disease. They cite several relevant studies from the literature (see Table 6-2), mostly isoniazid (INH) prophylaxis or bacille Calmette-Guérin (BCG) vaccine trials. In the two studies done on Eskimos, the average annual case

rates of 936 and 725 per 100,000 were much higher than rates seen in any other study, but there is no data on other risk factors. In studies predominantly involving Whites, the annual case rates varied from 29-79/100,000. The few BCG trials that included more than one race tended to show higher rates in Blacks than Whites, but both the absolute rates and the racial differences varied. In the Alabama study, the overall racial difference was predominantly due to very high rates in young Black women. The best single study was among Navy recruits, because the environment and follow-up were usually equivalent, at least once they were in the Navy. In that study, African Americans had an annual rate only 17 % higher than whites (91/78), but the Asians (195) had a rate more than double that of African Americans. It was also noted that upon entry into the Navy, highly positive purified protein derivative (PPD) reactions (> 20 mm) were more common in African Americans, which suggested that some may have entered with active disease.

Because of the variability of the rates in Whites, the small difference found in the Navy study, and the lack of data on other risk factors, the authors concluded, "*assertions that certain racial groups possess a "natural resistance" to TB are clearly unwarranted on the basis of available evidence*." The high rates in Asians and Eskimos compared to both Whites and Blacks seem less convincingly dismissed than the differences between Blacks and Whites, but risk factors, such as nutritional state, lack of a TB control program, or crowded and closed living conditions may explain the differences. In fact, after the implementation of intensive TB control measures in the Eskimo (Inuit) population in Canada, their TB rates, which had been the highest recorded in the world, showed the fastest rate of decline on record (Enarson 1986).

Stead attempted to eliminate exposure and environmental bias by studying 1,786 documented TST conversions among 13,122 residents of integrated nursing homes in Arkansas, with a similar analysis of approximately 2,000 inmates from integrated prisons in Minnesota and Arkansas (Stead 1990). The results were analyzed using multivariate analysis with a proportional-hazards model, adjusting for covariates of age, sex, and percentage of nursing home residents who were TST positive at entry. They found that Blacks had a higher rate of TST conversions (7.2 % in Whites vs. 13.8 % in Blacks overall, $P < 0.001$) regardless of the percentage of Black residents of the facility, and regardless of the race of the potential source patient. In fact, Blacks had higher rates of TST conversions even when the presumed source case was White (8.4 % vs. 15.3 %; $P < 0.001$).

Table 6-2: Race differences and TB rates

Location	Criteria for a positive reactor	Observation period (years)	Age on entry (years)	Racial group	Average annual case rates per 100,000 reactors
Muscogee, George and Russell Counties, Alabama (20,21)	> 5 mm induration to 5 T.U. PPD (Mantoux)	20	20-29	WM	78
				BM	74
				WF	32
				BF	**96**
			30-39	WM	49
				BM	75
				WF	10
				BF	**93**
			40-49	WM	104
				BM	97
				WF	32
				BF	83
			50-59	WM	141
				BM	131
				WF	107
				BF	105
Puerto Rico (22)	≥ 6 mm induration to 1 T.U. or 10 T.U. PPD-(Mantoux)	18,87	1-19	White	91
				Black	87
U.S. Navy Recruits (23)	≥ 10 mm induration to 5 T.U. (Mantoux)	4	17-22	White	78
				Black	91
				Asian	195

WM: white males T.U. = tuberculin units
BM: black males
WF: white females
BF: black females

Similar results were found in the prison populations. In contrast, however, there was no racial difference in the incidence of TB that developed in the nursing home

residents with positive skin tests. The authors interpreted this as evidence for the distinction of two aspects of TB, the initial infection and the development of disease, and concluded that Blacks have decreased resistance to the initial infection, but that once infected, they develop TB at the same rates as TST-positive Whites. This is consistent with the conclusions of the review by Kushigemachi *et al.* Although the nursing home setting convincingly controls for sources of bias, including age and sex, there is no data on the residents' weights, general health, or patterns of association and rooming. One other problem is that when no source patient was identified, the difference in TST conversion rates was greatest (4.4 % vs. 13.2 % $P < 0.001$), suggesting that the Blacks may have had some other source of infection, perhaps from visitors, which could explain all the differences. Even if African-Americans have a slightly increased rate of infection, the fact that there was no difference in the rate of progression to disease deflates the credibility of arguments that their immune system is less capable of controlling the infection. A separate study looked at TST conversion in school children exposed to a physical education teacher with TB. No racial differences were found, leading the authors to question the validity of the conclusions from the nursing home study (Hoge 1994).

The notion that the decline in TB in Europe was due to genetic selection runs counter to most thinking in the public health field. In the '70s, the historian Thomas McKeown (McKeown 1978) showed that the death rates in England and Wales from TB and other respiratory diseases declined precipitously from about 1830 to 1950, well before the advent of the BCG vaccine and anti-tuberculosis drugs (Figure 6-1). A similar decline also occurred in the United States.

McKeown concluded that improved nutrition was responsible for the decline in mortality and the increase in population, while others later argued that more important factors were the general improvements in living standards and such public health measures as improved housing, isolation of infectious individuals, clean drinking water, and improved sanitation (Szreter 2002). Nonetheless, it is generally accepted that this dramatic decrease was mainly the result of societal factors. This explanation appears plausible because: the decline was temporally linked to the improvements in living conditions and public health; the decline was too rapid and steep to be explained exclusively by genetic selection (Lipsitch 2002); the rate of decline remained steep even as the putative "selective pressure" decreased; and the dramatic decline in mortality was not limited to TB, but was also seen for many other infectious diseases (McKeown 1978). Although an element of genetic selection may also have played a role, the primacy of societal factors was demonstrated by the rise in TB rates in the US in the 1980s and '90s that accompanied the increase in homelessness and decrease in TB control measures, and set the stage for

the rampant spread of TB in the HIV-infected population (Frieden 1996). In New York City in the '90s, it was found that infection with a clustered TB strain, considered to be a marker of recent transmission, was associated with both homelessness and with being African-American. Can it then be argued that this demonstrates a genetic susceptibility to TB in the homeless? Taking into account the questionable hypothesis of extensive genetic selection for a TB-resistant population, and the lack of well-controlled, reproducible studies demonstrating that any racial group has either an increased susceptibility to infection or an increased propensity to develop disease, the notion of racial differences in susceptibility seems unproven. Nonetheless, it is certainly possible that distinct ethnic groups and populations may have different frequencies of polymorphisms that confer susceptibility or resistance to TB, and the frequency of alleles that conferred severe susceptibility in an endemic setting may be reduced over time. However, the danger in this line of thinking is that higher rates of TB in these populations may be accepted as the irremediable result of genetic make-up, rather than the consequence of lower socio-economic and health status along with poor TB control programs, which have the potential for improvement if, as demonstrated in New York City, sufficient financial and political resources are committed to the task.

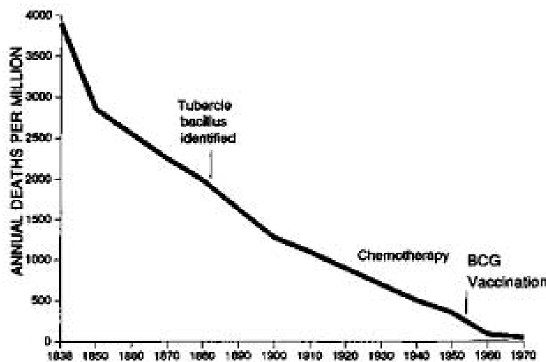

Figure 6-1: Fall in deaths from TB in England and Wales from 1838 to 1970. Most of the drop occurred before specific treatment or immunization was available. (Scrimshaw 1976). Available at: http://www.unu.edu/unupress/food/V181e/p07a.gif

6.2 Search for mutations and polymorphisms that increase susceptibility

6.2.1 Tuberculosis susceptibility in generalized immune deficiencies

Considerable insight has been obtained by studying humans with immunological deficiencies, and determining which genetic defects lead to increased risk of mycobacterial infections (Picard 2006). Undoubtedly, the largest group of highly susceptible persons are individuals infected with the HIV virus, who are prone to develop TB early in the course of the disease. After the onset of AIDS, they are also susceptible to atypical or environmental mycobacteria as well as many other pathogenic and opportunistic agents. TB takes the lives of a large percentage of AIDS patients in Africa (Cantwell 1996), and the early susceptibility underlines the overwhelming importance of CD4+ T cells in immunity to TB.

There are over 100 different primary genetic immunodeficiencies that predispose to infections with a variety of viruses, bacteria, fungi and protozoa, but only a few have been associated with severe mycobacterial infections (Casanova 2002). As might be imagined, children with severe combined immunodeficiency (SCID) who completely lack T cells are highly vulnerable to disseminated BCG infections after being vaccinated. Only a few cases of infections with atypical mycobacteria and *M. tuberculosis* have been described in these patients, but this may be due to lack of exposure, because without a bone marrow transplant, most of these children die within a year of birth.

Disseminated BCG infection, pneumonia with *M. intracellulare*, and a *M. tuberculosis* brain abscess (Metin 2004) have been described in individuals with hyper-IgE syndrome, a rare autosomal dominant disorder characterized by high serum IgE levels, eczema, and susceptibility to bacterial and fungal infections (Casanova 2002). Reports have described low levels of interleukin 12 (IL-12) and interferon gamma (IFN-γ) in several of these patients (Netea 2005), but this defect must be mild or variable, as many hyper IgE patients have been vaccinated with BCG and survived into adulthood without mycobacterial infections. A patient was recently described, who had been clinically diagnosed with hyper IgE syndrome and was unusually susceptible to various microorganisms including mycobacteria, as well as virus and fungi (Minegishi 2006). A mutation was found in the gene for tyrosine kinase 2 (Tyk2), a non-receptor tyrosine kinase of the Janus kinase family. The patient's cells showed defects in multiple cytokine signaling pathways, including IFN-γ, which were restored by transducing an intact Tyk2 gene.

The neutrophils of patients with chronic granulomatous disease lack the oxidative burst associated with ingestion of microorganisms, due to mutations in the NADPH oxidase complex. This defect in neutrophil killing makes them susceptible to severe recurrent bacterial and fungal infections. Both disseminated and local infections with BCG are fairly common in these patients (Jacob 1996), but disseminated infections with atypical mycobacteria (Moskaluk 1994, Ohga 1997), and TB (Barese 2004) have also been described, demonstrating that the phagocytic respiratory burst plays a role in the control of mycobacterial infections.

Mutations that impair signaling and activation of gene transcription promoted by Nuclear Factor kappa B (NF-κ-B) cause a rare disorder called anhydrotic ectodermal dysplasia with immunodeficiency. Affected patients are predisposed to disseminated infections with atypical mycobacteria, septicemia from pyogenic bacteria, and viral infections. This syndrome has been associated with X-linked hypomorphic (reduced function) mutations in NF-κ–B essential modulator (NEMO), and autosomal dominant hypermorphic mutations in the inhibitor of NF-κ–B (von Bernuth 2005).

Overall, mycobacterial infections occur in perhaps a third of patients with severe combined immunodeficiency and anhydrotic ectodermal dysplasia with immunodeficiency. However. it is only seen in a small percentage of patients with chronic granulomatous disease or hyper IgE, suggesting that the susceptibility is only partial. None of the other primary immunodeficiencies, including defects of HLA classes I and II, complement, B-cells, T-cells, or Toll-like receptor (TLR) signaling seem to predispose to mycobacterial infections (Ottenhoff 2005). Thus it appears that the TLR and NF-κ–B pathways are important for immunity to many types of pathogens, while, as described in the following section, the IL-12/23-IFN-γ pathway is critical specifically for immunity to mycobacteria and Salmonella.

6.2.2. Mendelian susceptibility to mycobacterial disease

Perhaps the most convincing evidence for genes involved in human susceptibility to mycobacteria has come from studying those rare patients with genetic mutations that selectively increase their susceptibility to mycobacteria, salmonella and occasionally virus (Casanova 2002, Fernando 2006, Ottenhoff 2005). Most of the mycobacterial infections in these unfortunate children and adolescents are not caused by *M. tuberculosis*, but rather by BCG after being vaccinated, or by the atypical mycobacteria that are ubiquitous in the environment (Petrini 2006). Presumably,

they are also very susceptible to *M. tuberculosis* but simply not exposed, because disease with *M. tuberculosis* has been reported in several of these patients.

The mutations responsible for this susceptibility have been identified in many afflicted individuals, and found to be transmitted by classic Mendelian inheritance. Although the defects are heterogeneous, they often occur in children of consanguineous parents, with several cases in the same family. The syndrome has been termed Mendelian susceptibility to mycobacterial diseases (MSMD). The mutations encountered are defining the human immunological response essential for controlling mycobacterial infections, which appears to be based upon the production of IFN-γ, the receptors for IFN-γ, and the subsequent downstream signal transduction that promotes the expression of the largely unidentified genes that confer immunologic protection.

Mutations causing MSMD have been found in five different genes: IFN-γR1 and IFN-γR2, the two chains of the IFN-γ receptor; IL-12B, encoding the p40 subunit of IL-12; IL-12RB1, the β1 subunit of the IL-12 receptor; and signal transducer and activator of transcription 1 (STAT1). The inheritance is most commonly autosomal recessive, but autosomal dominance has been reported in some families, and there is at least one example of X-linked recessive inheritance. In addition, the defects can be partial or complete, leading to at least 10 different disorders. Complete defects cause more severe disease than partial defects, and children with complete IFN-γR deficiencies are the most severely affected. The severity and prognosis correlate with the immune response to the infections: children who form lesions typical of lepromatous leprosy - poorly defined, with many mycobacteria but no epithelioid or giant cell - generally succumb to overwhelming infections that are often resistant to cure even with intensive antibiotic therapy. In contrast, patients who form granulomas similar to those of tuberculoid leprosy - paucibacillary, well defined, with giant and epithelioid cells - generally respond to therapy and survive (Ottenhoff 2005).

The first genetic defect identified in these patients was a complete deficiency of the IFN-γ receptor ligand-binding chain (IFN-γR1). Subsequently, kindreds were found with mutations in the IFN-γ receptor signaling chain (IFN-γR2). The recessive forms of these defects are null mutations – no IFN-γ receptor is found on the surface, and there is no response to IFN-γ in vitro. In other kindreds with complete defects, the children have IFN-γ receptors on the cell surface, but amino acid substitutions in the IFN-γR1 prevent binding of IFN-γ.

Table 6-3: MSMD, immune defects and TB susceptibility

Condition	Defect	Infections
Generalized Immune Deficiencies		
SCID	No T-cells	BCG / NTM / *M. tuberculosis* / virus / bacteria / fungi / protozoa
Hyper IgE	Low IFN-γ/IL-12	BCG / NTM / bacteria / fungi
EDA-ID		
NEMO	Low NF-κ-B function	BCG / NTM / *M. tuberculosis*
IkBa	Low NF-κ-B function	Other bacteria
CGD	No oxidative burst	BCG / NTM / *M. tuberculosis* / other bacteria / fungi
MSMD		
No response to IFN-γ		
IFN-γ R1	Complete	BCG / NTM / *M. tuberculosis* / Salmonella
IFN-γ R2	Complete	BCG / NTM / *M. tuberculosis* / Salmonella
Impaired response to IFN-γ		
IFN-γ R1	Partial	BCG / NTM / *M. tuberculosis* / virus
IFN-γ R2	Partial	BCG/NTM
STAT1	Complete	BCG/Virus
STAT1	Partial	BCG/NTM
Reduced production of IFN-γ		
IL-12p40	Complete	BCG / NTM / *M. tuberculosis* / Salmonella / other infections
IL-12RB1	Complete	BCG / NTM / *M. tuberculosis* / Salmonella
IL-12RB1	Partial	BCG
NEMO	Partial	NTM

Table modified from Casanova 2002, Ottenhoff 2005, and Fernando 2006

NTM: Non-tuberculous mycobacteria

Although patients with complete IFN-γR deficiencies have very high blood levels of IFN-γ (Casanova 2002), they all had disseminated atypical mycobacterial infections before reaching the age of 3 years; and all those who were BCG vaccinated developed disseminated BCG disease. The mycobacteria involved were both slow- and fast-growing species, and even included the generally innocuous *M. smegmatis* (Casanova 2002). These infections are life threatening and often incurable. Patients with complete INF-γR deficiencies can also be subject to severe viral infections (Dorman 1999).

Partial IFN-γR deficiencies have been attributed to mutations in both IFN-γR1 and IFN-γR2. In one form, a mutation in the extracellular segment of IFN-γR1 reduces the affinity of IFN-γ binding, while in other kindreds a mutation in the cytoplasmic domain perturbs the receptor's recycling/internalization and signaling. A single amino acid mutation in the extracellular domain of IFN-γR2 has been described that impairs but doesn't abolish the response to IFN-γ. A partial dominant IFN-γR1 deficiency, caused by a small frameshift deletion in IFN-γR1, has been described in several unrelated kindreds. The truncated proteins accumulate at the cell surface and bind IFN-γ, but lack an intracellular recycling site, so exert a dominant negative effect. Most of the IFN-γR1 dimers in heterozygotes will have at least one defective subunit and be nonfunctional, but the few normal dimers that are present are functional. The prognosis for patients with partial IFN-γR is relatively good, and many have survived into young adulthood, often without treatment.

Patients with IL-12B mutations have a complete IL-12p40 deficiency, with neither monocytes nor dendritic cells secreting IL-12 upon stimulation. Their lymphocytes secrete less IFN-γ than normal, but can be complemented by treatment with exogenous recombinant IL-12. BCG infections have occurred in all patients with complete IL-12 deficiency, and a minority also had infections with atypical mycobacteria or Salmonella.

Mutations in IL-12RB1 generally cause a complete lack of IL-12 receptor subunit IL-12Rβ1, resulting in low IFN-γ production that doesn't respond to exogenous IL-12. These patients have curable BCG and atypical mycobacterial infections, and about half have Salmonella infections. An X-linked recessive partial defect has also been described. Only one death has been reported, and there is wide variation in the clinical presentation between family kindreds and even among family members affected by the same mutation. Some individuals with documented mutations have been completely asymptomatic while their siblings have had disseminated BCG infections. This variation suggests that there may be other cytokine inducers of IFN-γ that can compensate in the absence of IL-12 signaling (Casanova 2002). The

p40 subunit of IL-12 is shared by IL-23, and the IL-23 receptor shares the IL-12β1 subunit, so defects in these genes also lead to deficiencies in IL-23 signaling (Ottenhoff 2005).

Complete STAT-1 deficiency has been described in two unrelated infants. Although they survived disseminated BCG infections, both died of severe viral infections, presumably the result of a defect in the STAT-1 mediated signaling of interferon alpha (IFN-α) through ISRE (IFN-α sequence response element), which is the key to anti-viral immunity. Partial STAT-1 deficiencies have been found to be caused by different mutations that impair either STAT-1 phosphorylation, DNA-binding (Chapgier 2006), or dimerization and translocation to the nucleus (Dupuis 2001). Some of these mutations confer susceptibility to mycobacterial infections in the heterozygous state (dominant trait), but susceptibility to viral infections only when homozygous (recessive trait).

Very recently, three kindreds were described, each with one male patient having sporadic mycobacterial infections and an X-linked recessive defect in the leucine zipper domain of NEMO (NF-κ-B essential modulator). Surprisingly, they did not display the classical features of anhydrotic ectodermal dysplasia with immunodeficiency mentioned above. In vitro studies showed a defect in IL-12 production after stimulation of monocytes and dendritic cells by CD40L-expressing T cells and fibroblasts (Filipe-Santos 2006), due to a defect in NEMO and NF-κB/c-Rel-mediated CD40 signaling.

There are other kindreds with marked susceptibility to mycobacterial infections whose genetic defect has yet to be identified, and it has been argued that genetic defects may be responsible for a much larger percentage of childhood TB than previously thought. TB in seemingly normal children in endemic areas can present as a disseminated form that is rare in adults, who tend to have only pulmonary disease. By estimating the frequency of disseminated disease in children (2×10^{-4}) the frequency of Mendelian type TB susceptibility mutations in the population (10^{-4} to 10^{-5}), and the cumulative incidence of disseminated TB among these individuals (0.5 to 0.9 cumulative penetrance), it has been postulated that mutations conferring Mendelian predisposition could be responsible for between 3 and 45 % of disseminated TB in children (Alcais 2005). While this remains unproven, the hypothesis could be experimentally tested. One recent, relevant study of children with non-tuberculous mycobacterial cervical lymphadenitis found no evidence of abnormalities in the IL-12/IFN-γ pathway (Serour 2006).

6.3. Candidate genes in common tuberculosis

The identification of the genes where mutations lead to extreme susceptibility has helped to identify the essential components of the human immune defense to mycobacteria. These genes, and several others thought to play a role in the human defense against TB, have also been studied to see if there might exist different alleles or polymorphisms that cause subtle changes in function that could account for individual variation in susceptibility to common TB. The polymorphic human leukocyte antigens (HLA) were the first proteins to be examined for associations with TB susceptibility, and reports continue to appear, making this the largest group of studies.

6.3.1 Human leukocyte antigens (HLA)

For a summary of HLA studies see Table 6-4 at
http://www.tuberculosistextbook.com/pdf/Table 6-4.pdf.

HLA alleles have been associated with susceptibility to several infectious diseases, including severe malaria, HIV progression, and hepatitis B and C persistence (Hill 2006, Yee 2004). HLA studies have also shown an association of HLA-DR2 with either leprosy per se or the type of leprosy - tuberculoid or lepromatous - in both case-control and family linkage studies, and in Asian, African, and American populations (Geluk 2006). Many studies have looked for associations of TB susceptibility with particular HLA alleles of the major histocompatibility complex (MHC). The MHC loci are divided into class I and class II alleles. The class I, HLA-A, B, and C, are thought to be principally involved in the presentation of peptides generated in the cytosol by virus-infected cells to CD8+ T cells, while the class II molecules, DR, DQ and DP, present antigens of phagocytosed pathogens, such as mycobacteria, to CD4+ T cells. In the human immune response to TB, CD4+ cells seem to be of primary importance (Flynn 2001), although CD8+ T cells probably also play a role.

While earlier studies found associations of TB susceptibility with class I alleles, there were several problems: the alleles that were found to be associated varied from study to study; the studies performed before the early ’90s determined the HLA phenotype using the lymphocytotoxic method, which had a 25 % misclassification rate compared with PCR-based techniques (Rajalingam 1996); and the studies often tested for associations to many different alleles without employing a correction for multiple testing (Bland 1995). A correction is necessary because when the statistical significance is defined at the 95 % level, as many as one in 20 alleles

tested can appear, by pure chance, to be associated. One means of correction is to multiply the probability of the association by the number of alleles tested. If the resulting probability is still below 0.05, there is more confidence that the association is real. This is termed a Bonferroni correction.

A recent meta-analysis (Kettaneh 2006) examined many of the earlier studies reporting HLA associations with TB susceptibility, but included only work involving mostly or exclusively adults, pulmonary TB, and serological determination of MHC alleles. The meta-analysis concluded that there was no significant association of pulmonary TB with class I antigens of either the A or C loci, but there was a protective effect for HLA B13 (OR 0.64, 95 % CI 0.50-0.81; $P = 0.0001$). OR is the odds ratio, which means that individuals carrying the HLA B13 allele have only a 64 % chance of developing TB compared to those without this allele. The confidence interval (CI) shows the boundaries in which the true value will be found 95 % of the time. The P value is the level of statistical confidence that the result obtained did not occur by chance. For the class II DR locus, lower risks of pulmonary TB were found for carriers of DR3 (OR 0.72, 95 % CI 0.59-0.89; $P = 0.002$) and DR7 (OR 0.65, 95 % CI 0.53-0.80; $P < 0.0001$), and a higher risk for carriers of DR8 (OR 1.72, 95 % CI 1.21-2.46; $P = 0.003$). In other words, individuals with DR8 have a 72 % greater probability of developing TB. The results for DR2 were heterogeneous, and evidence for it conferring a greater risk of pulmonary TB fell just short of statistical significance (fixed more stringently at 0.005 after a Bonferroni correction) (OR 1.67, 95 % CI 1.16-2.41; $P = 0.006$) (Kettaneh 2006). While this meta-analysis is very useful for analyzing the confusing early HLA literature, and questioning the validity of the varied associations, it is rather divorced from the recent literature because it excludes studies that use more accurate DNA-based methods for determining MHC alleles. The reason given for this is that the nomenclature varied in reports using the different methods, making comparisons very difficult. The HLA-B13, DR3, DR7, and DR8 associations have received scant attention in recent years, but the borderline DR2 association is frequently cited and is worth examining to illustrate the difficulties in unequivocally establishing associations.

The association with HLA-DR2 was reported in several studies of Asian subjects, mostly from a single group of investigators in New Delhi. In 1983, two studies from New Delhi reported an association of DR2 with TB (Singh 1983, Singh 1983). A case-control study then compared North Indian sputum-positive pulmonary TB patients with controls matched for age, sex and socioeconomic status. The difference in DR2 distribution between TB patients and controls was not significant after correction for the number of antigens tested, and the OR was only 1.6

(Bothamley 1989). A family study looked at HLA class I and II haplotype segregation in 25 multi-case families and found a significantly skewed transmission of DR2 from parents with pulmonary TB to offspring with pulmonary TB, using the method of Weitkamp (Weitkamp 1981). A re-analysis of the same family data with the LOD score method, published the following year (Singh 1984), found no evidence of HLA linkage to pulmonary TB. Because family studies generally have fewer subjects, they have less statistical power than case-control studies to find significant associations, and 100-200 families might be regarded as a minimum required to obtain reliable data.

Subsequently, a case-control study in an Indonesian population reported an association of TB with HLA-DR2 and DQw1 (Bothamley 1989), but it was not clear whether the controls came from the same community as the patient population. In addition, if only 10 % of the DR2 determinations were misclassified by the error-prone lymphocytotoxic method, the association disappears (Rajalingam 1996). These caveats are also relevant to other studies reporting an association with DR2 (Brahmajothi 1991).

A Russian study then looked at the presence of various HLA antigens in pulmonary TB patients and controls from six ethnic groups (Khomenko 1990). Different HLA antigens were found associated with pulmonary TB in the different ethnic groups, but in five of the six groups, a positive association was found for DR2, and a protective effect for the presence of DR3. After correcting the probabilities for the number of antigens tested (Bonferroni), the association with DR2 will probably maintain significance in only two of the groups.

A DR2 association was also reported in Tuvinian Mongol children (Pospelov 1996). Both HLA-DR2 and DRw53 were increased in children with TB when compared with healthy children, but not when compared to children with other chronic lung diseases. After correcting for the number of antigens tested, only the DRw53 association remained significant.

Using the more accurate oligonucleotide hybridization technique to identify the 11 subtypes of the DR2 antigen (Mehra 1995), a significant increase of DRB1*1501 was noted in pulmonary TB patients compared to controls ($p < 0.05$), and a subsequent report from the same group (Rajalingam 1996) found a slightly higher frequency of HLA-DR2 in pulmonary TB patients than in controls (Pc = 0.029, RR = 1.8 [Pc indicates a P value after a Bonferroni correction]) and a stronger association in drug-failure patients with extensive disease (Pc = 0.0001), but no association with any particular DR subtype. More recent studies have found DRB1*1501 associated with TB in India (Sriram 2001) and Mexico (OR~8) (Teran-Escandon 1999),

and with rapid onset of disease with *M. avium* in US patients with AIDS (LeBlanc 2000). The association of HLA-DR2 with pulmonary TB was not found in case-control studies of South Indians (Sanjeevi 1992), African Americans (Hwang 1985), Hong Kong Chinese (Hawkins 1988), Egyptians (Hafez 1985), Mexican-Americans (Cox 1988), Cambodians (Goldfeld 1998), or in a family study from Northern Brazil (Blackwell 1998). It is hard to reconcile the reports of associations found with DR2 and alleles such as DRB1*1501 with the many reports finding no association. Could the differences be attributable to ethnic differences, multiple testing, or study design?

In contrast to the questionable DR2 associations, a study in rural Cambodia (Goldfeld 1998) found a significant association of HLA-DQB1*0503 with pulmonary TB (P = 0.005), but no significant association with either DR2 or Tumor Necrosis Factor alpha (TNF-α) alleles. The study design strengthened the case for this association because it was done in two stages with two separate groups of patients. In the first stage, a large number of HLA alleles were tested but only HLA-DQB1*0503 appeared to be associated. Because the second stage only tested for this subtype, no statistical correction was necessary. In addition, the controls were patients seen for minor illnesses at the same hospitals, so were likely to be representative of the TB patient population. As this is a highly TB-exposed population, the authors interpreted the DQ allele as an association with development of clinical TB rather than susceptibility to infection (Goldfeld 1998). This association also appears more solid than others because there is a functional explanation for the increased TB susceptibility. The β subunit of DQB1*0503 has aspartic acid instead of alanine at amino acid 57 (Delgado 2006), which changes the charge in the cleft of the peptide binding pocket (Kwok 1996). This alters its binding affinity for antigenic peptides, reducing the affinity by five-fold for a peptide from the central region of the important TB antigen 6-kDa early secretory antigenic target (ESAT-6) (Brodin 2004). In a follow-up study, progression to TB was also found to be associated with homozygosity of other alleles with aspartic acid in position 57 of the HLA-DQB peptide (6-4): DQB1*0301, 0303; DQB1*04 (-0401, 0402); DQB1*0503; DQB1*0601, -0602, -0603. Compared with HLAβ 57-Ala alleles, presentation of the ESAT-6 peptide by HLA-DQβ 57-Asp resulted in less IFN-γ production by CD4+ T cells from TB patients (Delgado 2006). Remarkably, 41 HLA-DQB1*0503* alleles were found among TB patients, but the allele was not detected in any of the 107 TST-positive controls, suggesting that this allele in particular could have a near Mendelian effect. This allele has not been reported to be associated with TB in any other population, so could be specific for Cambodians.

However, a study of TB patients in the Venda population of South Africa (Lombard 2006) found an association of TB with some of the other β57-Asp haplotypes identified in the Cambodian study, DRB1*1302-DQB1*0602, DRB1*1302-DQB1*0603, DRB1*1101-1121-DQB1*0301-0304, and DRB1*1101-1121-DQB1*05. Seven other studies have also found some of these same alleles to be associated with TB susceptibility (Dubaniewicz 2000, Dubaniewicz 2005, Goldfeld 1998, Kim 2005, Pospelova 2005, Teran-Escandon 1999, Wang 2001), while three studies reported protective effects (Dubaniewicz 2005, Vejbaesya 2002, Wang 2001).

It has been suggested (Lombard 2006) that the haplotypes conferring increased susceptibility to TB may be maintained in the population because some of the same alleles also protect from severe malaria (DRB1*1302-DQB1*0501) (Hill 1991), from persistent hepatitis B (HLA-DRB1*1302) (Thursz 1995) (Hill 2001, Wang 2003), and from chronic hepatitis C (DRB1*1101 and DQB1*0301) (Hong 2005). Perhaps heterozygosity for these HLA alleles could protect Africans from both malaria and TB, as well as chronic hepatitis. Conversely, although the HLA-DQ β-57-Asp is associated with susceptibility to TB, it is also associated with resistance to autoimmune type-1 diabetes. Therefore, by the putative genetic selection in the European population for HLA-DQ β57-Ala alleles (HLA-DQ2 and -DQ8), Europeans, while increasing their resistance to TB, could have become more likely to develop autoimmune type-1 diabetes (Delgado 2006).

6.3.2. Cytokines and cytokine receptors

Many studies have looked for an association of TB susceptibility with polymorphisms in genes encoding other elements of the immune system thought to be important in controlling mycobacterial infections. These different polymorphisms, or alleles, which coexist in the population, are generally changes in a single nucleotide (Single Nucleotide Polymorphism, or SNP). They have mainly been evaluated in case control studies, but some have also been tested in family studies correlating the inheritance of particular parental alleles with the development of TB in both the parents and offspring.

For a summary of studies on candidate genes see Table 6-5 at
http://www.tuberculosistextbook.com/pdf/Table 6-5.pdf.

6.3.2.1. IFN-γ

From studies in mice (Flynn 1993) and investigations of humans with MSMD, it is clear that IFN-γ is critical for the defense against mycobacteria, and therefore, the

gene encoding it was an obvious candidate for polymorphisms that might slightly affect its function and alter susceptibility to common TB. At nucleotide +874, a SNP was identified that can present either a thymidine (T) or an adenine (A). In studies in Sicily (Lio 2002), South Africa (Rossouw 2003), Hong Kong (Tso 2005), and Spain (Lopez-Maderuelo 2003), the A allele, thought to produce less IFN-γ, was more common in patients with TB, whereas the T allele was more common in controls. The increase in the chances of having TB in an individual with an adenine at +874 compared to the chances in a patient with two thymidines at +874 (TT) varied from ~ 1.5 to 4.6 fold. In Croatia, an association was found only with microscopy or culture positive vs. negative TB cases (Etokebe 2006), and no association emerged from work performed in Turkey (Oral 2006), Malawi (Fitness 2004), Houston, Texas (Moran 2007) or West Africa (Cooke 2006). The study in West Africa found a minimal increase in susceptibility with two other IFN-γ alleles (OR ~ 1.45) (Cooke 2006). Thus, despite its importance in TB immunity, published studies can only suggest that polymorphisms in the IFN-γ gene might influence susceptibility to TB in some populations, but the data is inconclusive. There is even less evidence that different alleles of the IFN-γ receptor affect susceptibility. Only two (Cooke 2006, Fraser 2003) of six studies (Awomoyi 2004, Mirsaeidi 2006, Park 2004, Rosenzweig 2004) found an association of TB susceptibility with polymorphisms in the gene encoding the IFN-γ receptor 1 protein.

6.3.2.2. IL-12, IL-1, IL-10, TNF-α, IL-8

Other attractive candidate genes with contradictory studies are those encoding IL-12 and the IL-12 receptor. A study in Hong Kong Chinese (Tso 2004) found an association with two different polymorphisms in IL-12B, the gene encoding the p40 subunit of IL-12, while a study in Texas found no association with SNPs in the 3' untranslated region (3'UTR) (Ma 2003). In studies on the IL-12BR1 gene, encoding the beta subunit of the IL-12 receptor, associations with TB were found in both Morocco (Remus 2004) and Japan (Akahoshi 2003), but the associated IL-12RB1 SNPs were different in the two countries. A subsequent study confirmed the associations in Japanese patients (Kusuhara 2007), while a study in Korea tested several IL-12RB1 SNPs and found no association (Lee 2005).

Of several studies on polymorphisms in the IL-1B gene, encoding the beta chain of IL-1, two (Awomoyi 2005, Gomez 2006) found different SNPs to be associated with TB, while three found no associations (Bellamy 1998, Delgado 2002, Wilkinson 1999). A few studies have looked at polymorphisms in the IL-1 receptor, but just one found an association, and that was only with pleural TB (Wilkinson 1999).

Studies of polymorphisms in the gene for IL-10 found that in the -1082 SNP, the G allele was more common in TB patients in Cambodia (Delgado 2002), Sicily (Scola 2003), and Turkey (Oral 2006), with odds ratios of around 2. In Colombian patients, pleural TB was associated with SNPs at both -1082 and +874 (Henao 2006). No association for the -1082 SNP was found in studies in Gambia (Bellamy 1998), Korea (Shin 2005) or Spain (Lopez-Maderuelo 2003). In Korea, the C allele at IL-10 -592 and the ht2 haplotype (Shin 2005) showed slight protection (OR = 0.69). Overall, there is a suggestion of an association of TB with IL-10, especially the -1082 SNP, but the differences in susceptibility were quite modest.

The TNF-α -308 G-A polymorphism was found to protect against TB in Sicily (Scola 2003); and the -308A-238G haplotype was protective in Colombia (Correa 2005), but no association was found in studies from Turkey (Oral 2006), India (Selvaraj 2001) or Cambodia (Delgado 2002). An IL-8 polymorphism at -251 was found to confer 3.5 fold susceptibility to TB in Texas (Ma 2003), but no association with this SNP was found in the Gambia (Cooke 2004). Studies looking at other cytokines have failed to demonstrate convincing associations. Although it is always possible to suggest that differences in ethnic genetic make-up, or in study design or selection of controls can account for contradictory findings in distinct populations, the high degree of heterogeneity in the results, and the modest effects in most studies finding associations, make the putative influence of different cytokine SNPs on TB susceptibility less credible. One exception is MCP-1, described with genomic screens, below.

6.3.2.3. Vitamin D receptor

In the pre-chemotherapy era, TB was treated with vitamin D supplements, vitamin D-rich diets, and the sunlight that was the basis of the sanatorium movement (Evans 1994). As synthesis of vitamin D3 is dependent on cutaneous exposure to ultraviolet light, which is blocked by melanin, it was postulated that the putative increased susceptibility to TB (Liu 2006) in more pigmented races could be related to a relative vitamin D deficiency, especially when living in less sunny climes (Wilkinson 2000). In vitro studies have shown that the addition of vitamin D to infected macrophages augments their ability to eliminate *M. tuberculosis*, although it is not clear whether this is due to the induction of increased expression of reactive nitrogen intermediates (Rockett 1998), reactive oxygen intermediates (Sly 2001), or the anti-microbial peptide cathelicidin (Liu 2006).

Several polymorphisms were found in the gene for the Vitamin D Receptor (VDR) (Uitterlinden 2004). They were initially thought to influence bone density and osteoporosis (Sainz 1997), but subsequent studies found no convincing evidence

that they are associated with an increase in fractures (Uitterlinden 2006). However, studies have associated the different polymorphisms with susceptibility to osteoarthritis, diabetes, cancer, cardiovascular disease (Uitterlinden 2004), and TB (Bellamy 1999). Work on TB associations has focused on four polymorphisms that determine the presence or absence of four restriction enzyme sites, FokI, TaqI, BsmI, and ApaI, respectively, so that each of the four polymorphisms can have two possible alleles, designated F/f, T/t, B/b, and A/a (Bornman 2004).

An early study found that the homozygous tt genotype was underrepresented in Gambian patients with pulmonary TB, suggesting that it might be protective (OR = 0.53) (Bellamy 1999). This created sufficient interest to motivate at least eight other studies, which have reported diverse results. In Gujarati Indians in London, serum vitamin D deficiency was associated with susceptibility to TB, which appeared to be synergistically increased in individuals with the Tt or TT VDR genotypes, suggesting, again, that tt is protective (Wilkinson 2000). In contrast, the TT genotype was associated with decreased susceptibility in South Indian females (Selvaraj 2000), while the FF genotype was associated with resistance to TB in Han Chinese soldiers (Liu 2004). Six studies found no association of any individual vitamin D receptor with TB susceptibility (Bornman 2004, Delgado 2002, Lombard 2006, Roth 2004, Soborg 2007, Wilkinson 2000), although a study in Peru (Roth 2004) found that the Tt and FF genotypes were associated with faster sputum culture conversion after initiation of therapy.

Work in West Africa found that the SNPs had no independent associations with TB, but were in strong linkage disequilibrium, and the FA haplotype was transmitted more frequently than expected from TB parents to TB affected offspring (Bornman 2004). This means that the FA pair of SNPs was inherited together, perhaps with another linked gene that affects TB immunity. Finally, a study in the Venda population of South Africa found that the haplotype FbAT appeared to be protective (Lombard 2006). This would be consistent with the association of the f allele with TB in the Han Chinese (Liu 2004) and Gujarati Asians (Wilkinson 2000), and the protective effect of the TT genotype in South Indian women (Selvaraj 2000), but conflicts with the protective association of tt in the Gambia (Bellamy 1999) and Gujarati Asians (Wilkinson 2000), and the transmission of the FA haplotype to affected offspring in West Africa (Bornman 2004).

A meta-analysis of studies on the FokI and TaqI polymorphisms found the results to be inconclusive, and that the studies had too few participants (low statistical power) to prove the weak increases or decreases in susceptibility identified in those studies that found associations (Lewis 2005). In summary, while there is evidence that vitamin D promotes macrophage killing of *M tuberculosis* (Liu 2006, Rockett

1998, Sly 2001) the effector mechanism is not clear, and the association of VDR polymorphisms with susceptibility to TB remains unproven. Should there be an association of particular haplotypes with susceptibility to, or protection from TB, it seems likely to be the result of varying linkage disequilibrium with a nearby polymorphism that has functional significance in the human defense against TB. However, the relevant gene does not appear to be the vitamin D receptor, or else its effect is so minimal that it is easily obscured by other genetic or environmental factors. It is also possible that the affects of VDR polymorphisms on TB susceptibility are manifest only when the serum vitamin D levels are very low (Wilkinson 2000).

6.3.3. Pattern recognition receptors

One of the first lines of defense of the immune system is the recognition and uptake of microorganisms by professional phagocytes: macrophages and dendritic cells. On the surface of phagocytic cells are several different pattern recognition receptors, which, in the absence of adaptive immunity, bind to different patterns on microbes to promote phagocytosis and activate signaling that leads to cytokine production, antigen presentation, and the development of adaptive immunity. These pattern recognition receptors include Toll-like receptors (TLR), scavenger receptors, the complement receptors, the macrophage mannose-binding lectin (MBL), the dendritic-cell-specific intercellular adhesion molecule-3, called DC-SIGN, and others. Several of these have been shown to mediate the phagocytosis of *M. tuberculosis* (Ernst 1998), and have been studied to see whether different polymorphisms might affect TB susceptibility (Neyrolles 2006).

6.3.3.1. Toll-like receptors

Human TLRs are a family of proteins that recognize different pathogen-associated molecular patterns and stimulate signaling pathways that activate the innate immune response, cytokine production, and the process of adaptive immunity (Cook 2004). Mycobacteria are recognized by TLR1, 2, 4, and 6, which interact with the adaptor proteins MyD88 and Toll-interleukin 1 receptor (TIR) domain-containing adaptor protein (TIRAP), to activate macrophages and dendritic cells (Heldwein 2002). Signaling occurs when the TIR domain of the TLR interacts with the TIR domain of TIRAP (Yamamoto 2002). A few studies have looked at the possibility that polymorphisms in elements of the TLR system might influence susceptibility to TB. Three studies, in Turkey (Ogus 2004) Korea (Yim 2006), and Tunisia (Ben-Ali 2004) looked at different polymorphisms in TLR-2, and all found alleles that occurred more frequently in TB patients than in controls. A TLR4 polymorphism

was found to have no association with TB in a study in the Gambia (Newport 2004). A study in Vietnam found that the C558T polymorphism in TIRAP was associated with TB. The association was stronger in homozygote TT individuals, and was more pronounced when only patients with TB meningitis were considered (Hawn 2006). Interestingly, this TIRAP polymorphism appeared to impair signaling only with TLR2 ligands, but not those binding to TLR4 (Hawn 2006). These preliminary studies need to be repeated in other settings, but there is a clear suggestion that polymorphisms in the TLR2 pathway of innate immunity may influence how TB infections evolve.

6.3.3.2. Mannose-binding lectin (MBL)

MBL is a collagenous serum protein produced by the liver that participates in the innate immune system (Neth 2000). On binding to its ligands, it activates the complement cascade. Low levels of MBL in humans are caused by any of three structural mutations in codons 52, 54, or 57. The latter two polymorphisms are present at a fairly high frequency in sub-Saharan African and Eurasian populations, and have been associated with an increased risk of infection (Neth 2000). As mannose is abundant in the *M. tuberculosis* cell wall component manlam, and MBL has been shown to be a receptor for *M. tuberculosis*, studies have looked at the effect of the levels of MBL on susceptibility. While a study in India (Selvaraj 1999) found that more TB patients than controls were homozygous for low-producing polymorphisms, studies in Gambia (Bellamy 1998), China (Liu 2006), Poland (Druszczynska 2006), Turkey (Ozbas-Gerceker 2003) and Malawi (Fitness 2004) found no association. In contrast, although MBL was thought to aid in the immunity to infections, especially meningococcus (Abel 2002) (Neth 2000), studies in Denmark (Soborg 2003), Tanzania (Garred 1997) and South Africa (Hoal-Van Helden 1999) found that the lower MBL levels occurring with the alternative polymorphisms appear to be protective against TB. In the South African study, the protection was more pronounced against TB meningitis. Protection afforded by low levels of MBL is consistent with a recent finding that the binding of MBL by manlam during phagocytosis is key in limiting phago-lysosomal fusion (Kang 2005), which is thought to be important for intracellular survival of the bacteria. However, other work suggests that there has been no population selection either for or against the low producing alleles, and that MBL is probably redundant and not important in human defenses (Abel 2002, Verdu 2006).

6.3.3.3. DC-SIGN

DC-SIGN (dendritic-cell-specific intercellular adhesion molecule-3 (ICAM-3)-grabbing nonintegrin) is a lectin present on macrophages and monocyte derived

dendritic cells that recognizes many pathogens, and may modify the pathogenesis of HIV and dengue (Neyrolles 2006). It was shown to be a major receptor for *M. tuberculosis* on dendritic cells, presumably by binding to the mannose in the *M. tuberculosis* cell wall manlam (Tailleux 2003). Its expression is scant on alveolar macrophages from healthy individuals, but is variably induced after TB infection (Tailleux 2005). Two variants (-871G and -336A) have been identified in the promoter region of CD209, the gene for DC-SIGN, and the -336A allele has been shown to increase expression. In a study in South African Coloreds, these two variants were associated with a lower risk of developing TB, and the alternate nucleotides with an increased risk (-871A OR = 1.85; -336G OR = 1.48) (Barreiro 2006). The protective allele, -871G, was present in 21 % and 38 % of Asians and Europeans respectively, but was absent in Africans, which, it was postulated, could contribute to the putative increased TB susceptibility in this ethnic group (Barreiro 2006). A subsequent study from Colombia found no significant association of TB with the -336 allele, although the frequency of this allele was very low in the population studied (Gomez, Anaya et al. 2006).

DC-SIGN is an attractive candidate for influencing TB susceptibility, but more work is needed to prove an association, and there are inconsistencies in understanding how DC-SIGN might affect susceptibility. TB protection was associated with the high expression allele, -336A, suggesting that phagocytosis by DC-SIGN may somehow give the phagocytosing cell an advantage over the pathogen. In contrast, other studies suggest that phagocytosis mediated by DC-SIGN allows the pathogen to circumvent antigen processing. It was reported that DC-SIGN binding of ManLam prevents DC maturation through TLR-mediated signaling, thereby diminishing T-cell responses and fostering pathogen survival (van Kooyk and Geijtenbeek 2003). Interestingly, mice lacking SIGNR1, the murine homolog of DC-SIGN, show increased T cell activity early after infection, but no apparent alteration in susceptibility to TB (Wieland 2007).

6.3.3.4 The purinergic P2X7 receptor

Purinergic P2X7 receptors are cationic channels present on the cells in the blood and immune systems, and highly expressed on macrophages (Gu 2001). The P2X7 receptor is activated by extracellular ATP, which causes an opening of their cation-selective channel, leading to an influx of calcium and an induction of the caspase cascade, resulting in apoptosis. A calcium-dependent phospholipase D pathway is also activated, promoting phago-lysosomal fusion and mycobacterial killing.

A study of normal subjects found a polymorphism with a 1513 A-C change that causes the glutamic acid at residue 496 to be replaced by alanine, which results in a

marked decrease in the ability of the P2X7 pore to open after ATP activation (Gu 2001). Although this polymorphism was not associated with pulmonary TB in a case-control study in Gambia (Li 2002), this study identified five SNPs in the putative promoter region of the gene for P2X7, and in one, at -762, the presence of a C showed significant protection against TB, with an OR of 0.7 (C.I: 0.54 - 0.89; P = 0.003) for the heterozygote and 0.545 (CI: 0.318-.934 P = 0.027) for CC homozygotes. It was suggested that the C at -762 could affect the level of P2X7 expression by altering the binding of a transcription factor. Other loss of function polymorphisms have been identified in the P2X7 coding region, but their frequency is too low to be analyzed in association studies (Fernando 2005). However, the importance of P2X7 polymorphisms is not clear, as the differences in TB susceptibility appeared slight, at most two fold, and mice lacking P2X7 are as capable as wild-type mice in controlling pulmonary infections with *M. tuberculosis* (Myers 2005).

A study of two cohorts of Southeast Asian refugees in Australia found no association of the 1513 SNP with pulmonary TB, but, surprisingly, found a strong association of the C polymorphism with extrapulmonary TB (Fernando 2006). The odds ratio for a C at 1513 was 3.8 (CI 1.6 – 9.0; $p < 0.01$) in one cohort, and 3.7 (1.7 – 8.1; $p = 0.001$) in the second. Furthermore, in vitro studies showed that the ATP-mediated killing of mycobacteria was absent in macrophages from patients homozygous for the 1513 C allele, and impaired in macrophages from heterozygous subjects. There was a strong correlation between the capacity for mycobacterial killing and ATP-induced apoptosis. The authors postulated that decreased macrophage apoptosis leads to decreased killing of mycobacteria, permitting the bacillus to spread to other organs, both in recent infection as well as in reactivation. In one cohort, 35 % of reactive disease was extrapulmonary and showed a strong association with the 1513 C allele. While this association with extrapulmonary TB is interesting, it must be confirmed in other studies, along with the associations of MBL (Hoal-Van Helden 1999) and TIRAP (Hawn 2006) with TB meningitis, and SCL11A1 (Kim 2003), and IL-1Ra with pleural TB (Wilkinson 1999).

6.3.3.5. NOD2, surfactant proteins, complement receptor 1

Another pattern recognition receptor is the nucleotide oligomerization-binding domain 2 (NOD2). The caspase recruitment domain-containing protein 15 (CARD15) gene which encodes the NOD2 protein, is implicated in susceptibility to Crohn's disease, a granulomatous, chronic inflammatory gastrointestinal disorder for which *M. avium* subsp. *paratuberculosis* has been proposed as a causative agent (Behr 2006). The recognition of mycobacterial components by the NOD2 receptor was shown to be important for the induction of pro-inflammatory cytokines by mononuclear cells stimulated with *M. tuberculosis* (Ferwerda 2005). However,

large case-control studies in both Gambia (Stockton 2004) and South Africa (Moller 2006, Stockton 2004) failed to find an association between NOD2 and TB.

The surfactant proteins A (SP-A) and D (SP-D) are other pattern recognition elements of innate immunity that contribute to protection against virus, bacteria, and fungi (Kishore 2005). These lung surfactant-associated proteins are collagen containing calcium-dependent lectins, called collectins, and are structurally similar to MBL. They recognize many pathogens via their lectin domains and activate immune cells through their collagen regions. Surfactant protein A is a multi-chain protein encoded by the SFTP-A1 and SFTP-A2 genes, and several polymorphisms have been found in each. Polymorphisms in the SFTP-A2 gene were found to be associated with susceptibility to TB in Ethiopia (Malik 2006), Mexico (Floros 2000), and India (Madan 2002). It is likely that other studies will try to confirm these interesting associations.

Yet another of the many receptors on the surface of macrophages that have been shown to mediate the phagocytosis of *M. tuberculosis* (Ernst 1998) is the complement receptor 1 (CR1) (Schlesinger 1990). A recent large-scale study in Malawi found that homozygotes in one of five CR1 polymorphisms (Q1022H) had an increased TB risk (OR = 3.12). The SNP causes an amino acid change that may alter ligand binding, perhaps reducing the phagocytosis of *M. tuberculosis* (Fitness 2004).

6.4 Genes from mouse genetic susceptibility studies

6.4.1 *bcg/*NRAMP1/SLC11A1

Over half a century ago Lurie bred strains of rabbits that showed differing levels of resistance to infections with *M. tuberculosis* (Dorman 2004, Lurie 1952). While it is unfortunate that these rabbit strains were lost, it would have been difficult to identify the relevant genetic determinants. However, there are also susceptible and resistant strains of mice, and mouse genetics have developed sufficiently to have allowed some of the putative genes responsible for the differences to be identified.

The first gene identified was the NRAMP1 (natural resistance-associated macrophage protein 1), originally termed the *bcg* gene (Skamene 1994). It was found to be responsible for the abnormal sensitivity of a strain of mice to infections with BCG, *Salmonella,* and *Leishmania*. The encoded protein is a divalent cation transporter that appears to play a role in macrophage activation (Nevo 2006). It may also alter the phagosome environment to affect anti-microbial capacity, and regulate the levels of cations, especially iron. The gene was found only after a long

process that mirrored the development of mouse genetics (Liu 1995), but subsequently, the equivalent human gene, SLC11A1 (Solute carrier family 11, member 1), was identified rapidly (Cellier 1994). Since then, a number of studies have looked at genetic markers to see whether in humans, as in mice, there are variants that confer different levels of resistance to TB. Initial studies suggested a minor effect, with about a two-fold increase in susceptibility to TB for each of two polymorphisms, and a four-fold difference when both were present (Bellamy 1998). Results of subsequent studies have varied, and several have shown no effect (Li 2006). Doubts about the role of NRAMP1 in TB susceptibility increased after it was shown that mice that deleted for the NRAMP1 gene were as resistant to *M. tuberculosis* as wild-type mice (North 1999). Interest in the gene was renewed when a study found a SLC11A1 polymorphism to be strongly associated with susceptibility to TB in an extended indigenous Canadian family (Relative Risk = ~ 10). Stratifying the family members for TB exposure was critical in revealing the association (Greenwood 2000).

A recently published meta-analysis (Li 2006) analyzed 17 case-control studies on the association of TB susceptibility with four SLC11A1 polymorphisms (Figure 6-2):

- 5’ (GT)n (a micro-satellite with a variable number (n) of GT repeats immediately 5’ of the SLC11A1 gene
- INT4 (a single nucleotide change in intron 4: 469+14G/C)
- D543N (an aspartic acid to asparagine substitution at codon 543 in exon 15)
- 3’ UTR (a TGTG deletion located 55 bp downstream of the last codon in exon 15:1729+55del4)

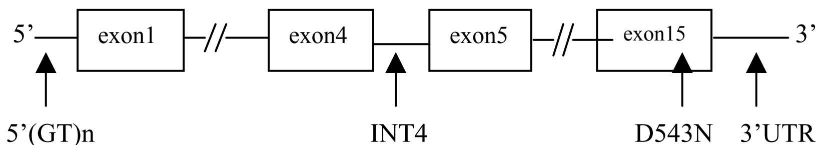

Figure 6-2: Sites of four frequently studied SLC11A1 polymorphisms

The results of the different studies were compared, the data were combined and analyzed as if they were a single group, as well as being analyzed separately for

associations in Africans, Asians, and Europeans. When the subjects in all the studies were considered as a whole, the less common alleles at the 3'UTR, D543N and 5' (GT)n showed significant associations with pulmonary TB, and individuals with these SNPs had a 27 %, 61 % and 25 % higher risk of developing TB than those with the common alleles. When only Asian populations were considered, these three polymorphisms were significantly associated with an increased risk of TB (39 %, 59 %, and 65 % respectively). Of the four polymorphisms, only the 3'UTR failed to show a significant association in African populations, but none of the four was associated with TB in Europeans. However, this lack of association may be due to the low proportion of Europeans (9.3 %) included in the studies analyzed (Li 2006). Although the association of SLC11A1 with TB is not found in all studies, there seems to be enough evidence to suggest that some of the variants have a mild effect on susceptibility. However, perhaps the true magnitude of the effect is apparent only if, as in the study of the large indigenous Canadian family, the subjects can be classified by exposure to TB, and other variables, including genetic background and general environment, can be controlled.

6.4.2 Ipr/sst1/SP110

Another potential gene candidate for genetic resistance was identified in a search for the genetic determinants responsible for the extreme sensitivity to *M. tuberculosis* shown by mouse strain C3HeB/FeJ (Kramnik 2000). This strain succumbs to infection within 4-5 weeks after infection, compared to 6-8 months for normal mice (Pan 2005). The TB lesions show pronounced necrosis, and infected macrophages display a pattern of necrosis rather than the apoptosis seen in macrophages from resistant strains (Pan 2005). This marked sensitivity, thought to involve innate immunity, was seen after infection with virulent *M. tuberculosis*, but there was little difference for infections with avirulent strains. The locus identified, termed sst1 (super susceptibility to TB), was located on mouse chromosome 1, 10 cM distal to the NRAMP1 gene.

The responsible gene was subsequently identified and termed *lpr1*, for intracellular pathogen resistance 1. Its expression is stimulated by IFN-γ and upregulated after infection in the *sst1* resistant mice, but it is not expressed in *sst1* sensitive strains of mice. Expression of *sst1* limits intra-macrophage replication of *Listeria monocytogenes* (Boyartchuk 2004) by a mechanism thought to involve innate immunity, which is dependent upon IFN-γ and reactive oxygen intermediates, but independent of nitric oxide.

When a transgenic strain of mice was constructed with the resistant form of *lpr1* replacing the sensitive form in the sensitive C3HeB/FeJ mouse, the level of resistance to *M. tuberculosis* was improved, but was not restored to that of the resistant mouse strains. This suggested that there are other genetic determinants responsible for the extreme sensitivity of the C3HeB/FeJ mouse. Transgenic studies have subsequently identified four other putative loci on chromosomes 7, 12, 15, and 17, confirming the multigenic determination of TB susceptibility in mice (Yan 2006).

In humans, the closest homologue of the predicted lpr1 protein is SP110, which is only 41 % identical but found in a region of human chromosome 2 that is syntenic to the *sst* region on mouse chromosome 1. SP110 has some of the same protein motifs as lpr1 (SP100 and SAND domains), and is a component of the nuclear body, a multi-protein complex believed to be involved in regulation of gene transcription (Thye 2006). It is predominantly expressed in leukocytes and spleen cells, with lower levels of expression in other tissues, and expression is regulated by interferon. SP110 interacts with viral proteins, and polymorphisms in SP110 have been associated with susceptibility to hepatitis C virus (Tosh 2006).

A study to determine whether variants in the SP110 gene are associated with susceptibility to TB was carried out in three West African countries (Tosh 2006). Families of TB patients were analyzed with transmission disequilibrium testing to see if certain variants were transmitted more frequently to affected offspring. Three SNPs were significantly associated with TB in Gambia, but only one of these, rs2114592, also showed a significant association in families from both Guinea-Bissau and the Republic of Guinea, while another, rs3948464, showed associations that were significant in the Republic of Guinea, but not in Guinea-Bissau. Several of the associations would not be significant after a Bonferroni correction for the 20 SNPs tested. The variants were found to be in strong linkage disequilibrium in a region of low haplotype diversity, so it is possible that it is actually another polymorphism in this region that has a functional role in altering TB susceptibility.

However, the role of SP110 in human susceptibility to TB remains unproven. A recent study described cohorts of children with mutations in SP110 who suffered from an autosomal recessive disorder of hepatic vascular occlusion, severe hypogammaglobulinemia, combined T and B cell immunodeficiency, absent lymph node germinal centers, and absent tissue plasma cells (Roscioli 2006). Their immunodeficiencies made them prone to infections with *Pneumocystis jerovici*, enterovirus, and mucocutaneous candidiasis, but not mycobacterial infections. While this would seem contradictory with the role of lpr1 in mice, it should be recalled that its effect in mice was only seen with virulent *M. tuberculosis*, and not avirulent BCG (Pan 2005). Thus, the unfortunate children with the SP110 mutations may not be

abnormally susceptible to BCG or atypical mycobacteria, and may not have been exposed to *M. tuberculosis*. Further doubts about SP110 come from a large case-control study in Ghana that examined 21 SNP variants and found no association (Thye 2006). Additional surveys are needed to determine whether SP110 is truly associated with TB susceptibility.

6.4.3. Genomic screens - family studies revisited

Blackwell *et al*. “scanned” the mouse genome for loci involved in susceptibility to leishmaniasis by comparing sensitive and resistant strains of inbred mice for hundreds of genetic markers across the entire genome (Bellamy 2006, Blackwell 1996). Five major chromosomal regions were identified, including the NRAMP1 locus. Possible associations with the homologous regions of the human chromosome were then examined in a large study in Belem, Brazil (Blackwell 1997), using multicase families for TB (98 families; 704 individuals), leprosy (72 families; 389 individuals), and leishmaniasis (89 families, 638 individuals), all from the same socio-economic strata (Shaw 1997). All relevant family members were studied using a combination of gene polymorphisms and microsatellite markers to trace the inheritance of the human chromosomal regions equivalent to those identified in mouse studies.

Leprosy was associated with both TNF-α and DR2 (Blackwell 1998), but there was no association of TB with TNF-α or any HLA locus, and only a weak association with NRAMP1, or a gene close to it. An association with TB susceptibility was found for the locus 17q11.2-q12 (LOD-score 1.3 P= 0.01), a region that is similar (syntenic) to a region on mouse chromosome 11 that is associated with susceptibility to leishmaniasis. Genes in this region encode several proteins that could be plausibly linked to TB immunology: NOS2A (Rockett 1998), encoding the inducible form of nitric oxide synthetase (Blackwell 1998); chemokines CCL2/MCP-1 (monocyte chemoattractant protein-1) (Lu 1998), CCL3/MIP-1α (macrophage inflammatory protein-1α), CCL4/MIP-1β, CCL5/RANTES (regulated on activation, normal T cell expressed and secreted), CCR7, the receptor for CCL19/21, and several genes encoding signal transducers and activators of transcription: *STAT3*, *STAT5A*, and *STAT5B*. A subsequent study of 92 multicase families looked for associations with particular genes in this region. Using 16 microsatellites and 69 SNPs, associations were found for four genes that are separated by fairly large intervals (NOS2A-8.4 Mb-CCL18-32.2 kb-CCL4-6.04 Mb-STAT5B). Conditional logistic regression using a case/pseudo-control data set showed that each gene contributed separately, suggesting that this is a cluster of susceptibility genes.

However, after correcting for the number of markers tested, the only significant association was with a SNP in cytokine CCL18 (Jamieson 2004). The authors calculated that in order to detect a gene that causes at least a two-fold or greater effect on TB susceptibility or resistance among variant alleles with < 0.2 frequency, a study would need 400 affected families composed of at least one patient and their parents. If the effect was less than two-fold, and the variant allele frequency < 0.1, the association would not be found even with 800 parent/child trios (Jamieson 2004).

A separate two-stage study of 16 Brazilian multi-case TB families first used 405 markers to scan the whole genome for regions associated with TB, and then further examined the 58 markers that produced a positive result, using a second set of 22 families and additional markers (Miller 2004). Associations were confirmed for three chromosomal regions, 10p26.13, 11q12.3, and 20p12.1, but no association was found for the 17q11.2-q12, perhaps because of the limited study size.

A large case-control study then looked for associations with different genes at the 17q11.2 locus in Mexican and Korean TB patients (Flores-Villanueva 2005). No association was found for NOS2A, RANTES or MIP-1α alleles, but a significant association was found for the adenine (A) to guanine (G) change in the -2518 promoter polymorphism of monocyte chemoattractant protein-1 (MCP-1), also known as, SCYA2 and CCL2. Compared with AA homozygotes, both Mexican and Korean AG heterozygotes had an increased risk for developing TB of 2.3 and 2.8 fold respectively, while GG homozygotes were 5.4 fold and 6.9 fold more likely to develop TB. The G allele increases production of MCP-1, and the higher blood levels of MCP-1 in the GG homozygotes were correlated with lower levels of IL-12 subunit IL-12p40, which has been shown in Mendelian disease to be critical for control of the infection. Furthermore, the hypersusceptible GG allele was present in 53 % of Mexican TB patients, compared with 27 % of controls, and 36 % of Korean cases, compared with 14 % of controls. This allele could thus be responsible for as much as 64 % of TB cases in the Mexican population, and the TB rates might be 64 % lower in a population without the G allele (Alcais 2005).

In mice, CCL2, the equivalent of MCP-1, and its receptor, CCR2, are important for protection from high dose *M. tuberculosis* infections (Peters 2001), but CCL2-/- or CCR2-/- mice infected with lower doses of *M. tuberculosis* have outcomes similar to wild-type mice, despite impaired macrophage recruitment to the lungs (Scott 2002, Kipnis 2003). Mice expressing high levels of MCP-1 show increased susceptibility to TB (Gu 1997).

Why was there no association with MCP-1 seen in the Brazilian study? Perhaps the ability to demonstrate this association in the Mexican/Korean study was aided by its strict criteria for study participants: sputum smear-positive new adult TB patients with culture-confirmed disease, excluding those with chronic illnesses, including malnutrition, or previous episodes of TB. The patients also had "*clinical and epidemiological features suggestive of active TB of recent evolution after recent exposure*" (Flores-Villanueva 2005). Controls were healthy TST-positive and TST-negative persons not vaccinated with BCG, who had had recent contact with a TB case. The three groups were similar in demographics and body mass index (before developing TB), household income, and consumption of cigarettes and alcohol. Using these strict inclusion criteria, the study could distinguish the predisposition for progression to clinical disease from susceptibility to infection. The MCP-1 G allele was as common in TST-positive as in TST-negative persons, showing that it had no effect on susceptibility to infection (Flores-Villanueva 2005).

A larger, two-stage genome wide study (Bellamy 2000) was performed by analyzing families from Gambia and South Africa that had at least two siblings with TB - 83 families in the first stage and 53 in the second. Associations were only found for two chromosomal regions, 15q and Xq, with LOD scores of 2.00 and 1.77 respectively. LOD scores of at least 3.0 are generally considered to indicate a strong association, so this, as well as the Belem work, suggested that there are no dominant genes responsible for TB susceptibility. Instead, perhaps many genes are involved, each exerting a small effect. This study found no association with NRAMP1 or the vitamin D receptor, both of which were found to increase susceptibility to TB about two fold in case control studies in the same Gambian population (Bellamy 1998, Bellamy 1998). However, as mentioned above, for a family association study to identify genes with such weak effects, an unreasonable number of families would be required (Bellamy 2000). A subsequent study fine-mapped the 15q region and found that the strongest association was with a region containing the gene UBE3A, which encodes a ubiquitin ligase involved in the ubiquinitination and degradation of specific proteins, including the T lymphocyte src kinase Lck (Cervino 2002). There have been no subsequent reports associating this gene with susceptibility to TB.

A different conclusion came from a whole genome scan of 96 multi-case Moroccan families, each having at least two siblings with pulmonary TB (Baghdadi 2006). No associations were seen for the 15q and Xq loci, the 17q11-q21 locus (Flores-Villanueva 2005, Jamieson 2004) or the 10p26.13, 11q12.3, and 20p12.1 loci (Miller 2004) found in the Brazilian studies, but a strong association (LOD = 3.49)

was found for a single region of chromosome 8q12-q13. Although this locus was not associated with TB in all families, the association was especially strong (LOD 3.94) when the families had at least one parent with TB, suggesting that predisposition to TB is inherited as an autosomal dominant trait. In contrast to the notion that TB susceptibility is determined by the sum of many genes, each exerting only small effects, this study suggests that there are genes with large, dominant effects. This model bridges the gap between Mendelian susceptibility mutations and determination of susceptibility by a quorum effect involving multigenic determinants (Casanova 2007).

6.5. The good, the bad and the maybe, in perspective

While the work on the Mendelian inheritance of genes responsible for extreme susceptibility to mycobacterial infections is clear, convincing and informative with respect to the human immune response to TB, the genetic components of common TB infections remain unclear. Considering all of the genes that have been tested for association with susceptibility to TB, in many diverse populations, using a variety of study designs and exclusion criteria, is there any way to make sense of the varied and often contradictory results? While the frequent lack of clear and reproducible associations may result from the difficulty in isolating the effects of a particular gene from the background of many genes involved in determining susceptibility to TB, there is also a suggestion that some genes may have large, dominant effects, at least in particular populations (Casanova 2007).

One approach to try to understand the literature might be to classify or stratify the genes into different categories. The first group would contain genes that have never or have only rarely shown an association, generally of small effect. This group would include most HLA alleles from the early studies, TNF, NOD, TLR-4, and probably MBL, although its association with TB meningitis deserves further study. There are also other genes, not reviewed here that have failed to show evidence of an association (Gomez 2006, Rajalingam 1997).

A second group, also fairly easy to identify, are those few genes with alleles that appear to confer important increases in susceptibility: HLA-DQB1*0503 and HLA-DQB1 alleles with an aspartic acid at position 57; MCP-1 (Flores-Villanueva 2005); and an as yet unidentified gene in locus 8q12-q13(Baghdadi 2006). In two carefully performed studies of adult pulmonary TB in Cambodia, the HLA-DQB1*0503 allele was found to have near Mendelian effects, being present in less than 10 % of patients with TB but in none of the controls (Delgado 2006, Goldfeld

1998). When this allele was excluded, individuals homozygous with other HLA-DQβ57Asp alleles had a 2.35-fold increased risk of developing TB, an effect also seen in South Africa (Lombard 2006) and several other populations (see Table 6-4). However, Asp/Asp homozygosity was present in only 26 % of the Cambodian TB patients, and was also found in 10 % of PPD+ healthy controls. While the Asp at aa 57 of the HLA-DQ beta chain reduces antigen presentation in a manner consistent with an increased risk of developing TB (Delgado 2006), the increase in susceptibility is only about two-fold, so other factors must be involved. The much greater effect on susceptibility conferred by the HLA-DQB1*0503 allele remains unexplained, and has only been reported in Cambodians.

MCP-1 (Flores-Villanueva 2005) is presumably the gene responsible for the association found at the 17q11.1-q12 locus (Jamieson 2004). Studies on the -2518 promoter SNP found that GG homozygotes and AG heterozygotes showed increased TB risk in both Mexican (OR = 2.3, 5.4) and Korean (OR = 2.8, 6.9) populations. The GG genotype was found in a striking 53 % of the Mexican TB cases (Alcais 2005) suggesting that the allele could have a major effect on the TB burden in the population. However, 27 % percent of PPD+ healthy controls were also GG homozygotes, and 48 % were AG heterozygotes. Although the GG genotype may confer a large increase in susceptibility, many TB infected GG homozygotes and AG heterozygotes don't develop TB, implying that other factors must be involved in determining who gets TB, both in the GG and the non-GG TB infected population.

The proposed autosomal dominant locus on chromosome 8q12-q13 gave a LOD score of 3.4 overall, and 3.9 in the Moroccan families with an affected parent. This association is stronger than reported for genes in any other affiliation study, although the locus was not associated with TB in all families examined (Baghdadi 2006). However, the reports describing the associations of this locus, and of MCP-1, are very recent and require confirmation, and the associated gene in the 8q12-q13 region remains to be identified.

The third group contains genes reported to be associated with susceptibility to TB, but whose associations either await confirmation or were not confirmed in all subsequent reports. These promising gene candidates requiring further study include: TLR-2, TIRAP, P2X7, DC-SIGN, Sst/SLC110, IL-12RB1, CR1 and the surfactant protein A subunits SFTPA1 and A2. More difficult to classify are the genes encoding IL-10, IL-8, IL-1, IL-12, VDR, and IFN-γR1, which were associated with minor changes in TB susceptibility in some reports, but no association in several others studies. Similarly, associations with HLA alleles, such as B13, D2, D3 (Kettaneh 2006), or DRB1*1501*, have been found in some studies but not in others (see Table 6-4), and an association with the MHC region has not been found

in any genome scan. Because MSMD has shown the importance of IFN-γ, it was tempting to think that its polymorphisms might affect susceptibility to common TB, but the heterogeneity of results with the IFN-γ polymorphisms, especially the +874 SNP, make it hard to come to any conclusion. In addition, the supposedly susceptible +874 AA genotype is present in more than 40 % of controls.

Finally, there is NRAMP1/SLC110, which was identified by comparing innately susceptible and resistant mouse strains, and found to have minor effects in some of the many case-control studies that looked for an association, and a minor association in one genome screen (Blackwell 1997). However, it appeared to have a major effect (RR = 10) in one carefully studied extended indigenous Canadian family (Greenwood 2000). A meta-analysis concluded that three polymorphisms showed significant associations with pulmonary TB, but only increased susceptibility by 27 %, 61 % and 25 %.

While many of the first gene candidates tested, such as VDR, IFN-γ, and IL-10 have given very heterogeneous results, perhaps more recent candidates, such as TLR2, DC-SIGN or SFTPA1 and 2 will prove more robust. However, most of their effects are not large, being generally less than three-fold. Is there any way to explain the difficulty in conclusively identifying the genes that determine why not all those exposed to *M. tuberculosis* become infected, and why only 10 % of those infected develop the disease? Is it possible to explain why genes associated with susceptibility in some studies often fail to demonstrate an association in others? Some possible explanations include:

- TB susceptibility is cumulatively determined by the sum of many different genes, each having small effects, and the genes may be different in different populations. This could certainly be possible, and is consistent with data in mice (Yan 2006), but proof would likely require the technical capacity to sequence hundreds of genes in hundreds or thousands of individuals (Hill 2006).
- Different ethnic groups have major different determinants of susceptibility. This seems appropriate for the marked effect of HLA-DQB1*0503 in Cambodians, and perhaps other loci, but can’t explain the varied results in similar ethnic groups, such as in different West African countries (Tosh 2006).
- Much of TB susceptibility is determined by the large effects of predominant genes, but most of these have not yet been identified (Casanova 2007).

While all of these explanations may be true to some extent, there are other important variables that could help account for the heterogeneity of results: exposure, strain virulence and general environment. These differences were recognized as nearly insurmountable confounding difficulties by the investigators of the early and mid 20th century, who knew that valid associations would only be detected if all epidemiologic variables were carefully controlled. Many of the molecular studies that showed the clearest associations had rigorous criteria for defining both cases and controls to ensure, for example, that the study was looking only at the development of pulmonary TB in recently infected adults of the same ethnic group, age, nutritional and socioeconomic status, with no complicating risk factors (Flores-Villanueva 2005, Moran 2007).

Even the most rigorous exclusion criteria can't control for all important variables. While in mouse experiments animals are infected with a uniform dose and delivery of a single strain of *M. tuberculosis*, human subjects in a study are generally infected with a variety of strains with varying levels of virulence (Lopez 2003). However, even if a study looking for associations were to perform molecular epidemiology on all the strains involved, and could assign a measure of relative virulence to each strain, how could it evaluate the differing intensities of exposure - the number of bacilli that each subject inhaled? Could it be possible that a particular genetic make-up would be able to avoid either infection or disease after a low-dose exposure to a low-virulence strain, but succumb to the same level of exposure to a more virulent strain, or a much higher dose of the less virulent strain? Perhaps there are alleles that make a person resistant to 80 % of the *M. tuberculosis* strains in a community but susceptible to the most virulent 20 %. While family studies should control for strain differences, the small effects of multiple genes would only be found if very large numbers of families were studied, and the most important genes may vary from family to family. To further complicate the analysis, the concordance rate in twin studies was, at most, about 50 % – so identical genes may not yield identical results at least half the time. Given the differences in the strain virulence and exposure within a population, and the genetic heterogeneity and apparent incomplete penetrance of the responsible genes, it should not be surprising that it is difficult to obtain clear, reproducible associations with specific alleles, even those that may have moderate effects.

While documenting or quantifying exposure to the bacillus, or strain virulence, may be difficult, their roles in pathogenesis are obvious. In contrast, environmental influences are not only difficult to document and quantify (Lienhardt 2001), but their effects have not been well studied and are poorly understood. Trudeau performed a classic study that demonstrated the importance of environment to the

development of TB. He compared two groups of infected rabbits: five animals were free to roam outdoors with ample food, while another five were kept in dark cages with minimal food. Within 3 months, four of the five caged rabbits died of TB, and the fifth developed serious illness. In contrast, only one of the free rabbits succumbed to TB, and the others remained healthy after six months (Smith 2003). The reasons for the difference - poor nutrition (Chan 1996, Dubos 1952), crowded living conditions, or emotional stress (Stansfeld 2002) - and the mechanism of their effects on the immune system, are unclear. Nonetheless, environmental influences and differences in exposure appear to affect susceptibility to TB, and their potential as confounders or effect modifiers can confuse, obscure, or invalidate attempts to identify genetic determinants.

The importance of environmental factors in human TB was illustrated by the change in TB mortality rates in Belgium and the Netherlands during the First World War. Before the war, in 1913, the rates were 118 and 142/100,000 for Belgium and the Netherlands, respectively, but by 1918, the rates had increased to 245 and 204/100,000 (Rich 1951). Another example was in Warsaw, Poland, where, in the early 20th century it was thought that there was a genetic explanation for the lower rates of TB mortality in the city's Jews, compared to non-Jewish Poles. However, the relative rates were inverted during the prolonged Nazi assault on the Warsaw Jewish Ghetto during World War II (Dubos 1952).

While the spread of TB, and the rates in a population - the likelihood that an individual will contract TB - are strongly influenced by general socioeconomic status, and possibly also stress levels (Farinpour 2003), perhaps the most important determinant is the quality of the local TB control program. It may be difficult to separate these factors however, because deteriorating and traumatic social conditions are often accompanied by a collapse of the healthcare system. A recent example is the dramatic rise in TB rates in Russia after the fall of the Soviet Union (Figure 6-3).

By the late '90s the death rate from TB for men aged 20-24 years was twice what it was in 1965, and while deaths from many causes began to fall in Russia after 1994, those from TB continued to rise. Part of this may be explained by falling living standards, prison TB, and mass migrations, but the most important component was likely to have been the crumbling healthcare system (Shilova 2001). While the Soviet system had kept TB under control, after its fall, as described by Professor Margarita Shilova, Head of the Tuberculosis Epidemiology Department at Moscow's Phthisiopulmonology Research Institute, "*suddenly, the money stopped. There were no drugs, communication with local hospitals broke down … the system broke down*". The result was a 7.5 % annual increase in new cases from 1991-99 (Shukshin 2006).

Figure 6-3 Increase in the reported incidence of TB in the Russian Federation, 1990-2001. Available at http://missinglink.ucsf.edu/lm/russia_guide/Russianhealth2.htm

Each of these examples illustrates how the TB rates in a population changed drastically due to changes in living conditions and TB control programs, while the genetic composition of the population remained constant. The point is that socioeconomic conditions and TB control programs can modify and perhaps override the effects of genetic composition in determining susceptibility to TB, except in cases of Mendelian inheritance of extreme susceptibility. Will identifying genetic determinants of susceptibility contribute to the control of TB? Given that susceptibility seems to be determined by a complex interplay of strain virulence, intensity of exposure and environmental factors, as well as human genetic composition, would it be feasible or advisable to target vaccines, prophylaxis, treatment, or control efforts based on the genetic composition of individuals, families or ethnic groups, instead of simply improving control programs (and socioeconomic status, although more difficult) for the entire population? In the resource-poor countries where TB is endemic, would it be feasible or ethical to screen the population to decide who to vaccinate, or who to treat with prophylaxis? Might it be more efficient and less costly simply to concentrate on diagnosing and effectively treating cases, and using extra funds for contact tracing? In populations with alleles that are highly associated with increased susceptibility, such as HLA-DQB1*0503 in Cambodians, the results of a cost-benefit analysis could depend upon the frequency of the relevant

alleles, the cost of detecting them, their importance in reactivation TB, and the target population to be tested - contacts, PPD positives, children, or the general population.

The identification of genes responsible for Mendelian inheritance of extreme susceptibility has helped identify the essential elements of human immune defense against mycobacteria, and the discovery of genetic determinants of susceptibility to common TB could further this knowledge and perhaps lead to the development of better vaccines, more precise evaluations of candidate vaccines, new diagnostic tests for active and latent disease, and therapeutic strategies for immunological intervention. In light of the continuing presence of multi-drug resistant strains (Raviglione 2006), and the difficulties in finding and bringing new drugs and vaccines into clinical use, further investigation in the field may be justified, despite the relatively disappointing results obtained so far.

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Chapter 7: Global Burden of Tuberculosis

Ernesto Montoro and Rodolfo Rodriguez

7.1. Global epidemiology of tuberculosis

The consequences of tuberculosis (TB) on society are immense. Worldwide, one person out of three is infected with *Mycobacterium tuberculosis* – two billion people in total. TB accounts for 2.5 % of the global burden of disease and is the commonest cause of death in young women, killing more women than all causes of maternal mortality combined. TB currently holds the seventh place in the global ranking of causes of death. Unless intensive efforts are made, it is likely to maintain that position through to 2020, despite a substantial projected decline in disease burden from other infectious diseases (Dye 1999, Smith 2004).

Effective drugs to treat and cure the disease have been available for more than 50 years, yet every 15 seconds, someone in the world dies from TB. Even more alarming: a person is newly infected with *M. tuberculosis* every second of every day. Left untreated, a person with active TB will infect an average of 10 to 15 other people every year (Dye 2005).

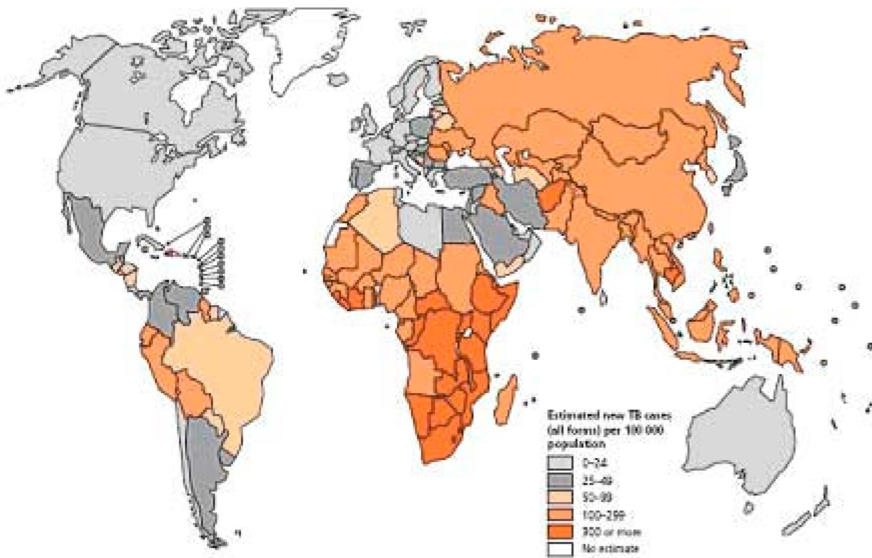
TB hinders socioeconomic development: 75 % of people with TB are within the economically productive age group of 15-54 years. Ninety-five per cent of all cases and 99 % of deaths occur in developing countries, with the greatest burden in sub-Saharan Africa and South East Asia. Household costs of TB are substantial (Dye 2006, World Health Organization 2006a).

In most countries, more cases of TB are reported among men than women. This difference is partly due to the fact that women have less access to diagnostic facilities in some settings, but the broader pattern also reflects real epidemiological differences between men and women, both in exposure to infection and in susceptibility to disease. In regions where the transmission of *M. tuberculosis* has been stable or increasing for many years, the incidence rate is highest among young adults, and most cases are caused by recent infection or reinfection. As transmission falls, the caseload shifts to the older age groups, and a higher proportion of cases come from the reactivation of latent infection (Borgdorff 2000).

While the human immunodeficiency virus (HIV) infection has clearly had a profound effect on TB epidemiology, other potentially important risk factors have been somewhat neglected. In the coming years, more attention needs to be given to the interaction between chronic diseases and TB, including diabetes, undernutri-

tion, and respiratory illnesses caused by tobacco and air pollution (Corbett 2003, World Health Organization 2004).

Although the “direct costs” of diagnosis and treatment are significant for poor families, the greatest economic loss occurs as a result of “indirect” costs, such as loss of employment, travel to health facilities, sale of assets to pay for treatment-related costs, and in particular, lost productivity from illness and premature death (Smith 2004, Floyd 2003, World Health Organization 2005a).



Source: WHO report, 2006

Figure 7-1: Estimated TB incidence rates, 2004

The World Health Organization (WHO) estimated 8.9 million new cases of TB in 2004 (140/100,000 population). About 3.9 million cases (62/100,000) were acid fast bacilli (AFB) sputum smear-positive, the most infectious form of the disease. There were 14.6 million prevalent cases (229/100,000), of which 6.1 million were AFB sputum smear-positive (95/100,000). An estimated 1.7 million people (27/100,000) died from TB in 2004, including those co-infected with HIV (248,000). The WHO African region has the highest estimated incidence rate (356/100,000), but the majority of patients with TB live in the most populous countries of Asia; Bangladesh, China, India, Indonesia, and Pakistan together ac-

count for half (48 %) of the new cases that arise every year (Figure 7-1). In terms of the total estimated number of new TB cases arising annually, about 80 percent of new cases occur in the 22 top-ranking countries (Dye 2006, World Health Organization 2006a).

In 2004, the estimated TB incidence per capita was stable or falling in five out of six WHO regions, although it was still growing at 0.6 % per year globally. The exception is the African region, where the incidence of TB was still rising, in line with the spread of HIV. However, the rate of increase in the number of cases notified from the African region is slowly decreasing each year, probably because the HIV epidemic in African countries is also slowing. In Eastern Europe (mostly countries of the former Soviet Union), the incidence per capita increased during the '90s, peaked around 2001, and has since fallen. The average downturn in case notifications in Eastern Europe is mainly due to data from Russia and the Baltic States of Estonia, Latvia, and Lithuania; however, incidence rates might still be increasing in the central Asian republics of Tajikistan and Uzbekistan (Dye 2006, World Health Organization 2006a).

In all other regions (Table 7-1), the incidence rate was stable or decreasing continuously between 1990 and 2003. The downfall was relatively quick in Latin America, Central Europe and the established market economies. In summary, the global trend in incidence rate was increasing most quickly at 1.5 % per year in 1995 but has since been decelerating. If the trends suggested by the case notifications are correct, and if these trends persist, the global incidence rate will reach about 150 per 100,000 in 2015, resulting in more than 10 million new cases in that year (Dye 2006, World Health Organization 2006a, World Health Organization 2006 b).

Global efforts to control TB were reinvigorated in 1991, when a World Health Assembly resolution recognized TB as a major global public health problem. Two targets for TB control were established as part of this resolution – detection of 70 % of new AFB smear-positive cases, and cure of 85 % of such cases by the year 2000. Despite intensified efforts, these targets were not met; more than 80 % of known cases are successfully treated, but only 45 % of cases are detected (World Health Organization 1993, World Health Organization 1994, World Health Organization 2006a).

Table 7-1: Estimated incidence, prevalence and TB mortality, 2004

WHO region	Incidence				Prevalence		TB Mortality	
	All forms		Smear-positive					
Africa	2 573* (29§)	356$	1 098*	152$	3 741*	518$	587*	81$
The Americas	363* (4§)	41$	161*	18$	466*	53$	52*	5.9$
Eastern Mediter-ranean	645* (7§)	122$	289*	55$	1 090*	206$	142*	27$
Europe	445* (5§)	50$	199*	23$	575*	65$	69*	7.8$
South East Asia	2 967* (33§)	182$	1 327*	81$	4 965*	304$	535*	33$
Western Pacific	1 925* (22§)	111$	865*	50$	3 765*	216$	307*	18$
Global	**8 918* (100§)**	**140$**	**3 939***	**62$**	**14 602***	**229$**	**1 693***	**27$**

* number (thousands) Source: WHO, 2006
§ % of global total
$ per 100,000 pop.

Since 2000, the United Nations Millennium Development Goals have provided a framework for evaluating implementation and impact under target 8 (among 18), which is to "*have halted by 2015 and begun to reverse the incidence of malaria and other major diseases*" (including TB). Although the objective is expressed in terms of incidence, the Millennium Development Goals also specify that progress be measured in terms of the reduction in TB prevalence and deaths. The target for these two indicators, based on a resolution passed at the 2000 Okinawa (Japan) summit of G8 industrialized nations, and subsequently adopted by the Stop TB Partnership (www.stoptb.org), is to halve TB prevalence and death rates between 1990 and 2015 (evolution TB control). These additional targets are much more of a challenge, especially in Africa and Eastern Europe (World Health Organization 2000, World Health Organization 2005b, United Nations Statistics Division 2006).

High-burden countries

In March 2000, the Ministers of Health and Finance from 20 countries harboring 80 % of the world's TB cases met in Amsterdam and issued the Amsterdam Declaration. This stated that the global situation was "*both alarming and unacceptable*", and that "*We commit ourselves to accelerate action against TB through expansion of DOTS*" (World Health Organization, International Union Against Tuberculosis

and Lung Disease 2001, World Health Organization 2002a, World Health Organization 2006a).

There are 22 high-burden countries, which account for approximately 80 % of the estimated number of new TB cases (all forms) arising worldwide each year. These countries (Table 7-2) are the focus of intensified efforts in Directly Observed Treatment, Short-course (DOTS) expansion (www.who.int/tb/dots/whatisdots/en/index.html). The high-burden countries are not necessarily those with the highest incidence rates per capita; many of the latter are medium-sized African countries with high rates of TB/HIV co-infection (Dye 2006, World Health Organization 2006a, World Health Organization 2006b).

TB death rates in high-burden countries varied dramatically, from 9 per 100,000 population in Brazil to 139 per 100,000 in South Africa. In these two countries, the overall case fatality rates for TB were 13 % and 27 %, respectively, and the difference was due largely to the difference in HIV infection rates (Dye 2006, World Health Organization 2006a).

In 2004, only six high-burden countries (Democratic Republic of Congo, Myanmar, the Philippines, South Africa, Thailand and Viet Nam) reached the detection rate of new AFB smear-positive cases (70 %) by DOTS, and the estimate for at least one of these countries (Democratic Republic of Congo) is uncertain (Dye 2006, World Health Organization 2006a).

Eight high-burden countries met the 85 % target for treatment success based on the 2003 cohort. All of them are in South-East Asia or Western Pacific regions, with the exception of Afghanistan, where the case detection rate by the DOTS program is relatively low. Among high-burden countries, only the Philippines and Viet Nam had met the targets for both case detection and treatment success by the end of 2004 (Dye 2006, World Health Organization 2006a).

The progress made in global TB control by the end of 2005 depended greatly on what had been previously achieved in eight countries, which were inhabited by 61 % of the patients who were undetected in 2004. For this reason, Bangladesh, Ethiopia, Nigeria, Pakistan, and the Russian Federation will be under close scrutiny, in addition to China, India, and Indonesia (Dye 2006, World Health Organization 2006a).

Table 7-2: Estimated TB burden, 2004

	Incidence		Prevalence, all forms per 100,000 pop. per year	Mortality, all forms per 100,000 pop. per year	HIV prevalence, in incident TB cases %
	All forms per 100,000 pop.	**Smear positive per 100,000 pop. per year**			
1. India	168	75	312	30	5.2
2. China	101	46	221	17	0.9
3. Indonesia	245	110	275	46	0.9
4. Nigeria	290	125	531	82	27
5. South Africa	718	293	670	135	60
6. Bangladesh	229	103	435	51	0.1
7. Pakistan	181	81	329	40	0.6
8. Ethiopia	353	154	533	79	21
9. Philippines	293	132	463	48	0.1
10. Kenya	619	266	888	133	29
11. DR Congo	366	159	551	79	21
12. Russian Fed.	115	51	160	21	6.8
13. Viet Nam	176	79	232	22	3.0
14. UR Tanzania	347	147	479	78	36
15. Uganda	402	175	646	92	19
16. Brazil	60	26	77	7.8	17
17. Afghanistan	333	150	661	92	0.0
18. Thailand	142	63	208	19	8.5
19. Mozambique	460	191	635	129	48
20. Zimbabwe	674	271	673	151	68
21. Myanmar	171	76	180	21	7.1
22. Cambodia	510	226	709	94	13
High Burden Countries	**178**	**79**	**301**	**34**	**0.0**

Source: WHO, report 2006

Community participation in TB control is part of the National Tuberculosis Control Programme strategy in 14 high-burden countries. The number of high-burden countries with national strategies for advocacy, communication, and social mobili-

zation has increased from two in 2002 to 11 in 2005, and is expected to reach 19 by 2007 (Dye 2006, World Health Organization 2006a).

High-burden countries are in various stages of developing collaborations within and among public and private health sectors (through PPM-DOTS). While Bangladesh, China, India, Indonesia, Kenya, Myanmar, and the Philippines have already improved links between National Tuberculosis Control Programmes, hospitals and other healthcare providers, PPM-DOTS is still at an early stage in most other high-burden countries (Dye 2006, World Health Organization 2006a).

The total cost of TB control, which includes the general health system staff and the infrastructure used for TB control, in additional to the National Tuberculosis Control Programme budget requirements, is projected to be US$ 1.6 billion in the 22 high-burden countries in 2006, compared with US$ 876 million in 2002. The Russian Federation and South Africa have by far the largest costs, with a combined total of US$ 810 million. Assuming that health systems have had the capacity to manage a growing number of TB patients in 2006, the funding gap for total TB control costs in 2006 will have been the same as for the National Tuberculosis Control Programme budgets, i.e. US$ 141 million. Total costs increase to US$ 2.0 billion, and the funding gap increases to US$ 180 million when all 74 countries that reported data are included. These 74 countries represent 89 % of TB cases globally (Dye 2006, World Health Organization 2006a).

All but one of the 22 high-burden countries that increased spending between 2003 and 2004 also increased the number of new AFB smear-positive cases that were detected and treated in DOTS programs. Cambodia increased spending, but did not increase the total number of AFB smear-positive patients treated by DOTS (Dye 2006, World Health Organization 2006a).

Among the 22 high-burden countries, five (India, Indonesia, Myanmar, the Philippines, and Viet Nam) were in the best financial position to reach the World Health Assembly targets in 2005; two (Cambodia and China) were well placed to do so, if able to make up funding shortfalls (Dye 2006, World Health Organization 2006a).

7.2. Tuberculosis and the interaction with the HIV epidemic

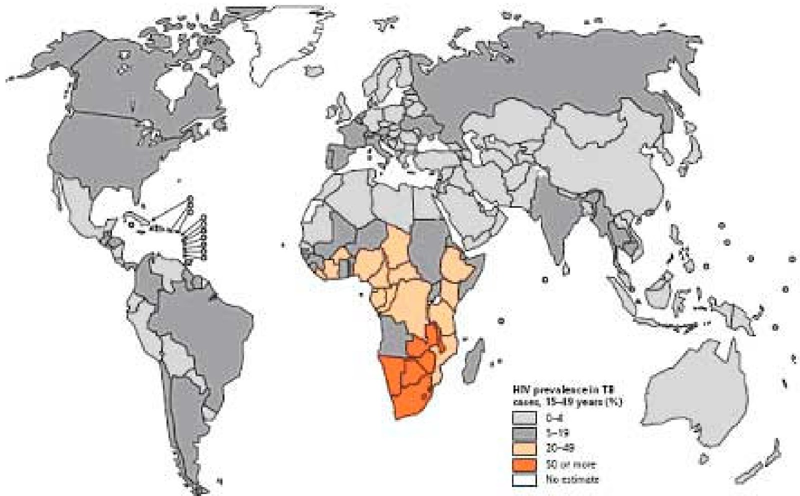
HIV and TB form a lethal combination, each speeding the other's progress. HIV infection is a potent risk factor for TB. Not only does HIV increase the risk of reactivating latent *M. tuberculosis* infection, it also increases the risk of rapid TB progression soon after *M. tuberculosis* infection or reinfection. In persons infected with *M. tuberculosis* only, the lifetime risk of developing TB ranges between 10 %

and 20 %. In persons co-infected with *M. tuberculosis* and HIV, however, the annual risk can exceed 10 %. The TB burden in countries with a generalized HIV/AIDS epidemic has therefore increased rapidly over the past decade, especially in the severely affected countries of eastern and southern Africa. TB is one of the most common causes of morbidity and the most common cause of death in HIV-positive adults living in less-developed countries, yet it is a preventable and treatable disease (Corbett 2003, Aaron 2004, World Health Organization 2006b).

It is possible that, in addition to increasing individual susceptibility to TB following *M. tuberculosis* infection, a high burden of HIV-associated TB cases also expands *M. tuberculosis* transmission rates at the community level, threatening the health and survival of HIV-negative individuals as well. In several countries, HIV has been associated with epidemic outbreaks of TB. Many of the reported outbreaks involved multidrug-resistant (MDR) strains, which respond poorly to standard therapy - the growing burden of TB (Corbett 2003, Aaron 2004, World Health Organization 2006a, World Health Organization 2006b).

According to a study published by Corbett et al., an estimated 8.3 million new TB cases were reported in 2000 worldwide. Nine percent (7 %-12 %) of all new TB cases in adults (aged 15-49 years) were attributable to HIV infection, but the proportion was much greater in the WHO African Region (31 %) and some industrialized countries, notably the United States (26 %). There were an estimated 1.8 million deaths from TB, of which 12 % were attributable to HIV. In turn, TB was the cause of 11 % of all adult AIDS deaths. The worldwide prevalence of *M. tuberculosis*-HIV co-infection in adults was 0.36 % (11 million people). Co-infection prevalence rates equaled or exceeded 5 % in eight African countries. In South Africa alone there were 2 million co-infected adults (Corbett 2003, Corbett 2004).

Other studies published by Dye *et al*. reported that much of the observed increase in the incidence of global TB since 1980 is attributable to the spread of HIV in Africa. Globally, an estimated 13 % of adults with newly diagnosed TB were infected with HIV in 2004, but there was great variation among regions — from 34 % in the African region to 1.4 % in the Western Pacific region. Rates of HIV infection in patients with TB have so far remained below 1 % in Bangladesh, China, Indonesia, and Pakistan. In African populations with high rates of HIV infection, a relatively high proportion of patients with TB are women aged between 15 and 24 years. The rise in the number of TB cases is slowing in Africa, almost certainly because HIV infection rates are beginning to stabilize or fall. HIV has probably had a smaller effect on TB prevalence than on incidence because the virus significantly reduces the life expectancy of patients with TB (Figure 7-2) (Asamoah-Odei 2004, Dye 2005, Dye 2006).



Source: WHO report, 2006

Figure 7-2: Estimated HIV prevalence in new adult TB cases, 2004.

In regions where HIV infection rates are high in the general population, they are also high among patients with TB; estimates for 2004 exceeded 50 % in Botswana, South Africa, Zambia, and Zimbabwe, among other countries (Corbett 2003, Dye 2005, Dye 2006, World Health Organization 2006a).

The survival rate of HIV-positive TB patients varies according to AFB smear status and regimen. It is generally higher for AFB smear-positive than for smear-negative patients, and it is lowest with rifampicin-based regimens (Corbett 2003, Dye 2005, Dye 2006, World Health Organization 2006a).

In summary, the HIV pandemic presents a massive challenge for global TB control. The prevention of HIV and TB, the extension of WHO DOTS programs, and a focused effort to control HIV-related TB in areas of high HIV prevalence are matters of great urgency (World Health Organization 2002a,b; Aaron 2004, World Health Organization 2006a). The WHO and its international partners have formed the TB/HIV Working Group (www.stoptb.org/wg/tb_hiv), which is developing a global policy on the control of HIV-related TB, providing advice on how those fighting against TB and HIV can work together to tackle this lethal combination. The temporary policy on collaborative TB/HIV activities describes steps to create mechanisms of collaboration between TB and HIV/AIDS programs, to reduce the burden of TB among people with HIV and the burden of HIV among TB patients.

These activities should be included in national TB control plans (Aaron 2004, World Health Organization 2002a, World Health Organization 2002b; World Health Organization 2006a).

7.3. Progress of the DOTS strategy

In 1994, the internationally recommended control strategy, later named DOTS, was launched. It stands for Directly Observed Treatment, Short-course, and its key components include:

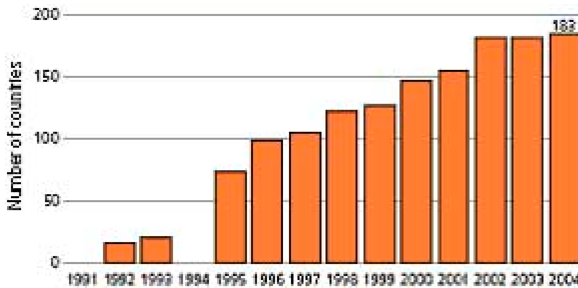
- government commitment;
- case detection by predominantly passive case finding;
- standardized short-course chemotherapy for, at least, all confirmed sputum AFB smear-positive cases, provided under proper case management conditions;
- a system of regular drug supply; and
- a monitoring system for program supervision and evaluation.

A six-month supply of drugs for DOTS costs less than US$ 10 in some parts of the world. The World Bank has ranked the DOTS strategy as one of the “most cost-effective of all health interventions”. Countries that employ DOTS have been able to prevent an expected increase in drug resistance. Some countries using DOTS, such as Cuba and Nepal, have even begun to see declining levels of drug resistance (World Health Organization 1994, World Health Organization 2002c).

A total of 183 countries and territories were implementing the DOTS strategy in 2004. By the end of 2004, 83 % of the world’s population lived in countries covered by DOTS. DOTS programs notified 4.4 million new and relapse TB cases in 2004, of which 2.1 million were new AFB smear-positive. In total, 21.5 million TB patients, and 10.7 million AFB smear-positive patients, were treated in DOTS programs over the 10-year period 1995-2004 (Sharma 2006, World Health Organization 2006a).

Globally, the case detection rate by DOTS programs increased almost linearly from 11 % in 1995 to 28 % in 2000, and then accelerated to 45 % in 2003. If the 7 % global increase in detection between 2002 and 2003 was maintained, it would have reached approximately 60 % by 2005, 10 % below target. Comparing different parts of the world in 2003, case detection was highest in the Latin American (48 %)

and Western Pacific regions (50 %), and lowest in Eastern Europe (22 %). The recent acceleration has been mostly due to rapid implementation in India, where case detection increased from 1.7 % in 1998 to 47 % in 2003, and in China, where case detection increased from 30 % in 2002 to 43 % in 2003. India and China together accounted for 63 % of the increase in case notification by DOTS programs between 2002 and 2003. With this display of growing coverage, governments, donors, and other supporting agencies were beginning to ask for evidence that DOTS is having the expected epidemiologic impact (Figure 7-3) (De Cock 1999, World Health Organization 2002b, World Health Organization 2002c; Dye 2005, Frieden 2005, Sharma 2006, World Health Organization 2006a).



Source: WHO report, 2006

Figure 7-3: Number of countries implementing DOTS (out of a total of 211 countries), 1991-2004.

The global treatment success rate under DOTS has been high since the first observed cohort in 1994 (77 %). Since 1998, it has remained above 80 %, even though the cohort size has increased 6-fold to 1.4 million patients. There is, however, much variation between regions. Treatment success exceeded the 85 % target in the Western Pacific region, largely because China reported a 93 % success rate. Clearly, the biggest failure of DOTS has been in Africa, where rates of TB continue to rise, seemingly unabated. In 2002, the African region showed less than 75 % cure rates, and death rates were as high as 8 % in patients co-infected with *M. tuberculosis* and HIV. Whether this statistic indicates a failure of DOTS, or is the result of the rapid spread of the HIV epidemic, is debatable. Eastern Europe, another region plagued by poor health systems and an expanding HIV epidemic, witnessed continued increases in TB incidence rates throughout the 1990s, though the increase now seems to have peaked. Increases in incidence rates of disease are

also noted in Central Asian countries, though the death rate in DOTS recipients remains stable at 5 %. Both Eastern Europe and Central Asia are also hotspots of MDR-TB (Dye 2005, Frieden 2005, Sharma 2006, World Health Organization 2006a).

Although the decline in TB has almost certainly been accelerated by good chemotherapy programs in countries such as Chile, Cuba, and Uruguay, there have only been few recent, unequivocal demonstrations of the impact of DOTS in high-burden countries. Two examples come from Peru and China. In Peru, the incidence rate of pulmonary TB has decreased annually by 6 % since the nationwide implementation of DOTS in 1991. In 13 provinces of China that implemented DOTS, the prevalence rate of culture-positive TB was cut by 30 % between 1990 and 2000 (Dye 2005, World Health Organization 2006a).

Despite substantial success with DOTS expansion, most countries will probably not meet the target of the United Nations Millennium Development Goals of halving the prevalence of TB and the associated death rates between 1990 and 2015. Further innovative steps need to be taken as the public-health community moves beyond DOTS expansion to global TB control. The main objectives are: to continue DOTS expansion with more funding and oversight, build on existing DOTS programs to pursue DOTS-Plus, increase funding for research into improved diagnostics, therapeutics, and vaccines, revisit strategies of chemoprophylaxis and active case finding, and use DOTS to strengthen public-sector infrastructure and community-based health programs and insurance schemes (Sharma 2006, World Health Organization 2006a).

DOTS-Plus

Based upon DOTS, DOTS-Plus is a comprehensive management strategy under development and testing that includes the five tenets of the DOTS strategy. DOTS-Plus takes into account specific issues (such as the use of second-line anti-tuberculosis drugs) that need to be addressed in areas where there is high prevalence of MDR-TB. These drugs should be stored and dispensed at specialized health centers with appropriate facilities and well-trained staff. Thus, DOTS-Plus works as a supplement to the standard DOTS strategy. By definition, it is impossible to conduct DOTS-Plus in an area without having an effective DOTS-based TB control program in place. It is vital that DOTS-Plus pilot projects follow WHO recommendations in order to minimize the risk of creating drug resistance to second-line TB drugs (which are more toxic and expensive, and less effective, than first-line drugs). The regimen includes two or more second-line TB drugs to which the isolate is susceptible, including one drug given parenterally for six months or

more. The total duration of treatment is 18-24 months. This treatment is directly observed and should be either individualized according to drug susceptibility test results of *M. tuberculosis* isolate identified on culture, or given as a standardized regimen to patients who fail supervised re-treatment (for example, when culture and drug susceptibility testing are not performed) (World Health Organization 2002d, Sharma 2006).

DOTS-Plus is not intended to be a universal strategy, and is not required in all settings. DOTS-Plus should be implemented in selected areas with moderate to high levels of MDR-TB in order to combat an emerging epidemic. Via the Green Light Committee review process, DOTS-Plus is already being implemented in Bolivia, Costa Rica, Estonia, Haiti, Karakalpakstan (Uzbekistan), Latvia, Malawi, Mexico, Peru, Philippines and the Russian Federation (Arkhangelsk, Ivanono, Tomsk and Orel Oblasts). More recently, DOTS-Plus projects have also been approved in Georgia, Honduras, Jordan, Kenya, Kyrgyzstan, Lebanon, Nepal, Nicaragua, Romania, and Syria (Gupta 2002, Sharma 2006, World Health Organization 2002d).

The Working Group on DOTS-Plus for MDR-TB identified the lack of access to second-line anti-tuberculosis drugs as one of the major obstacles to the implementation of DOTS-Plus pilot projects. The working group has made arrangements with the pharmaceutical industry to provide concessionally-priced second-line anti-tuberculosis drugs to DOTS-Plus pilot projects that meet the standards outlined in the Guidelines for Establishing DOTS-Plus Pilot Projects for the Management of MDR-TB. Currently, prices have been reduced by up to 99 % compared with prices on the open market. It is the task of the Green Light Committee to review the applications from potential DOTS-Plus pilot projects and to determine whether or not they are in compliance with the Guidelines for Establishing DOTS-Plus Pilot Projects (Gupta 2002, World Health Organization 2002c, World Health Organization 2002d; Sharma 2006).

7.4. The new Stop TB strategy

The first Global Plan to Stop TB set out the actions that were needed in TB control over the period 2001-2005 and helped to steer global TB control efforts during that time. Current rates of progress are insufficient to allow the targets of halving TB mortality and prevalence by 2015 to be achieved. Particularly urgent action is needed in regions where the epidemic is worsening, notably in Africa but also in Eastern Europe (Dye 2005, World Health Organization 2001, World Health Organization 2006c).

As a global movement to accelerate social and political action to stop the spread of the disease, the Stop TB Partnership provides the platform for international organizations, countries, donors (public and private sector), governmental and non-governmental organizations, patient organizations, and individuals to contribute to a collective and concerted campaign to Stop TB. Making the most of partners' efforts, in terms of effectiveness and efficiency, requires a plan. The Stop TB Partnership has developed a Global Plan to Stop TB that covers the period 2006–2015 (Squire 2006, World Health Organization 2006d).

Within the Partnership's strategic approaches for the next decade, the Plan sets out the activities that will make an impact on the global burden of TB. This involves reducing TB incidence - in line with the Millennium Development Goals - and reaching the Partnership's targets for 2015 of halving TB prevalence and deaths compared with 1990 levels. TB is a long-haul disease: the Plan represents a step towards the elimination of TB as a global public health problem by 2050, and the realization of the Partnership's vision of a TB-free world. It sets out the resources needed for actions, underpinned by sound epidemiological analysis with robust budget justifications; and it supports the need for long-term planning for action at the regional and country level (United Nations Statistics Division 2006, World Health Organization 2006a, World Health Organization 2006c).

The Plan will serve to stimulate political commitment, financial support, effective intervention, patient involvement, and community participation; and it will also indicate the potential of the new tools to control TB, which are currently under development (improved drugs, diagnostics and vaccines). There is no truly effective vaccine against TB, and the limitations of the available tools for diagnosis and treatment (smear microscopy testing and ¨short-course¨ chemotherapy) make standard TB care demanding for both patients and healthcare providers. The need to rely on the available tools has substantially hindered the pace of progress in global TB control. Facilitating the concerted efforts of the Stop TB Partnership's Working Groups on New Diagnostics, Drugs, and Vaccines for TB is thus, a key component of the Stop TB Strategy. In the spirit of partnership, TB control programs should actively encourage and participate in this process. Countries should advocate the development of new tools, help to speed up the field testing of new products, and prepare for swift adoption and roll-out of new diagnostics, drugs and vaccines as they become available (Squire 2006, World Health Organization 2006d).

The development of the Plan has relied on contributions from the Stop TB Partnership's seven working groups — DOTS expansion, DOTS-Plus for MDR-TB, TB-HIV, new TB diagnostics, new TB drugs, new TB vaccines, advocacy, communi-

cation and social mobilization — coordinated by the Partnership Secretariat (Squire 2006, World Health Organization 2006d).

The Working Groups have contributed to the two key dimensions of the Plan:

- regional scenarios (projections of the expected impact and costs of activities oriented towards achieving the Partnership's targets for 2015 in each region), and
- the strategic plans of the working groups and the Secretariat (Squire 2006, World Health Organization 2006c, World Health Organization 2006d).

The Stop TB Strategy is divided into four major sections (World Health Organization 2006c, World Health Organization 2006 d):

The Stop TB Strategy at a glance. Provides an overview of the strategy.

- **Vision, goal, objectives, targets and indicators**. Explains the goal and related objectives, targets and indicators of the Stop TB Strategy, as well as the overall vision to which the Strategy will contribute.
- **The six principal components of the Stop TB Strategy**. (see below).
- **Measuring global progress and impact**. Explains how progress towards TB control targets will need to be measured and evaluated.

The six components of the Stop TB Strategy are (World Health Organization 2006a, World Health Organization 2006c, World Health Organization 2006d):

1. **Pursuing high-quality DOTS expansion and enhancement.** Making high-quality services widely available and accessible to all those who need them, including the poorest and most vulnerable, requires DOTS expansion to even the remotest areas. In 2004, 183 countries (including all 22 of the high-burden countries which account for 80 % of the world's TB cases) were implementing DOTS in at least part of the country.
2. **Addressing TB/HIV, MDR-TB and other challenges.** Addressing TB/HIV, MDR-TB and other challenges requires much greater action and input than DOTS implementation and is essential in order to achieve the targets set for 2015, including the United Nations MILLENNIUM DEVELOPMENT GOALS relating to TB (Goal 6; Target 8).

3. **Contributing to health system strengthening.** National Tuberculosis Control Programmes must contribute to overall strategies to advance financing, planning, management, information and supply systems, and innovative service delivery scale-up.

4. **Engaging all care providers.** TB patients seek care from a wide array of public, private, corporate and voluntary healthcare providers. To be able to reach all patients and ensure that they receive high quality care, all types of healthcare providers are to be engaged.

5. **Empowering people with TB, and communities.** Community TB care projects have shown how people and communities can undertake some essential TB control tasks. These networks can mobilize civil societies and also ensure political support and long-term sustainability for National Tuberculosis Control Programmes.

Enabling and promoting research. While current tools can control TB, improved practices and elimination will depend on new diagnostics, drugs and vaccines.

With the implementation of the strategy:

- equitable access for all to quality TB diagnosis and treatment will be expanded,
- over the ten years of this Plan, about 50 million people will be treated for TB under the Stop TB Strategy, including about 800,000 patients with MDR-TB; in addition, about 3 million patients who have both TB and HIV will be enrolled on antiretroviral therapy (in line with UNAIDS plans for universal access) (www.unaids.org/en),
- some 14 million lives will be saved from 2006 to 2015,
- for the first time in 40 years, a new TB drug will be introduced in 2010, with a new short TB regimen (1–2 months) shortly after 2015,
- by 2010, diagnostic tests at the point of care will allow rapid, sensitive, and inexpensive detection of active TB. By 2012, a diagnostic toolbox will accurately identify people with latent TB infection and those at high risk of progression to disease, and
- by 2015, a new, safe, effective, and affordable vaccine will be available with the potential to have a significant impact on TB control in later years (World Health Organization 2006 a, World Health Organization 2006c, World Health Organization 2006d).

The total cost of the Plan — US$ 56 billion — represents a three-fold increase in the annual investment in TB control compared with the first Global Plan. This total includes US$ 9 billion for research and development and US$ 47 billion for implementation of current interventions (US$ 44 billion are country-level costs, representing about 80 % of the Plan's total cost (Squire 2006, World Health Organization 2006c, World Health Organization 2006d).

In a resolution adopted by the 58th World Health Assembly in 2005, on 'sustainable financing for TB prevention and control', all countries made a commitment to ensure the availability of sufficient domestic and external resources to achieve the Millennium Development Goals relevant to TB. National governments and donors must fulfill this commitment by mobilizing the funds to increase current levels of funding and fill the US$ 31 billion gap. With the will, the funds and the action, together we can Stop TB (World Health Organization 2005a, World Health Organization 2006b; Squire 2006).

If the Stop TB Strategy is implemented as set out in the Global Plan, the resulting improvements in TB control should reverse the rise in the incidence of TB by 2015, and halve the prevalence and death rates in all regions except Africa and Eastern Europe (Squire 2006, World Health Organization 2006a, World Health Organization 2006c, World Health Organization 2006d).

The 2006 WHO report Global TB Control concluded that three (of six) WHO regions – namely the Americas, South-East Asia, and the Western Pacific - are likely to have met both the 2005 targets. Seven of the 22 high-burden countries are likely to have met the 2005 targets: Cambodia, China, India, Indonesia, Myanmar, the Philippines and Viet Nam (World Health Organization 2006a).

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Chapter 8: Tuberculosis caused by Other Members of the *M. tuberculosis* Complex

Angel Cataldi and Maria Isabel Romano

8.1. *Mycobacterium bovis* disease in humans

Bovine tuberculosis (TB) is caused by *Mycobacterium bovis,* a mycobacterium highly similar to *Mycobacterium tuberculosis* and belonging to the *M. tuberculosis* complex. The main host of *M. bovis* is cattle (*Bos taurus*) but it affects many other mammalians including man. In man, it is the most frequent cause of zoonotic TB, i.e. TB transmitted from animals to humans, which is clinically indistinguishable from TB caused by *M. tuberculosis*. Before milk pasteurization, *M. bovis* was an important cause of human TB, especially intestinal TB in children. After the generalized adoption of pasteurization of milk and other dairy products, the occurrence of zoonotic TB dropped sharply.

Very few studies are published on zoonotic TB. In the last 50 years, research on zoonotic TB was influenced by scientific trends, societal worries such as human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) and contaminated food, as well as by the availability of tools for the identification of the bovine TB bacillus. For example, the development of the polymerase chain reaction (PCR) and other molecular tools to identify *M. bovis* and differentiate it from other members of the *M. tuberculosis* complex have allowed the discovery of more cases in retrospective studies and have suggested new forms of transmission. The medical literature on the incidence of zoonotic TB is marked by numerous clinical descriptions of cases of *M. bovis* at the regional or nosocomial level, but there are very few systematic surveys of *M. bovis* diagnosis on a national level (Anon 2003, Barrera 1987, Cousins 1999, Pavlık 1998). There are three main explanations for the absence of an accurate and methodical estimation of the contribution of *M. bovis* to the global TB burden. First, at the clinical or radiological level, there is no difference between TB caused by *M. tuberculosis* or that of *M. bovis*. Second, most laboratories use Löwenstein-Jensen culture medium with glycerol, which does not promote *M. bovis* growth. Furthermore, cultivation is always an expensive option for many low-income countries compared to the cheaper and faster acid-fast staining. Third, and perhaps most important, in most cases the treatment of TB caused by *M. tuberculosis* or *M. bovis* was the same; therefore, there was no clinical interest in differentiating the causative agent.

There is a direct correlation between the prevalence of human TB of bovine origin

and that of TB in livestock (Cosivi 1998). At the global level the situation of bovine TB is disparate. In many developed countries bovine TB was eradicated 30-40 years ago by strong campaigns based on tuberculin skin testing (TST) and mandatory sacrifice of animals at the slaughterhouse. In these countries, human TB caused by *M. bovis* accounts for around 1 % of all TB cases, and sporadic cases occur in elderly people by reactivation of ancient infections or in immigrants from countries where bovine TB has not been eradicated. Importantly, some developed countries, including England or New Zealand, could not completely eliminate bovine TB, or worse, there is a re-emergence of the disease (Thoen 2006). The persistence of *M. bovis* in wildlife is frequently indicated as the main cause of this reemergence. On the other hand, in many low-income countries, bovine TB continues to be an important animal health problem. Different epidemiological scenarios can be observed. Meat and bovine products are important resources in some countries, such as Argentina, Brazil, Mexico, and Venezuela, where the number of cattle equals or exceeds that of the human population, and the risk of zoonotic TB could be higher. Less clear is the situation in countries where livestock industry is less developed and intensive, and cattle farming is a family affair for milk consumption or retail commercialization; on the other hand, if the total number of cattle is highly reduced, people live near the animal folds and sometimes consume milk raw. In Central American and African countries, as well as in China, cows are preserved for milk production and meat is consumed from other species such as sheep and swine that are less susceptible to *M. bovis*. Finally, in India, a high proportion of people do not eat cattle meat but do consume milk and are in close contact with cattle, increasing the risk. Some low-income countries, including Cuba, Mongolia, and Costa Rica are remarkable exceptions, because they have eradicated bovine TB, probably because the cattle population is relatively small.

The epidemiology of zoonotic TB, was recently examined by Thoen *et al.* (Thoen 2006), who reviewed publications from 1966 on. In this chapter we will concentrate on the most recent findings.

In Africa, there are several reports about the incidence of zoonotic TB. Many of these studies involved research on pre-existing mycobacterial collections or in limited clinical settings, such as in Egypt, Nigeria, Madagascar, Zaire, and Tanzania (Table 8-1). Other authors looked for *M. bovis* in cattle, as in Ghana and Zambia, where high incidences were described. Another study demonstrated the presence of *M. bovis* in milk in Tanzania (Kazwala 1998). An excellent review about bovine TB was published by Ayele (Ayele 2004). Genotyping analysis demonstrates different clonal populations depending on the geographical region under study. Due to the consumption of raw milk in regions where AIDS is highly

prevalent, many studies concentrated on patients having lymphadenitis. One study in Djibouti showed a low prevalence of *M. bovis* in those patients (Koeck 2002), while others in Ethiopia (Kidane 2002) and Tanzania (Mfinanga 2004) demonstrated 17 % and 10 % prevalence respectively (Table 8-1).

In Asia, where a policy of bovine TB control was adopted in few countries, there are very few publications on zoonotic TB (Table 8-1). Clearly, a more active search for *M. bovis* is needed on the Asian continent, where the burden of TB is high.

In Latin America, most of the studies were published in Argentina describing incidence ranging from 0.7 % to 6.2 % in a main milk region, with a much lower national prevalence. In Brazil and Mexico, only one publication was available per country (Table 8-1). In a small collection of human isolates from Chile, *M. bovis* was not found (Mancilla 2006), a finding that merits a more extended survey in that country (Table 8-1). Genotyping of *M. bovis* from humans in Argentina showed that the predominating genotype in cattle also predominates in humans, strongly indicating that infection is transmitted from cattle. Furthermore, the majority of patients are related to the farming or meat industry (Zumárraga 1999). In contrast, Romano *et al*. recently selected isolates with scarce growth in Löwenstein-Jensen media from different hospitals in Argentina, and many of them were *M. bovis*. Genotyping of these unsuspected *M. bovis* isolates showed that they belong to a spoligotype that is not predominant in cattle, suggesting that a clone circulates among humans (M. Romano, unpublished observations). The situation of zoonotic TB in Latin America has recently been reviewed by Ritacco *et al.* (Ritacco 2006).

In the United States, the description of an outbreak of *M. bovis* TB cases in San Diego was of special interest (Table 8-1). The ingestion of raw milk products by immigrant children was suspected as the source of the infection (Dankner 1993, Dankner 2000). No recent cases were informed from Canada. Other reports also describe a higher incidence of *M. bovis* among third world immigrants residing in industrialized countries (Cousins 1999, Jalava 2007).

In Europe, in the last decades, *M. bovis* in humans was reported sporadically (Thoen 2006). The prevalence of TB cases caused by *M. bovis* is around 1.0 % of all TB cases in the United Kingdom. A similar figure is found in Germany (where *Mycobacterium caprae* is relevant) and in Spain (Table 8-1). A recent paper described that in Lyon, France, there was no genetic relatedness among nine *M. bovis* isolates collected from patients over five years, strongly indicating that there is no active transmission (Mignard 2006). In contrast, another typing study in Italy described spoligotyping and Mycobacterial Interspersed Repetitive Units (MIRU) patterns in a collection of 42 isolates, with one genotype accounted for 32 % of all

isolates, while the others were unique (Lari 2006). Importantly, a study in the United Kingdom showed that there is no increase in *M. bovis* disease in humans in spite of an important increase in the incidence of bovine TB (Jalava 2006).

In New Zealand, *M. bovis* accounts for 2.7 % of laboratory-confirmed human TB cases. Many of the genotypes were identical to patterns from farmed and wild animals (Baker 2006). In contrast, there has been no publications on zoonotic TB in Australia during the last six years.

Table 8-1: Bovine TB in humans

Country	Target of study	Main findings	Reference
Egypt	Identification of *M. bovis* in humans using cultures.	6 % of the total TB cases were caused by *M. bovis*	Elsabban 1992
Egypt	Identification of *M. bovis* in humans using cultures	High incidence of *M. bovis*	Nafeh 1992
Nigeria	Identification of *M. bovis* in humans using cultures	3.9 % of TB cases caused by *M. bovis*	Idigbe 1986
Madagascar	Identification of *M. bovis* in humans using cultures	1.25 % of TB cases caused by *M. bovis*	Rasolofo-Razanamparany 1999
Madagascar	Genotyping of *M. bovis* collection	A genotype is prevalent in humans and cattle	Rasolofo-Razanamparany 2006
Zaire	Identification of *M. bovis* in humans using cultures	High incidence of *M. bovis*	Mposhy 1983
Zambia	Large field diagnostic test of cattle	33 % of positive herds	Cook 1996
Tanzania	Detection of *M. bovis* in milk	6 % of samples positive for *M. bovis*	Kazwala 1998
Burundi	Culture of mycobacteria from human and cattle samples.	No *M. bovis* in humans, 38 % in cattle.	Rigouts 1996
Ghana	Large field diagnostic test of cattle	13.8 % of positive animals.	Bonsu 2000
Tanzania	Screening of TB patients from rural communities	16 % of TB cases caused by *M. bovis*	Kazwala 2001

Country	Target of study	Main findings	Reference
Tanzania	Genotyping of *M. bovis* collection	Low clustering of cases in humans and cattle	Kazwala 2005
Djibouti	Biopsies of lymph nodes from TB patients	Low prevalence of *M. bovis*	Koeck 2002
Ethiopia	Biopsies of lymph nodes from TB patients	17.1 % of samples positive for *M. bovis*	Kidane 2002
Tanzania	Biopsies of lymph nodes from TB patients	10 % of samples positive for *M. bovis*	Mfinanga 2004
Nigeria	Genotyping of *M. bovis* collection	No common patterns of *M. bovis* from cattle and humans	Cadmus 2006
China	Clinical report	Case of disseminated TB due to *M. bovis*	Wei 2004
India	Screening of CSF from patients	Molecular evidence of *M. bovis*	Prasad, 2005
Argentina	Identification of *M. bovis* in humans using cultures	8 % of *M. bovis* in extrapulmonary TB	Peluffo 1982
Argentina	Identification of *M. bovis* in humans using cultures	*M. bovis* identified in 0.47 % of sputum samples	Barrera 1987
Argentina	Identification of *M. bovis* in humans using cultures	Annual variations of *M. bovis* going from 0.7 % to 6.2 % of human TB	Sequeira 1990
Argentina	Identification of *M. bovis* in humans using cultures	7 % of extrapulmonary TB due to *M. bovis*	Solda 2005
Brazil	Mycobacterial cultures from children	*M. bovis* provoked 3.5 % of cases of pediatric TB	Correa 1974
Chile	Identification of *M. bovis* in humans using cultures	No *M. bovis* isolation	Mancilla 2006
Mexico	Identification of *M. bovis* in humans using cultures	3/19 isolates were *M. bovis*	Toledo Ordoñez 1999
Argentina	Genotyping of *M. bovis* collection	A genotype is prevalent in humans and cattle	Zumarraga 1999

Country	Target of study	Main findings	Reference
USA	Diagnostic of TB in workers from a dairy farm	Risk factor for zoonotic TB, but no cases demonstrated.	Winthrop 2005
USA	Diagnostic of TB in immigrant children	Pediatric TB due to *M. bovis*	Dankner 1993, 2000
Australia	Identification of *M. bovis* in humans using cultures	*M. bovis* present in immigrant workers	
United Kingdom	Identification of *M. bovis* in humans using cultures	No increase of zoonotic TB in spite of increase in cattle.	Jalava 2007
United Kingdom	Identification of *M. bovis* in humans using cultures	*M. bovis* provoked 1.0% of TB cases	Yates 1988
France	Genotyping of *M. bovis* collection	No genetic relatedness among *M. bovis* isolates collected from patients	Mignard 2006
Italy	Genotyping of *M. bovis* collection	A genotype accounts for 32 % of human isolates	Lari 2006
Germany	Identification of *M. bovis* in humans using cultures	*M. bovis* represents 1 % of all TB cases. 31 % of the isolates were *M. caprae*	Kubica 2003
United Kingdom	Clinical report	An intrafamilial spread of *M. bovis*	Smith 2004
United Kingdom	National survey for *M. bovis*	*M. bovis* represents between 0.5 % and 1.5 % of TB cases	de la Rua-Domenech 2006
Spain	Identification of *M. bovis* in humans using cultures	9 *M. bovis* cases in patients in 4 years	Remacha 2006
Spain	Identification of *M. bovis* in humans using cultures	0.95 % of all cases of tuberculosis due to *M. bovis*	Esteban 2005
New Zealand	Identification of *M. bovis* in humans using cultures	2.7 % of TB cases due to *M. bovis*. Many of the genotypes were identical to patterns from animals	Baker 2006

In the last 10 years, human disease due to drug-resistant *M. bovis* has been described (Blazquez 1997, Hughes 2003, Sechi 2001). One case was of special concern because it affected many patients, most of them HIV-positive (Blazquez

1997). This strain spread over Europe and into Canada, and affected 141 patients (S. Samper, personal communication). This fact highlights the high risk of spread of MDR *M. bovis*, especially in parts of Africa where *M. bovis* animal disease and HIV human infection co-exist.

In humans, the disease caused by *M. bovis* or *M. tuberculosis* is clinically indistinguishable. However, if the physiopathology of TB in humans and cattle are compared, some differences are observed. In humans, the apical lobes of the lungs are most affected. In cattle, lesions are most frequently observed in the dorsal caudal lung regions (Cassidy 2006). This part is the most distant from the mouth and nostrils, meaning that the droplets must travel the longest possible route. In bovines, on the other hand, the lesions are frequently located in lymph nodes associated with the respiratory tract, and not in the lung parenchyma. This observation may be related to the fact that the detection of infected cattle is made in the early stages of disease progression, before the presentation of advanced cavitary lesions. At the histological level, the differences are related to the cell types intervening in the immune response and granuloma formation. For example, the content of $\gamma\delta$ T cells is much higher in cattle and these cells, as well as neutrophils, participate in granuloma and lesion formation (Cassidy 2001). Nowadays, and due to control campaigns, large liquefied lesions are less frequently observed in cattle in contrast with findings in wildlife where it is possible to observe advanced lesions (Cassidy 2006).

Sheep and horses are rarely infected. The infection in goats shows extreme variation according to the geographic location. There are less reports of TB in domestic than in feral pigs. The direct transmission of *M. bovis* from wildlife animals to humans is much less frequent. Transmission from deer to humans has been reported in Canada (Long 1999). Cats, but not dogs, have been reported in several countries as the source of human TB (Fernandez 1999, Underwood 1999, Monies 2000). Importantly, there are no reports of human infection by *M. bovis* coming from a direct environmental source (Biet 2005). The fact that in situations where the prevalence of *M. bovis* in cattle is high but does not seem to be associated with a higher incidence in humans may suggest that humans are less susceptible to *M. bovis* than to *M. tuberculosis* (de la Rua-Domenech 2006).

After the introduction of milk pasteurization, there was a clear impact on the death rate of children under five years of age (Thoen 2006). A recent review (de la Rua-Domenech 2006) described the survival of *M. bovis* in different foods. *M. bovis* survives well in cows' milk. Viable bacilli can be found in yogurt and cream cheese made from unpasteurized milk for up to 14 days after preparation, and in butter for up to 100 days. The consumption of unpasteurized raw milk or milk

products is still allowed in many European countries. In low-income countries, consumption of raw milk or dairy products is common in rural areas.

The detection of *M. bovis* in milk from infected cattle is problematic because *M. bovis* is usually present in low amounts. As contaminating microbiota exist in raw milk, other bacteria and fungi overgrow *M. bovis*. Decontamination methods applied to other clinical samples with higher bacillary loads, such as sputum or necropsy samples, kill the few *M. bovis* that may exist in tested milk. This has led to a worrying situation in which there are no validated methods for its detection in milk or milk products. The main problem is the failure of culture as a gold standard. PCR methods for the detection of members of the *M. tuberculosis* complex in clinical specimens were developed in the mid-90s. Although the first developed PCR method used primers directed at a *M. bovis* specific sequence (Rodriguez 1995), most PCR protocols use primers derived from the insertion sequence IS*6110* insertion sequence, present in all members of the *M. tuberculosis* complex. The detection limit in artificially contaminated milk is generally low: 10-1,000 colony forming units (cfu) (Zanini 1998, Zumárraga 2005, Antognoli 2001). The sensitivity among tuberculin skin test (TST)-positive cows also varies in different studies, from 11-50 % (Cornejo 1998, Romero 1999, Sreevatsan 2000, Zumárraga 2005). One study in Argentina did not find *M. bovis* in cattle milk (Perez 2002). This variation is expected, as excretion of *M. bovis* in milk is sporadic and not all infected animals excrete bacilli. There are no published studies on the detection of *M. bovis* by PCR in cheese. In summary, PCR is powerful in detecting *M. bovis* in milk but there is an urgent need to validate this technique on a wider level.

8.2. The BCG vaccine: adverse reactions

The bacille Calmette-Guérin (BCG) is a live, attenuated vaccine derived from *M. bovis*. BCG is known to cause local reactions consistent with primary infection with an attenuated strain (i.e. a small localized ulcer and possible regional lymphadenopathy), and more severe reactions are thought to be rare. Deep ulcers, prolonged drainage, lymphadenitis (1 %), abscess (2 %) (Turnbull 2002), osteitis (0.04 %) (Kroger 1995), and rarely disseminated infection have all been reported (Albot 1997).

The age of the recipient and the dose of vaccine affect the incidence of local complications. Disseminated disease is thought to be rare, in the order of 1/1,000,000 doses and directly related to immune dysfunction (Turnbull, 2002). The major worldwide concern about the risk of disseminated infection has been connected to the risk of HIV-related immunosuppression in the recipient. BCG is given routinely

to newborns in many countries. However, this practice is under active review because of concerns that the vaccine's problems may outweigh its efficacy. Some authors recommend that BCG vaccination should be confined to groups of infants with a high risk of TB infection, and should be given at six months of age, in order to reduce severe disease and deaths among infants with immunodeficiency disorders (Romanus 1993).

From 1993 to 2001, 20 adverse events of BCG vaccination were reported in members of TB-endemic Aboriginal communities in Canada. Six of these were disseminated disease and five were in children from Aboriginal communities (the sixth one was vaccinated as an infant outside Canada). All of these cases were confirmed as being caused by BCG. One of these children was HIV-infected and the other four had congenital immunodeficiencies, which presented for the first time as disseminated BCG infection. All of these children died as a result of their underlying immunodeficiency. This rate of disseminated infection indicates a higher rate of underlying congenital immunodeficiency in this population and an unanticipated serious risk in this population of BCG recipients (Hutmacher 2002). Health Canada recommends administration of BCG vaccine to all newborn infants who are members of TB-endemic Aboriginal communities because of the high rate of TB infection and the high risk of serious disease in young children after primary infection. The debate about BCG vaccine in Canada has accelerated as a result of concerns about adverse events (Clark 2006).

Several studies have shown clear benefits of BCG vaccination when the risk of tuberculous infection is higher than 1 % per year (Rouillon 1965, Immunization Practices Advisory Committee 1988, Health Canada 2002). These benefits become less clear when the risk of infection is lower than 0.1 %, as rates of severe TB disease and deaths are quite low, regardless of the BCG vaccination policy. Results of these studies are therefore consistent with recommendations of the IUATLD and World Health Organization (WHO), which state that discontinuation of BCG can be considered in populations with an annual risk of tuberculous infection lower than 0.1 % (IUATLD 1994; World Health Organization 2001). The vaccine may be considered in select situations where exposure to TB infection cannot be readily controlled with anti-tuberculous chemotherapy, particularly where multidrug resistance is documented. The recipients in this situation may include household contacts as well as laboratory personnel and travelers (National Advisory Committee on Immunization 2002). A study in which the vaccine was administered to high risk newborn infants before environmental exposure to mycobacteria could have occurred, showed an overall efficacy of 73 % (range 59 % to 80 %) for disease and 87 % for death (Rosenthal 1961, Fordham von Reyn 2002). The overall trends are

that newborns are better protected and primary disease, miliary TB, and TB meningitis are better prevented.

Another concern about the administration of BCG is its effect on the TST. Because administration of BCG induces a positive skin test of variable size in a large proportion of vaccinated individuals, this reaction will affect the interpretation of TST results in contact tracing, thus, jeopardizing the use of a valuable tool in the control of TB transmission in the community.

The place of BCG vaccination in TB control programs is being carefully reassessed because of the significant risk of dissemination in immunocompromised patients. BCG has to be administered to newborns from endemic countries in Africa, Asia, and Latin America, because the vaccine is effective for the prevention of disseminated TB and meningitis. However, a careful review and identification of underlying risks for immunodeficiency should also be performed. This should include a careful family history for immunodeficiency and prenatal HIV screening.

On the other hand, in non-endemic countries, BCG could be discontinued, but if the vaccine is no longer to be given routinely to newborns from endemic communities in these countries, then possible consequences must be anticipated. The rates of miliary TB and meningitis in these infants will increase if ongoing exposure of young infants continues. In the meantime, if the routine infant BCG vaccine program is abandoned in these communities, this must be compensated for by support of enhanced TB detection and treatment programs. An effective TB prevention and control program requires effective assessment of active disease, effective therapy including DOTS, finding and screening of contacts of infectious cases, and identification and management of latently infected individuals. Otherwise, there is little doubt that infants from these endemic communities will be at increased risk of disseminated primary TB. For example, Sweden moved from the mass vaccination of newborns with BCG to a selective vaccination program for high risk groups. This strategy met with some success, measured at 82 % effectiveness (Romanus 1992). This was accompanied by a higher rate of atypical mycobacterial infection in the non-BCG-vaccinated population (Romanus 1995). In some countries, such as Canada, the high risk population is already being vaccinated, but higher selectivity may be required given the identified risk of the vaccine; possibly limiting newborn BCG use to communities with active cases until the outbreak can be brought under control.

Safer and more effective vaccines for TB prevention may soon be available (Doherty 2005). Alternative vaccines to BCG are on the horizon and it is hoped that they will have better efficacy, be more standardized, and have fewer side effects,

especially in immunocompromised individuals, including the HIV-infected population worldwide, which is at a high risk of TB co-infection. (see chapter 10). One alternative intervention already exists in the form of the early detection and treatment of tuberculous infection. The administration of isoniazid is highly effective in reducing the risk of disease (International Union Against Tuberculosis Committee on Prophylaxis 1982) and protection may last for up to 30 years (Hsu 1984). Treatment of infection is generally well tolerated by children (Kopanoff 1978), and compliance is usually much higher than in adults (Wobeser 1989, McNab 2000). Considering the safety issues outlined in this report, the best course of action may be the removal of the BCG vaccine combined with improvements in TB programming in non-endemic countries (Vaudry 2003). Such improvements must include early case finding in adults to prevent transmission, and early detection and treatment of infection in children through contact tracing and screening in high-risk communities.

8.3. *Mycobacterium africanum* subtypes

M. africanum is predominantly isolated in Africa and, in certain areas of the continent, it is thought to produce a significant proportion of the cases of pulmonary TB (Frothingham 1999, Haas 1997). Reports on the sporadic isolation of *M. africanum* in Europe and the United States (Desmond 2004) have also been made, including one outbreak of multidrug-resistant (MDR) *M. africanum* (Schilke 1999).

Based on biochemical characteristics, two major subgroups of *M. africanum* have been described, corresponding to their geographic origin in Western (subtype I) or Eastern (subtype II) Africa. Numerical analyses of biochemical characteristics revealed that *M. africanum* subtype I is more closely related to *M. bovis*, whereas subtype II more closely resembles *M. tuberculosis* (Niemann 2002, Sola 2003). *M. africanum* subtype II was classified by its resistance to thiophen-2-carboxylic acid hydrazide (TCH). It is the main cause of human TB in Kampala, Uganda (East Africa).

Spoligotyping does not lead to a clear differentiation of *M. tuberculosis* and *M. africanum*, but all *M. africanum* subtype II isolates lack spacers 33 to 36, differentiating them from *M. africanum* subtype I. In an IS*6110* restriction fragment length polymorphism (RFLP)-based dendrogram, *M. africanum* subtype II isolates were clustered into two closely related strain families (Uganda I and II) and clearly separated from *M. tuberculosis* isolates. An additional characteristic of both *M. africanum* subtype II families is the absence of spoligotype spacer 40. In addition, all strains of the *M. africanum* subtype II family Uganda I also lack spacer 43 (Nie-

mann 2002, Viana-Niero 2001). Lack of spacers 40 and 43 are not exclusive markers for the *M. africanum* subtype II family Uganda I, but might represent a useful additional criterion for *M. africanum* subtype identification in combination with biochemical test results (Brudey 2004, Mostowy 2004) (see figure 8-1).

The *gyrB* desoxyribonucleic acid (DNA) sequence allows the differentiation of *M. africanum* subtype I strains from *M. bovis*, *M. caprae*, and *M. microti*. *M. africanum* subtype I and *M. pinnipedii*, however, display identical *gyrB* DNA sequences and the same occurs with *M. africanum* subtype II and *M. tuberculosis* (Niemann 2000). Thus, differentiation of *M. africanum* subtype II from *M. tuberculosis* continues to be based on phenotypic characteristics such as growth on bromocresol purple medium.

In recent studies, based on the regions of difference (RD) to distinguish *M. africanum*, three groups were identified: *M. africanum* subtype II isolates that have deletion of TbD1 and have retained RD9, RD7, RD8 and RD10 intact. Some have suggested that these organisms should be included in the species *M. tuberculosis*. A second group consists of *M. africanum* subtype I with deletion of RD9, RD7, RD8, and RD10, called 1a. Finally, *M. africanum* subtype I with deletion of RD9 but not RD7, RD8, and RD10, and called 1b, forms the third group. Both subtype I branches of *M. africanum* have indistinguishable *gyrB* sequences. In addition, Mostowy *et al.* (Mostowy 2004) have recently described several novel RD loci within *M. africanum* organisms. Among these were RD711 and RD713, deleted in *M. africanum* subtype Ib, and RD701and RD702, found deleted in a larger study of strains *M. africanum* subtype Ia (Mostowy 2004).

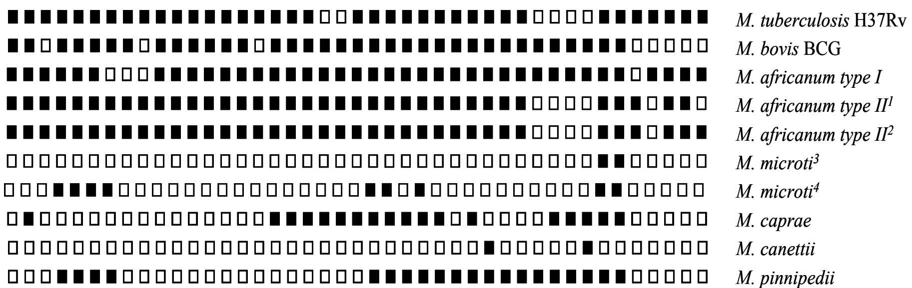

Figure 8-1: Spoligotypes of *Mycobacterium tuberculosis* complex strains. [1]*M. africanum* subtype II Uganda 1; [2]*M. africanum* subtype II Uganda 2; [3]*M. microti* vole type; [4]*M. microti* llama type

8.4. *Mycobacterium microti* disease

M. microti is a member of the *M. tuberculosis* complex and was first isolated in 1937 as the causative agent of pulmonary TB in the wild vole (*Microtus agrestis*) (Wells 1937). It was considered to be avirulent for humans, cattle and laboratory animals and was therefore proposed as a live vaccine against TB. The efficacy of vaccination with *M. microti* was assessed in clinical trials in the United Kingdom (Hart 1977) and the Czech Republic (Sula 1976), and indeed the strain was used as a vaccine in Africa for more than 15 years (Fine 1995). In all cases, *M. microti* proved to be safe and effective in preventing disease, showing a protective efficacy similar to that of BCG.

However, *M. microti* has been recently identified as the causative agent of pulmonary TB in both immunocompromised and immunocompetent humans (van Soolingen 1998, Horstkotte 2001). Genotypic analysis of *M. microti* showed the existence of two different variants of *M. microti*: vole and llama types. The vole type, isolated from voles, ferrets, and pigs, shows hybridization with only two of the 43 spacers in spoligotyping; whereas in the llama-type, the spoligotype-PCR product hybridizes with nine spacer sequences (figure 8-1). The first four *M. microti* isolates from humans in the Netherlands showed spoligotype patterns of the vole-type. Three of these four human *M. microti* isolates were obtained from immunocompromised patients (two had undergone kidney transplantation; one was HIV-infected). Two of the patients with *M. microti* infection had a history of contact with mice, which was found to be suggestive of zoonotic transmission (van Soolingen 1998, Brodin 2002). The first case of human infection with *M. microti* of the llama-type was reported in Germany: the patient was HIV-infected and presented with pulmonary TB (Horstkotte 2001). The time span required for cultivation of vole-type strains (3 and 4 months) is significantly longer than that required for growth of *M. microti* llama-type strains. The patient with *M. microti* llama-type infection was successfully treated with isoniazid, rifampin, and pyrazinamide, which indicates that the standard TB therapy is sufficient for treatment of patients with *M. microti* infection. A possible source of infection could not be identified in this patient.

Recent data demonstrated that *M. microti* can cause severe pulmonary TB in immunocompetent patients (Niemann 2000a). *M. microti* has been isolated in Germany from two HIV-negative immunocompetent patients with pulmonary TB. According to spoligotype patterns, one of the isolates belonged to the llama type and the other to the vole type. These findings emphasize the relevance of *M. microti* as a pathogen in immunocompromised as well as immunocompetent patients.

The prevalence and clinical importance of the different types of *M. microti* may have been underestimated so far because of difficulties with primary isolation and differentiation. Hence, further studies applying molecular methods are necessary to analyze the epidemiology of *M. microti* more thoroughly.

Genomic differences between *M. microti* and the other strains of the *M. tuberculosis* complex revealed novel deletions specific to *M. microti*. A surprising finding was that one of these deletions overlaps RD1, a locus that is absent from BCG substrains but present in *M. tuberculosis* and *M. bovis* and, therefore, assumed to be involved in the attenuation of BCG. The deletion found in *M. microti*, however, was found to be extended further to additional contiguous genes, and was therefore called RD1mic. Subsequent work has shown that complementation of *M. microti* with the RD1 locus increased the virulence of the recombinant strain in the mouse model (Pym 2002), suggesting that the loss of this region may have contributed to the attenuation of *M. microti*. The deletion of RD1 or RD1mic removes the genes esxA/esxB, which belong to the early secretory antigenic target 6 (ESAT-6) family and have been shown to be potent T-cell antigens. The ESAT-6 family may play a role in the attenuation of *M. microti*. In a genomic analysis using microarrays to compare *M. tuberculosis* and *M. microti*, 13 deletions were identified in 12 strains of *M. microti*, including regions RD1 to RD10, which are also missing in *M. bovis* BCG. In addition, four new deleted regions, MiD1, RD1mic, MiD2 and MiD3, were identified (Frota 2004). With regard to deleted regions and virulence, this study showed that it is difficult to ascribe virulence to any particular pattern of deletion. We have also used microarrays to extend the analysis of the *M. microti* genome (Garcia-Pelayo 2004). An *M. microti* of the vole-type, another of the llama-type, and a third isolate with an unusual type were used in this study. Using the improved resolution of this technique, a new deletion was described from *M. microti* that removes genes encoding ESAT-6 antigens and PE/PPE proteins, and it was shown that this locus may be prone to deletion. This region, called MiD4, was deleted from all *M. microti* strains tested suggesting that this region was deleted in a common ancestor of the *M. microti* lineage. Intriguingly, MiD4 was also found to be deleted from *M. pinnipedii*. As *M. pinnipedii* is closely related to *M. microti*, it is possible that deletion of MiD4 occurred in a common ancestor to both strains (Cousins 2003, Brosch 2002). The use of deletions as evolutionary markers demands that they are not generated at a hypervariable locus, since if this were the case, the deletion could appear independently in multiple lineages. The highly repetitive nature of the DNA that flanks MiD4 suggests that it may be prone to deletion, hence offering an alternative explanation as to why both *M. microti* and *M. pinnipedii* lack this locus. Sequence analysis of the *M. pinnipedii* junction, however, showed that it is identical to that of *M. microti*, suggesting that the loss of

MiD4 was a unique event that occurred in an ancestor of both strains. PE and PPE genes also appear overrepresented in deletions from *M. microti*, with RD1Mic, RD8, MiD3, and MiD4 removing genes for four PE and five PPE proteins. The genes encoding many PE or PPE proteins show a high degree of variation in the members of the *M. tuberculosis* complex and, indeed, among strains of the same species. Cole *et al*. were the first to speculate that the PE/PPE proteins could be of immunological importance, as a source of antigenic variation (Cole 1998). Further work has shown that some PE_PGRS proteins are surface-associated and immunogenic (Brennan 2001, Banu 2002). Using a signature-tagged mutagenesis approach, Camacho *et al*. showed that inactivation of PPE46 (Rv3018c) attenuated *M. tuberculosis* for the murine model (Camacho 1999). Interestingly, inactivation of a gene of the MiD4 produces attenuation of *M. tuberculosis*, and lends further evidence to suggest that loss of MiD4 could have attenuated *M. microti*.

8.5. *Mycobacterium caprae* and *Mycobacterium pinnipedii*

The *M. tuberculosis* complex traditionally consisted of four members: *M. tuberculosis, M. bovis, M. africanum, and M. microti.* More recently, three novel species have been described:

- "*M. canettii*": less virulent than the classical *M. tuberculosis* H37Rv
- *M. caprae*: a species that occurs primarily in Spanish goats, and also found in humans
- *M. pinnipedii*: responsible for TB in marine hosts.

8.5.1. *Mycobacterium caprae*

The names proposed for *M. caprae* are *M. tuberculosis* subspecies *caprae* (Aranaz 1999) and *M. bovis* subspecies *caprae* (Niemann 2002a). This species was originally described as preferring goats to cattle as hosts (Gutierrez 1995, Aranaz 1996) and has been found in Spain, Austria (Prodinger 2002), France (Haddad 2001), Germany (Erler 2003, Erler 2004), Hungary (Erler 2004), Italy, Slovenia (Erler 2004), and the Czech Republic (Pavlik 2002). In addition, *M. caprae* was isolated from humans and wildlife species such as red deer (Prodinger 2002) or wild boar (Erler 2004, Machackova 2004). In Central European regions, where *M. caprae* is the major cause of TB in cattle it is also the predominant agent of TB in humans (Kubica 2003, Prodinger 2002).

The major phenotypic difference between the caprine mycobacterial isolates and *M. bovis* is the sensitivity to pyrazinamide (PZA), which has been used as a major criterion for separation of *M. bovis* from the other members of *the M. tuberculosis* complex. Growth of *M. bovis* is not inhibited by PZA, while other *M. tuberculosis* complex species are susceptible to this antimycobacterial drug. The sequencing of the pyrazinamidase gene (*pncA*) demonstrated a single point mutation at nucleotide 169, a G to C substitution, which appears to be unique to *M. bovis* (Scorpio 1996). The sequence of the *pncA* gene of the *M. caprae* reveals that it has the wild-type *pncA* gene, and it can be used to differentiate between *M. caprae* and *M. bovis*. However, *M. caprae* is similar to *M. bovis* in its preference for pyruvate for growth, which differentiates both species from other members of the *M. tuberculosis* complex. In addition, it is possible to differentiate *M. caprae* from all other *M. tuberculosis* complex members by *gyrB* sequencing or amplification followed by restriction analysis (Chimara 2004). *M. caprae* also has specific fingerprinting patterns obtained by IS*6110* RFLP, as well as a spoligotype pattern that is very different from those obtained for other members of the complex. By spoligotyping, *M. caprae* forms a homogeneous cluster easily recognizable by the absence of spacers 1, 3–16, 30–33, and 39–43 (figure 8-1). The lack of spacers 39–43 has also been described in *M. bovis, M. microti,* and *M. pinnipedii*. However, the fingerprinting patterns obtained with IS*6110* and spoligotyping segregated *M. caprae* isolates from the other members of the complex (Liebana 1996, Aranaz 1998).

As reported in the original description of *M. tuberculosis* subsp. *caprae* (Aranaz 1999), isolates that displayed the caprine spoligotype pattern have also been found in humans, and these clinical cases have been linked with goat farming (Gutierrez 1997). *M. bovis* isolates from cattle and humans, described by Niemann *et al.* (Niemann 2000a, Niemann 2000b), are likely to be caprine isolates, because they share features such as susceptibility to PZA, the substitution described in the sequence of the *gyrB* gene, and the spoligotype pattern defined by the typical absence of spacers. Further evidence for the independence of the caprine mycobacterial isolates from *M. bovis* is derived from two recent studies that have examined the evolution of the *M. tuberculosis* complex (Brosch 2002, Mostowy 2002, see Chapter 2).

In Germany, *M. bovis* subsp. *caprae* has been described (Niemann 2002) as the causative agent of almost one-third (31 %) of the human *M. bovis*-associated TB cases analyzed. This proportion was surprisingly high, especially when compared with the prevalence of *M. bovis* subsp. *caprae* strains in human or animal isolates in other countries: a study on *M. bovis* TB in France revealed no *M. bovis* subsp. *caprae* strains among more than 1,000 animal isolates (Haddad 2000). *M. bovis*

subsp. *caprae* strains were not found in the United Kingdom (Sales 2001, Roring 1998), Ireland (Costello 1999), South America (Zumarraga 1999), and Cameroon (Njanpop-Lafourcade 2001). Outside Germany, small numbers of *M. bovis* subsp. *caprae* strains have been identified only in Spain (3.6 % of *M. bovis* isolates from humans and 12 % of isolates from goats and sheep) (Gutierrez 1997), and in Austria (12 cases in humans and animals in seven years) (Prodinger 2002). It might be assumed that *M. bovis* subsp. *caprae* represents a newly emerging genotype in Germany and is now spreading to other European countries. However, because the overall mean age for patients in Germany infected with *M. caprae* was 66.1 years, cases are probably due to reactivation rather than recently acquired infection. It is therefore likely that the patients were infected before effective control measures for bovine TB were introduced in the '50s. Consequently, *M. caprae* must have been present in Germany at that time and is not just emerging. Prior to the introduction of molecular tools for the identification and differentiation of *M. bovis* strains, *M. caprae* isolates might have been misclassified due to their susceptibility to PZA, resulting in false low notification rates. Susceptibility to PZA, however, was also observed in three *M. bovis* subsp. *bovis* strains that were obtained from two patients and a cow. These isolates showed no particular spoligotype patterns and were not related in the similarity analysis. From a phylogenetic point of view, these strains may represent ancestral *M. bovis* strains, from which both subspecies might have diverged. The only marked difference between the two patient groups was revealed in the spatial analysis of the inner-German origin of the patients: the regional proportion of *M. bovis* subsp. *caprae* showed a large difference between Southern (up to more than 80 %) and Northern parts of the country (less than 10%).

This observed geographic shift in the regional proportion of both subspecies might have resulted from a similar shift in the animal population, as indicated by the finding that animals infected with *M. bovis* subsp. *caprae* strains were mainly from Southern Germany. This is further supported by the presence of *M. bovis* subsp. *caprae* strains in wild and livestock animals in Western Austria, in a region located at the Southern German border (Prodinger 2002).

8.5.2. *Mycobacterium pinnipedii*

M. pinnipedii was first isolated from captive and wild sea lions and fur seals from New Zealand and Australia (Cousins 1993, Cousins 2003). Similar organisms were subsequently recovered from the same mammal species in South America (Bernardelli 1996, Romano 1995, Bastida 1999) as well as from a Brazilian tapir (Cousins 2003). Recently, their ability to cause disease in guinea pigs and rabbits has been

demonstrated by experimental inoculation (Cousins 2003). This fact, together with the finding of a human isolate from a seal trainer, who worked in an affected colony in Australia (Thompson 1993), and a bovine isolate in New Zealand (Cousins 2003), suggests that *M. pinnipedii* can cause infection across a wide host range. Many of the isolates obtained in Australia, Uruguay, and Argentina have been well characterized (Romano 1995, Romano 1996, Cousins 1993, Bernardelli 1996, Cousins 1996, Alito 1999, Zumarraga 1999a, Zumarraga 1999b, Castro Ramos 1998). This information, together with preliminary tests on seal isolates from Great Britain and New Zealand, suggested that the seal bacillus (Cousins 1993), isolated from pinnipeds from all continents, might be a unique member of the *M. tuberculosis* complex.

The results of biochemical tests clearly confirmed that the seal isolates belong to the *M. tuberculosis* complex. The negative reactions in the nitrate reduction and niacin accumulation tests were consistent with the identification of *M. bovis*, a fact that led to their initial identification as such in Australia (Forshaw 1991), Argentina (Bernardelli 1996), and Great Britain. Some seal isolates produced varying amounts of niacin, as do some *M. africanum* isolates. Most seal isolates grew preferentially on media that contained sodium pyruvate, although some also grew on Löwenstein–Jensen medium containing glycerol. In contrast to *M. bovis,* the seal isolates were susceptible to PZA. Isolates inoculated into guinea pigs produced significant lesions or death within six weeks and those inoculated into rabbits caused death within six weeks, confirming that the isolates were fully virulent for both laboratory animals.

Spoligotypes of mycobacteria isolated from seals (Romano 1995) showed the formation of a cluster that is clearly different from those of all other members of the *M. tuberculosis* complex (figure 8-1). All seal isolates lacked spacers 1 to 3, 8 to 22, and 39 to 43. The absence of these latter spacers is a characteristic shared with *M. bovis* isolates.

Mycobacteria isolated from seals were also tested for polymorphisms in the *oxyR* and *pncA* genes. Similarly to *M. tuberculosis, M. microti and M. africanum*, *M. pinnipedii* was found to contain CAC (His) at codon 57 in the *pncA* gene, and the *oxyR* gene showed G at nt 285. In addition, these mycobacteria had the same sequence polymorphisms of *gyrA* and *katG* as *M. bovis* and as some *M. tuberculosis* (Group 1 of Sreevatsan 1997). The MPB70 antigen, which is always detected in *M. bovis*, was not detected in the mycobacteria from seals. In contrast, their genomes contained the *mtp40* fragment present in the RD5 region described by Brosch *et al*. (Brosch 2002). The RD5 region is present in seal isolates, but is not present in *M. bovis* and BCG.

To extend the repertoire of these deletion markers, we therefore undertook a whole genome microarray analysis of the recently defined *M. pinnipedii* (Bigi 2005). In this study, we evaluated the extent of genetic variability in *M. pinnipedii* by microarray-based comparative genomics. This is a powerful method that allows genomes to be rapidly screened for deletion events. Using a DNA microarray that included both sequenced *M. tuberculosis* strains (H37Rv and CDC1551) and *M. bovis* AF2221/97, we identified two regions exclusively absent from *M. pinnipedii*. The PiD1 deletion was identified in this study for the first time as being absent from all isolates of *M. pinnipedii*. The coding sequences at the junction points are truncated, indicating that it is a deletion. Its bordering genomic regions do not contain repetitive sequences, suggesting that the deletion was the result of an irreversible event in a common progenitor strain. This deletion removes Rv3531c and parts of Rv3530c, encoding a hypothetical protein and possible oxidoreductase involved in cellular metabolism, respectively. The significance of these missing functions, if any, to the seal bacillus host tropism and phenotype is unknown at present. The second specific deletion, PiD2, has been recently defined as RD2seal by Marmiesse et al (Marmiesse 2004), since it overlaps the 10.7 kbp RD2 region. Interestingly, a region encompassing Rv1978 and part of Rv1979 is also missing in some *M. microti* isolates. However this deletion, called RD2mic, maps to a slightly different locus to that of RD2seal. This information, together with the fact that the RD2 region is deleted from some BCG sub-strains, strongly suggests that these deletions have occurred as independent events in an unstable region. These strain-specific deletions could serve as markers for phylogenetic and evolutionary studies, and also as a signature for rapid identification and diagnosis. Thus, these findings, together with previous studies, support the unique taxonomic position of *M. pinnipedii* within the *M. tuberculosis* complex.

8.6. Identification of species within the *M. tuberculosis* complex

The high degree of sequence conservation among members of the *M. tuberculosis* complex makes differentiation of species in the clinical mycobacteriology laboratory a difficult task. Routine differentiation is still based on phenotypic characteristics, such as oxygen preference, niacin accumulation, nitrate reductase activity, colony morphology, and resistance to two compounds, TCH and PZA.

M. tuberculosis is the most frequent cause of human TB, but some cases are caused by *M. bovis*. It is necessary to differentiate between *M. bovis* and *M. tuberculosis* in order to know the prevalence and distribution of human TB due to *M. bovis*. This may contribute to knowledge about the risk factors associated with the transmission

of *M. bovis* to the human population *M. bovis* differs from *M. tuberculosis* in having a low growth rate on egg media supplemented with glycerol, but a faster growth on egg media supplemented with pyruvate (Stonebrink medium). *M. bovis* isolates are resistant to PZA, while *M. tuberculosis* strains are generally considered PZA-sensitive.

Several molecular techniques were designed to differentiate *M. tuberculosis* complex, including methods to detect mutations in *pncA* and *oxyR* genes (Scorpio 1996), *mtp40*-PCR (Del Portillo 1991, Liébana 1996), and PCR-amplification of regions of difference (RD) (Parsons 2002, Huard 2003), among others. Some techniques are useful for the differentiation of *M. tuberculosis* and *M. bovis*, such as *pncA* and *oxyR*. A species specific mycobacterial DNA element in the *M. tuberculosis* complex has been described by Del Portillo (1991), the *M. tuberculosis mpt40* fragment. Mpt40 protein was originally described as being produced only by *M. tuberculosis.* Now, it is well know that this protein is encoded by the *plcA* gene, contained in RD5. This region is present in most, but not all, isolates of *M. tuberculosis, M. africanum, M. pinnipedii,* and *M. microti*, and is consistently absent from *M. bovis* and *M. bovis* BCG isolates. Given the high polymorphism in this region, the use of the *mpt40* sequence as a genetic marker for *M. tuberculosis sensu stricto* is very restricted (Viana-Niero 2004).

Spoligotyping can also be used for differentiation of members of the *M. tuberculosis* complex (Kamerbeek 1997). For instance, the spoligotypes of "modern" *M. tuberculosis* strains typically lack spacer sequences 33-36 in the direct repeat (DR) region (see Figure 8-1). Similarly, *M. bovis* and *M. caprae* strains are known to lack spacers 3, 9, and 16. All *M. bovis, M. caprae,* and *M. microti* strains are known to lack spacers 39 to 43 in their spoligotypes (Zumarraga 1999b). It should also be noted that all *M. tuberculosis* complex organisms along the *M. africanum* type I to *M. bovis* evolutionary track lack spacers 9 and 39. Therefore, spacers 9 and 39 are potential markers for the differentiation of *M. tuberculosis* from the remaining *M. tuberculosis* complex species by spoligotyping. Although their absence has been noted in *M. africanum* subtype I isolates, they are present in *M. africanum* subtype II.

For more details, see Table 8-2 at http://www.tuberculosistextbook.com/pdf/Table 8-2.pdf.

RD analysis is currently used for differentiation between species of the *M. tuberculosis* complex. TbD1 is a deletion found only in *M. tuberculosis*, all other *M. tuberculosis* complex strains, including some *M. tuberculosis* have TbD1. Based on the presence or absence of this *M. tuberculosis*-specific deletion 1 (TbD1), *M.*

tuberculosis strains can be divided into ancestral and ''modern'' strains, respectively; the latter comprise representatives of major epidemics, for example, the Beijing, Harlem, and other epidemics (Brosch 2002). TbD1 is always absent in *M. africanum* type II strains.

Previously, based on *katG* codon 463 (*katG*463) and *gyrA* codon 95 (*gyrA*95) sequence polymorphisms, Sreevatsan *et al*. (Sreevatsan 1996, Sreevatsan 1997) defined three groups among the tubercle bacilli: group 1 with *katG*463 CTG (Leu), *gyrA*95 ACC (Thr); group 2 with *katG*463 CGG (Arg), *gyrA*95 ACC (Thr); and group 3 with *katG*463 CGG (Arg), *gyrA*95 AGC (Ser). *M. tuberculosis* organisms belonging to group 1 have *katG* and *gyrA* sequences indistinguishable from those of *M. microti*, *M. africanum,* and *M. bovis*.

M. tuberculosis strains containing the TbD1 region belong to group 1, and are considered ancestral strains. However, *M. tuberculosis* with TbD1 deletion can also be in group 1, although most strains presenting TbD1 deletion belong to groups 2 and 3. This finding suggests that during the evolution of *M. tuberculosis*, the *katG* mutation at codon 463 CTG (Leu) occurred in a progenitor strain that had the region TbD1 deleted. This proposal is supported by the finding that strains belonging to group 1 may or may not have deleted region TbD1, whereas all strains belonging to groups 2 and 3 lack TbD1.

Furthermore, a subsequent loss of DNA, reflected by the deletion of DR9 was identified for an evolutionary lineage that diverged from the progenitor *M. tuberculosis* strains. It is represented by *M. africanum*, *M. microti*, *M. caprae*, *M. pinnipedii,* and *M. bovis* (Brosh 2002). Thus, RD9 allows differentiation between *M. tuberculosis* and the other strains of the *M. tuberculosis* complex. Other regions of difference, such as RD7, also allow differentiation between *M. tuberculosis* and the other species. RD7 deletion was observed in *M. bovis*, *M. microti*, some *M. africanum,* and *M. pinnipedii.*

In a previous report, we described a PCR protocol for the differentiation of *M. tuberculosis* from *M. bovis (*Zumarraga 1999c). This differential strategy is based on the amplification of the region designated as RD7. The deletion removes most of the *mce-3* operon, one of four highly related operons that may be involved in cell entry, and therefore it may contribute to differences in virulence or host specificity within the species of the *M. tuberculosis* complex.

Human beings can be infected by *M. caprae* or *M. bovis* from infected livestock, and infection with both species remains a serious public health problem in some countries. Differentiation of these species is important for epidemiological reasons. *M. pinnipedii, M. microti, M. bovis,* and *M. caprae* show a single nucleotide poly-

morphism in the TbD1 region at codon 551 (AAG) of the *mmpL6* gene relative to "*M. canettii"*, *M. africanum*, and *M. tuberculosis* strains, which are characterized by codon AAC. This polymorphism, which is associated with deletion of RD12 and RD13 loci, differentiates the group comprised of *M. bovis* and *M. caprae* from other species of the *M. tuberculosis* complex. On the other hand, it is now known that *M. caprae* can be genetically differentiated from *M. bovis* on the basis of a positive amplification of the RD4 locus, as well as SNP analysis of the *gyrB* nucleotide 1311-1410 and *pncA*169 (see Table 1). *M. bovis* BCG strains possess a specific polymorphism – the RD1 deletion. This deletion allows the differentiation between BCG and all the other species of the *M. tuberculosis* complex. *M. pinnipedii* and *M. microti* are very closely related microorganisms.

Some deletions that are useful for the differentiation of isolates of the *M. tuberculosis* complex are summarized in Table 8-3. These strain-specific deletions could serve as markers for phylogenetic and evolutionary studies, and also as a signature for rapid identification and diagnosis.

Table 8-3: Differential distribution of some regions of difference (RD) loci among *Mycobacterium tuberculosis* complex

	TbD1	**RD1**	**RD7**	**RD12 and RD13**	**RD4**	**RD2**	**PiD1**
M. tuberculosis ancestral	+	+	+	+	+	+	+
M. tuberculosis modern	-	+	+	+	+	+	+
M. africanum	+/-	+	+/-	+	+	+	+
M. microti	+	RD1mic	-	+	+	RD2 mic	+
M. pinnipedii	+	+	-	+	+	RD2seal	-
M. caprae	+	+	-	-	+	+	+
M. bovis	+	+	-	-	-	+	+

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Chapter 9: Molecular Epidemiology: Breakthrough Achievements and Future Prospects

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9.1. Introduction

Our understanding of the transmission of tuberculosis (TB) has been greatly enhanced since the introduction of desoxyribonucleic acid (DNA) fingerprinting techniques for *Mycobacterium tuberculosis* in the early '90s. Historical enigmas have been solved in the last decade and classical dogmas are being evaluated. This review summarizes the most important and recent findings in the molecular epidemiology of TB and discusses essential knowledge still lacking. Furthermore, current developments in the introduction of typing techniques are described, as well as future challenges to improve the usefulness of molecular markers in the epidemiology of TB.

Because the number of publications on the molecular epidemiology of TB has become too large to summarize in detail in a single review, only relatively new findings and subjects currently in the centre of attention are reviewed.

In the '90s, a wide variety of genetic markers for *M. tuberculosis* were identified (Kremer 1999). However, only a minor number of these appeared to offer enough discrimination and reproducibility for wide scale implementation (Table 9-1) (Kremer 1999, Kremer 2005a). In 1993, IS*6110* Restriction Fragment Length Polymorphism (RFLP) typing was adopted as the standard method for routine typing of *M. tuberculosis* (van Embden 1993). In this method, chromosomal DNA is digested with restriction enzyme *Pvu*II. The digested DNA is separated on an agarose gel and, after Southern Blotting, hybridized with a DNA probe. This DNA probe is directed to the IS*6110* insertion sequence and labelled with peroxidase, enabling enhanced chemiluminesence (ECL) detection of IS*6110*-containing restriction fragments (van Soolingen 1994). Another typing method, 'spoligotyping' has been used extensively as a secondary typing method (Bauer 1999, Kamerbeek 1997, Kwara 2003) and as a marker to study the phylogeny of the *M. tuberculosis* complex (Filliol 2002, Filliol 2003, Goyal 1997, Smith 2003). Spoligotyping exploits the polymorphism in the direct-repeat region of *M. tuberculosis* complex strains. This region consists of direct repeats interspersed with unique spacer sequences, and is amplified by Polymerase Chain Reaction (PCR) with primers directed to the repeats. The PCR-product is subsequently hybridized to known spacer sequences which are immobilized on a membrane through reversed-line blotting.

Because one of the primers, and hence the PCR product, is labelled with biotin, ECL detection is achieved after incubation with peroxidase-labelled streptavidin (Kamerbeek 1997). Another DNA typing method frequently used for *M. tuberculosis* is Variable Numbers of Tandem Repeats (VNTR) typing. Typing results of this method are expressed as numerical codes. Each number of the code represents the number of tandem repeats at a particular repeat locus. The number of repeats varies by strain and is determined through PCR amplification of the repeat locus with primers directed to the regions flanking that repeat locus and determination of the PCR-product size. After an extended period of improvement and validation, VNTR typing is now ready to become the next gold standard for typing of *M. tuberculosis* complex isolates (Supply 2006).

Table 9-1: Reproducibility and number of types obtained by using various DNA typing methods for differentiation of 90 *M. tuberculosis* complex strains and 10 non-*M. tuberculosis* complex mycobacterial strains (Kremer 1999, Kremer 2005a)

DNA target	Method used [a]	Reference	Reproducibility (%) [b]	No. of types obtained
IS*6110*	RFLP (*Pvu*II)	(van Soolingen 1994)	100	84
IS*6110*	Mixed-Linker PCR	(Haas 1993)	100	81
IS*6110*	FLiP	(Reisig 2005)	97	81
IS*6110*	IS*6110* inverse PCR	(Otal 1997)	6	nd [c]
IS*6110*	LM-PCR	(Prod'hom 1997)	81	73
IS*6110*/MPTR	IS*6110* ampliprinting	(Plikaytis 1993)	39	nd
IS*6110*/PGRS	DRE-PCR	(Friedman 1995)	58	63
15 loci	VNTR typing	(Supply 2006)	nd	89
12 MIRUs	VNTR typing	(Supply 2001)	100	78
ETRs A-E	VNTR typing	(Frothingham 1998)	97	56
5 QUBs [d]	VNTR typing	(Roring 2004)	87	82
DR locus	Spoligotyping	(Kamerbeek 1997)	94	61
DR locus	2nd gen. spoligotyping	(van der Zanden 2002)	90	61
DR locus	RFLP (*Alu*I)	(van Soolingen 1993)	100	48
PGRS	RFLP (*Alu*I)	(van Soolingen 1993)	100	70

(GTG)5	RFLP (*Hinf*I)	(Wiid 1994)	94	30
Total genome	APPCR	(Palittapongarnpim 1993)	71	71
4 conserved loci	Amadio PCR	(Amadio 2005)	74	13
*EcoR*I/*Mse*I sites	FAFLP typing	(Ahmed 2003)	7	nd
*EcoR*I/*Mse*I sites	FAFLP typing	(Sims 2002)	0	nd
*BamH*I/*Pst*I sites	FAFLP typing	(Kremer 2005a)	0	nd

[a] RFLP; Restriction Fragment Length Polymorphism, FLiP; Fast Ligation Mediated PCR, LM-PCR; Ligation-Mediated PCR, DRE-PCR; Double Repetitive Element PCR, VNTR; Variable Numbers of Tandem Repeats, APPCR; Arbitrarily Primed PCR, FAFLP; Fluorescent Amplified Fragment Length Polymorphism.

[b] Fraction of duplicates showing identical types (31)

[c] nd, not done

[d] Results indicated exclude QUB locus 3232

The disclosure of suitable genetic markers to study the epidemiology of infectious diseases in the last decades has led to the widespread use of a new phrase; 'molecular epidemiology'. In fact, as pointed out by Foxman (Foxman 2001), this phrase is used in many articles on DNA fingerprinting (strain typing) of bacterial isolates, regardless of the inclusion of epidemiological data. Often, the availability of bacterial isolates dictates the design of the study, and not a fundamental, relevant epidemiological question in a given area. In many published studies, microbiologists with an interest in molecular techniques were the main driving forces behind the described research. This was understandable in the initial stage of the implementation of molecular typing techniques, when the main emphasis was on the evaluation of genetic markers. However, now that the value of genetic markers for *M. tuberculosis* has become clear, it is important to involve researchers of different disciplines in the design of any molecular epidemiological study, in order to ensure the validity of the research question, the sample size, the selection of cases and the interpretation of the results.

9.2. Historical context

DNA fingerprinting of *M. tuberculosis* has been applied since the early '90s to study transmission of TB at various scales. The first report on the use of IS*986* RFLP to examine transmission of TB was published in September 1990 (Hermans 1990, McAdam 1990). Nine isolates with identical fingerprint patterns all originated from an outbreak of TB among individuals who were all treated by the same

physician, specialized in the treatment of arthritis patients. This finding led to the understanding that DNA polymorphism even in the genetically conserved *M. tuberculosis* complex isolates could be applied as a strain-specific marker. In the years thereafter, the disclosure of many other genetic makers for *M. tuberculosis* complex would follow.

Many investigators have tried to evaluate the reliability of strain typing by comparing the clustering of *M. tuberculosis* isolates based on DNA fingerprints with the findings on the respective TB patients in contact tracing. However, this was highly cumbersome, as contact tracing by interviews in itself is not at all capable of finding even a quarter of the epidemiological links between sources and follow-up cases. Thus, contact tracing cannot serve as a gold standard to evaluate DNA fingerprint results. In contrast, DNA fingerprinting seems to be a much more sensitive tool to visualize epidemiological links between cases than conventional contact tracing.

In the beginning, strain typing was mainly used to study outbreaks of TB and institutional transmission. Soon thereafter, in multiple population-based studies, the rate of recent transmission and risk factors for transmission were determined (Diel 2002, Small 1994, van Soolingen 1999). Active transmission of TB in low-prevalence settings appeared to be associated for a large part to particular risk groups such as drug abusers, homeless people, and certain immigrant groups (Diel 2002, Small 1994, van Soolingen 1999). Transmission of drug resistant bacteria could be compared to that of drug-susceptible strains (van Doorn 2006, van Soolingen 2000). These findings are discussed in Section 9.5.

With DNA fingerprinting, laboratory cross-contaminations were identified to occur at a considerable rate of 3-5 % of the positive cultures in low-prevalence settings, even though less than 10 % of the inoculated cultures were found positive in these areas (de Boer 2002, Small 1993). It is still not clear what the magnitude of this problem is in high-throughput laboratories in high-prevalence settings. Also, nosocomial infections by bacille Calmette-Guérin (BCG) have been disclosed by DNA fingerprinting and this contrasts the previous assumption that all *M. bovis* BCG infections are (late) complications of vaccination (Vos 2003b, Vos 2003a). Chemotherapeutics for the treatment of cancer patients were prepared in the same, non-disinfected biosafety cabinets that were used earlier to prepare BCG suspensions to treat bladder carcinoma patients. In this way, BCG bacteria were directly inoculated into cancer patients, in some cases with dramatic consequences.

More recently, hypotheses on the infectiousness of individual patients have also been tested (see below). Another important finding in molecular epidemiology is that exogenous re-infections after curative treatment play a much larger role than

previously anticipated (Das 1995, Sonnenberg 2001, van Rie 1999a). In the light of the description of exogenous re-infections it is interesting to read the recent observations on the detection of mixed infections (see Section 9.7). Can a part of the exogenous re-infections be explained by the initial presence of more than one strain in diagnosed TB patients?

Although *M. tuberculosis* may be one of the most widespread infectious agents in humans, not much is known about the evolution of this bacterium and whether there is an ongoing selection towards better adapted strains under the pressure of the measures introduced against TB in the last century. Because of the introduction of genetic markers for *M. tuberculosis,* the phylogeny of this bacterium can be studied in detail and the changes in the population structure can be disclosed. This has led to the recognition of a wide variety of genotype families worldwide (Bhanu 2002, Douglas 2003, Kremer 1999, Niobe-Eyangoh 2004, van Soolingen 1995, Victor 2004). In particular, the international database of spoligotyping patterns has been used most extensively for this purpose (Brudey 2006, Filliol 2002, Filliol 2003, Sola 2001).

Although it has become clear that the phylogeny of *M. tuberculosis* differs significantly in several geographic areas, not much is known about the dynamics of the population structure and the reasons for the genetic conservation observed among *M. tuberculosis* isolates in high-prevalence areas. If particular genotypes of *M. tuberculosis* are selected, how fast does a shift towards more adapted variants occur? Are we influencing the spread of particular genotypes of *M. tuberculosis*? Best studied in this respect is the Beijing genotype family of *M. tuberculosis*. There are indications that there is indeed a dramatic and relatively fast change in the composition of the worldwide population of *M. tuberculosis* (see Section 9.6). If the current observations hold true, we may be facing a recurrent TB epidemic caused by bacteria with a higher level of evolutionary development. However, more research is needed to draw better conclusions.

9.3. Infectiousness of tuberculosis patients

In most low-incidence settings, the majority of TB transmissions are limited to one or two persons. However, especially in high risk groups, such as the homeless and drug abusers in urbanized areas, ongoing transmission may take place for years and DNA fingerprint clusters sometimes grow over a hundred cases (unpublished observations in the Netherlands). In these clusters, primary, secondary, and tertiary sources can usually not be distinguished. This makes it difficult to know how many cases are derived from individual sources. DNA fingerprinting, however, has dis-

closed new information on the infectiousness of individual patients. For instance in San Francisco, 6 % of the TB cases in a two-year period seemed to have derived from a single source (Small 1994). In the Netherlands, a large outbreak in the small city of Harlingen was traced back to a single case diagnosed with a large doctor's delay (Kiers 1996, Kiers 1997).

It is only partly known what determines the transmissibility of TB. It is known that large patient- and/or doctor-originated delays play a significant role in the magnitude of transmission. Furthermore, a more extensive pulmonary process and a bad coughing hygiene clearly contribute to disease transmission. However, the bacteriological factor has not yet been established very well. It is, for instance, still not clear whether *M. tuberculosis* strains associated with large clusters on the basis of DNA fingerprinting are transmitted more easily than non-clustered strains. Is large-scale transmission only facilitated by risk factors, or do the bacterium's characteristics also contribute to a more efficient transmission and breakdown to disease?

Although there is a correlation between the smear status of a source case and the rate of transmission, smear-negative patients can also transmit TB. In San Francisco smear-negative, but culture-positive cases were found to be responsible for 17 % of the cases (Behr 1999). This indicates that smear-negative pulmonary TB suspects should be considered infectious.

9.4. DNA fingerprinting, contact investigation and source case finding

Case finding and treatment are the most important measures to inhibit the spread of TB in a community. In low-prevalence settings, where contact tracing has been routinely used for decades, a lot is known on how transmission of TB takes place. Prolonged exposure to an infectious source enhances the chance of transmission. Hence, direct and close contact with a TB patient is a main cause of infection in low-prevalence settings. However, in high-prevalence areas the transmission routes are less clear. What is the chance of acquiring an infection from an intimate contact in comparison to the chance of contracting TB from a casual contact in an environment with a high risk of infection? A recent study in South Africa (Verver 2004) pointed out that only 46 % of 313 TB patients had a matching fingerprint with an isolate of another member of the household they were living in. The proportion of transmission in the community that took place in the household was found to be only 19 %. This suggests that in this area, and presumably also in other high-incidence settings, TB transmission mainly occurs outside the household.

In settings in Western countries where the incidence of TB has become very low, the role of contact investigation remains highly important. In each area, the risk factors for the transmission of TB may differ. Factors such as being homeless, a drug abuser, living in urban areas, and low age have commonly been found to increase the risk of transmission (Borgdorff 1999, Borgdorff 2001, Diel 2002, Small 1994, van Soolingen 1999).

Usually, contact investigation is performed on the basis of the stone-in-the-pond principle and uses the Mantoux skin test (Veen 1990, Veen 1992) as an indicator of infection. Depending on the number of contacts found positive in the first ring of close contacts, the contact investigation is extended to the next ring of less intimate contacts. If again the ratio of positive contacts in that ring is high, the number is extended to the next circle of contacts. In many molecular epidemiological studies, it has been found that only a minority of the epidemiological links between TB cases disclosed by DNA fingerprinting, are also found by conventional contact tracing on the basis of interviews (Diel 2002, Lambregts-van-Weezenbeek 2003, Sebek 2000, Small 1994, van Deutekom 2004). This suggests that a large part of the TB transmission takes place through casual contacts in public places, such as bars, discothèques, public transportation, or other crowded settings. These contacts will generally not be found by interviews. Furthermore, in low incidence areas, where the skills of physicians to recognize TB adequately are waning, sources of transmission often spread the disease for extended periods and typing of isolated bacteria can help to find the source of an outbreak.

In the Netherlands, nationwide DNA fingerprinting of *M. tuberculosis* has supported contact investigations since 1993 (Lambregts-van-Weezenbeek 2003, Sebek 2000, van Soolingen 1999). All *M. tuberculosis* cultures are subjected to standardized IS*6110* RFLP typing, and clustered cases are systematically reported to the regional TB services involved (cluster feedback). In an evaluation of six years of routine DNA fingerprint surveillance, it was found that among 2,206 clustered cases, 462 (21 %) of the epidemiological links between patients were expected on the basis of contact tracing information. After cluster feedback, an additional 540 (24 %) epidemiological links were established. Epidemiological links based on documented exposure increased by 35 % (Lambregts-van-Weezenbeek 2003) (Figure 9-1).

Routine molecular typing also appears highly useful for evaluating the performance of TB control in a given area. In the Netherlands, each regional TB service quarterly receives an overview of the growth of the active-transmission clusters of patients to visualize in which populations ongoing transmission occurs and at what rate. In this way, municipal health services are able to deduce how much active

transmission is ongoing in their region. Sometimes this leads to new measures, such as active screening of particular risk groups.

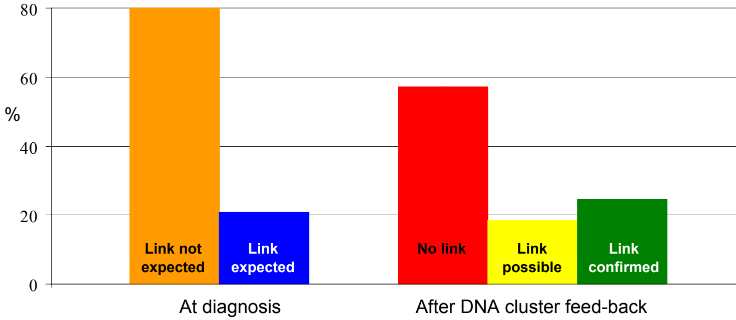

Figure 9-1: Epidemiological linkage at diagnosis and after cluster-feedback, the Netherlands 1994-2004. The bars indicate the percentage of cases with a certain level of epidemiological linkage. Source: KNCV/RIVM DNA fingerprint surveillance project.

One of the significant disadvantages of IS*6110* RFLP typing is that it requires extended culture incubation periods to obtain sufficient quantities of DNA. In the Netherlands, the typing results become available for contact tracing, on average, two months after the diagnosis of TB in a patient. At that time point, the contact investigation has usually already been finalized and not many TB services decide at that stage to re-open the contact investigations, even if the typing results provide new clues. However, the DNA fingerprint analysis clearly helps to evaluate the contact tracing process, and has therefore become an indispensable tool in TB control in the Netherlands. It is expected that the yield of molecular typing in resolving epidemiological links between patients will sharply increase when faster fingerprinting methods are implemented in the near future. In any case, nationwide molecular epidemiological analysis contributes significantly to the evaluation of contact tracing and the performance of a TB control program. It clearly indicates the rate of recent transmission and to what extent, and in which populations and areas it occurs. Figure 9-2, available at http://www.tuberculosistextbook.com/pdf/Figure 9-2.pdf, summarizes the surveillance of active transmission of TB in the Netherlands, 1997-2005.

9.5. Transmission of drug resistant tuberculosis

In a recent paper by Zignol *et al*. (Zignol 2006), the global incidence of multidrug-resistant TB (MDR-TB) was described. The estimates of the World Health Organization (WHO) on the global rate of MDR-TB have been updated from 272,906 MDR-TB cases in the year 2000 to 424,203 in the year 2004 because of the inclusion of countries that had previously not been surveyed. Zignol *et al*. underline the importance of expanding appropriate diagnostic and treatment services for MDR-TB patients, especially in countries with the highest burden of MDR-TB such as China, India, and the Russian Federation. Recently, the WHO also expressed its concern about the occurrence of extensively drug resistant (XDR) strains; *M. tuberculosis* isolates resistant to at least isoniazid (INH), rifampicin (RIF), to one of the fluoroquinolones, and to one of the injectable anti-tuberculosis drugs (Anonymous 2006). These alarming observations trigger the question; are resistant strains as transmissible as susceptible ones?

In as early as the ’50s, Mitchison observed that a large part of the INH resistant *M. tuberculosis* isolates revealed a lower degree of virulence in a guinea pig model (Mitchison 1954). For decades, it remained unclear whether resistant strains caused less transmission of TB than susceptible ones. This is important with respect to hygienic measures to prevent transmission from patients infected by MDR strains. Furthermore, for models predicting the development of the future TB epidemic, it is important to know if and how resistance interferes with transmission of TB. If resistant strains would be able to spread as efficiently as, or even better than susceptible ones, the global rates of anti-tuberculosis drug resistance would rise steadily. Indeed, transmission of highly resistant strains has been reported in, for example, New York (Bifani 1996) and South Africa (van Rie 1999b, Gandhi 2006). However, observations of transmissibility of particular (multidrug) resistant strains should not be generalized to resistance in general. In a review by Cohen *et al*., describing the effect of drug resistance on the fitness of *M. tuberculosis*, it was concluded that the fitness estimates of drug-resistant *M. tuberculosis* strains are quite heterogeneous and that this confusion makes it difficult to predict the influence of resistance on the trend of the TB epidemic (Cohen 2003). Indeed, various bacterial characteristics may influence the interference of resistance in transmissibility, including the drug susceptibility profile, the combination of mutations underlying drug resistance, presumably the genotype family the *M. tuberculosis* bacteria represent, and possibly bacterial DNA repair mechanisms. In addition, non-bacterial factors may influence the interference of resistance and transmissibility, such as the immune status of the humans exposed, and the treatment regimen ap-

plied. Because the above-mentioned factors have not been studied much, no meaningful conclusions can be drawn on the influence of the development of resistance on the worldwide TB epidemic. Yet, because of the contribution of DNA fingerprinting studies, some pieces of the puzzle have been unravelled in the last decade (van Doorn 2006, van Soolingen 2000).

In a recent study in the Netherlands, in which 8,332 patients from the period 1993-2002 were included, the drug susceptibility profiles and transmissibility of the respective isolates were studied with the aid of DNA fingerprinting (van Doorn 2006). In total, 592 isolates were resistant to INH, of which 323 carried a mutation at amino acid position 315 (Δ315) of the catalase-peroxidase gene (*katG*). The remaining INH resistant strains had other mechanisms underlying INH resistance. As predicted by Mitchison (Mitchison 1954), in general INH resistant strains were less transmissible (i.e. less frequently present in DNA fingerprint clusters) than susceptible ones. However, strains with the Δ315 were as frequently part of active transmission as susceptible ones. Moreover, the INH resistant strains with the Δ315 had a higher level of INH resistance and were associated with multidrug resistance (van Doorn 2006, van Soolingen 2000). This suggests that the type of genetic mutation underlying INH resistance is an important factor in the fitness of the bacterium. Thus, particular strains may be the cause of MDR-TB transmission in both high and low-incidence settings, even though INH resistant strains in general are less fit than susceptible ones. In South Africa, most of the childhood contacts of adults with MDR-TB were more likely to be infected from these than other (drug susceptible) TB sources (Schaaf 2000). It would be highly interesting to know the mutations underlying resistance in these cases.

In the Netherlands, transmission of MDR-TB is usually limited to incidental single person-to-person transmission. However, in the period 2003/2004 a single MDR-TB case infected nine other persons, of which two developed active disease. The respective MDR-TB strain had a mutation at amino acid position 315 of *katG* and exceptional mutations underlying RIF resistance (unpublished observations). It is not clear whether this type of resistant variant influences the epidemiology of TB in low and high-incidence areas. Therefore, further, more detailed and representative investigations into the basis of resistance in combination with the behaviour of the bacterium are needed.

9.6. Resistance and the Beijing genotype

Another important factor that may determine the transmissibility of resistant strains is the genetic background of the bacterium. Based on several genetic markers, various *M. tuberculosis* genotype families have been identified, such as the Beijing family (van Soolingen 1995), the Haarlem family (Kremer 1999), Family 11 (Victor 2004), the Manila family (Douglas 2003), the Delhi family (Bhanu 2002), the Cameroon family (Niobe-Eyangoh 2004), the Latin American Mediterranean (LAM) family, the Central Asian clade, and the East African Indian clade (Brudey 2006, Filliol 2002, Filliol 2003, Sola 2001). It is important to study genotypic and phenotypic characteristics of the genotype families that fuel the worldwide TB epidemic. Up until now, the Beijing genotype has been studied most extensively. The Beijing genotype was first described in 1995 (van Soolingen 1995), and strains belonging to this genotype family appeared to be genetically highly conserved, which suggests that the spread of these strains started relatively recently. Moreover, in several areas, Beijing genotype strains are more frequently isolated from young patients than from older patients (Anh 2000, Borgdorff 2003, Glynn 2006). If, in high incidence areas, active transmission of TB is associated with lower age of the patients, as it is in low incidence settings (van Soolingen 1999), this suggests that Beijing genotype strains are emerging. The fact that Beijing strains have more often been found recently where population-based molecular epidemiological studies have been ongoing for several years points in that direction (Borgdorff 2003, Glynn 2006). Furthermore, the Beijing strains are associated with drug resistance in some areas (Glynn 2002, Glynn 2006). Thus, strains of the Beijing family may have a genetic background that favours their transmission, despite their drug resistance.In 2006, a large worldwide survey was published on the spread of the Beijing genotype of *M. tuberculosis* and its association with drug resistance (Glynn 2006). In this study, which included 29,259 patients from 35 countries, the overall prevalence of Beijing strains was 9.9 %, and the proportion of TB due to the Beijing genotype ranged from 0 % to over 72.5 % per area. The Beijing genotype was endemic in East Asia and parts of the USA. In Cuba, the former Soviet Union, Vietnam, South Africa, and in parts of Western Europe this genotype was epidemic and associated with drug resistance (Glynn 2006).

Previously, in New York outbreaks of MDR-TB were also caused by one of the evolutionary branches of the Beijing genotype family; the W strains (Bifani 1996, Kurepina 1998). The W strains, however, are a relatively minor branch on the evolutionary tree of the Beijing genotype family.

It is to be determined to what extent the worldwide prevalence of MDR-TB is influenced by the success of particular genotype families of *M. tuberculosis* in absolute terms, such as the Beijing strains. It is at least striking that in many areas with a high rate of MDR-TB, the Beijing strains are also highly prevalent (Glynn 2006, Kruuner 2001, Pfyffer 2001, World Health Organization 2004, Zignol 2006). It has yet to be determined whether there is a causal correlation between these observations.

It remains unclear whether transmission of highly resistant strains in high incidence settings are exceptions to the rule that resistance in general costs fitness of the bacterium, or that particular genotypes of *M. tuberculosis* have developed efficient ways to become resistant to anti-tuberculosis drugs and maintain or even increase their ability to spread in a community. In the latter case, these genotypes will spread in the coming years and will influence the development of the worldwide TB epidemic.

9.7. Genetic heterogeneity of *M. tuberculosis* and multiple infections

When talking about multiple *M. tuberculosis* sub-populations in sputum of TB patients, two phenomena are often confused, although they should be clearly distinguished:

- multiple strain populations derived from a single ancestral strain displaying genetic drift
- multiple infections by more than one strain.

In the case of multiple (or mixed) infections, the presence of more than one *M. tuberculosis* strain is demonstrated on one occasion of culturing from clinical material. This should not be confused with re-infection, usually after curative treatment, as this refers to a new episode of the disease caused by another strain. In South Africa, where the prevalence of TB is very high, the contribution of re-infection to new episodes of TB after curative treatment is considerable, and has been estimated at 75 % (van Rie 1999a, Verver 2005).

Numerous observations in the molecular epidemiology of TB have pointed out that bacteria are subject to evolutionary change. Sometimes minor rearrangements of IS*6110* RFLP profiles are noticed in epidemiologically related- and serial patient isolates. The rate of change of IS*6110* RFLP patterns in such isolates has been studied by several investigators (de Boer 1999, Niemann 1999, Niemann 2000, Yeh

1998). However, also within clinical *M. tuberculosis* isolates, sub-populations of bacteria with minor genomic differences co-exist (de Boer 2000, Shamputa 2004, Shamputa 2006). For example, low-intensity bands in IS*6110* RFLP profiles are a reliable indication of a sub-population of bacteria with, for example, a one-band difference in IS*6110* RFLP. Preparation of single colony cultures and subsequent IS*6110* RFLP typing of isolates with such low-intensity bands showed the co-existence of separate sub-populations of bacteria, either with or without a normal-intensity band at the position where the low-intensity band occurred in the original clinical isolate (de Boer 2000).

Several recent papers describe the finding of multiple *M. tuberculosis* populations in sputum specimens of TB patients (Richardson 2002, Shamputa 2004, Shamputa 2006, van Rie 2005, Warren 2004). These findings point out that multiple infection of *M. tuberculosis* may be more prevalent than previously assumed. In the study by Warren *et al.*, a PCR technique was used to specifically identify *M. tuberculosis* bacteria of the Beijing genotype family and other evolutionary lineages in sputum specimens of patients from South Africa (Warren 2004). These authors concluded that at least 19 % of the patients included were infected by both Beijing and non-Beijing strains. Multiple infections were more frequently observed in re-treatment cases than in new cases. The same group also explored IS*6110* RFLP typing to detect multiple strain infections; a minor part of the IS*6110* RFLP patterns exhibited background patterns suggestive of mixed infections (Richardson 2002). This was confirmed in three (2.3 %) of the cases. In addition, another interesting approach was followed to study the occurrence of multiple infections in TB patients; by investigating *M. tuberculosis* strain diversity in autopsy material in South Africa (Plessis, 2001). In two out of 12 patients, pulmonary infection by two strains was demonstrated. The question remains about how this relates to the practical bacteriology: if this study had been performed at the time of diagnosis of TB in these patients, would one or two strains have been isolated from the sputum? Is the presence of multiple strains in autopsy material related to time-spaced infections, and do they represent re-infections? Is it possible that *M. tuberculosis* bacilli from a first infection are present in the body in a dormant state, and that a super-infection can lead to disease caused by the second infection without reactivation of the dormant bacteria? Therefore, although it is now clear that mixed infections do occur in TB patients, more research is needed to understand this phenomenon.

In the Netherlands, where from 1993 to 2006 about 15,000 *M. tuberculosis* isolates (of which 60 % were derived from patients from high-incidence regions) were subjected to IS*6110* RFLP analysis, double IS*6110* RFLP patterns were observed on only one occasion (de Boer 2000). During an episode of laboratory cross-

contamination in a peripheral laboratory in the Netherlands, clearly the RFLP pattern of the control strain was present as a background pattern in several isolates originating from that laboratory (Van Duin 1998). No other double IS*6110* RFLP patterns with different intensities were observed in any of the typing results. However, the sensitivity of IS*6110* RFLP typing to detect multiple infections is limited; at least 10 % of the DNA of a tested strain needs to be from another strain to be able to see this as a low-intensity, background pattern (de Boer 2000). Thus, multiple infections probably occur more often.

In the study by Shamputa *et al*., the clonality of 97 *M. tuberculosis* isolates was analyzed by first preparing a limited number (mostly 10) of single colony cultures and analyzing them by IS*6110* RFLP typing, spoligotyping, and VNTR analysis (Shamputa 2004). Different subpopulations of bacteria, including the ones representing evolutionary drift, were found in eight (8.2 %) of the isolates, while the frequency of confirmed mixed infections by different strains was 2.1 % (Shamputa 2004). In this study, it was found that the predominant strains and the primary isolates always had concordant drug susceptibility profiles, which suggests that the practical implications for the treatment of the respective cases were limited. However, in the study by Van Rie *et al.,* it was reported that re-infection and mixed infection do cause changes in drug susceptibility patterns of *M. tuberculosis* isolates and that treatment with second-line drugs may lead to re-emergence of drug-susceptible strains in patients with mixed infections (van Rie 2005). If mixed infections are common in high prevalence settings, this may be of concern for the clinician, as pointed out by Behr (Behr 2004); it may be that drug-resistant bacteria are not detected and cause a relapse after an apparent ‘curative’ treatment. With the current knowledge, such a case would probably be classified as exogenous re-infection, because no representative studies have been undertaken to combine investigations on mixed infections during the first episode of the disease and the presentation of relapses after treatment in the same patients.

Although the study by Shamputa *et al.* (Shamputa 2004) is so far the most extensive study on this subject published so far, one has to realize that the analysis of 10 colonies of a primary isolate is a very limited number. The chance of detecting a mixed infection is limited by the ratio of the strain variants in the isolates and the coincidence of picking the right colonies. When the ratio of a mixture is 1:1, 5 colonies need to be analyzed to identify both strains with a 95 % confidence interval. However, if the ratio of the mixture is 1:10, 29 colonies should be analyzed to detect a mixture with the same reliability. The ratio of mixed infections may be much less balanced in clinical samples; particular strains may predominate over other strains with a ratio of 1:100, 1:1,000, or even less.

It is also not clear whether individuals suffering from mixed infections (or re-infections) constitute a human population hypersensitive to *M. tuberculosis* infections with regard to their immunological and/or genetic background. More studies focusing on the immunological aspects and genetic predispositions possibly associated with re-infections would be highly interesting.

So far, only anecdotal observations on mixed infections have been reported. However, the current observations of mixed and re-infections in any case merit more representative studies to determine the magnitude of this problem. To critically evaluate the results and to check for possible laboratory cross-contamination, at least two culture-positive clinical samples should be analyzed.

9.8. The new standard genetic marker: VNTR typing

IS*6110* RFLP typing (van Embden 1993) has gained recognition as the gold standard in the molecular epidemiology of TB since 1993. However, this method is technically demanding and labor intensive, requires weeks of incubation for culturing of the isolates to obtain sufficient quantities of DNA, and suffers from problems of interpretability and portability of the complex banding patterns. In addition, it provides insufficient discrimination among isolates with a low number of IS*6110* copies (< 6); a problem that is only partly overcome by using additional typing methods, such as spoligotyping (Cowan 2005). Variable Number of Tandem Repeats (VNTR) typing is increasingly used to solve these problems (Frothingham 1998, Le Fleche 2002, Roring 2002, Skuce 2002, Smittipat 2000, Supply 1997, Supply 2000). This method is based on PCR amplification of multiple repeat loci, using primers specific for the flanking regions of each locus and on the determination of the sizes of the PCR products. The sizes of the amplicons reflect the number of tandem repeats present at the respective loci. Sizing can be done using a capillary system (Allix 2004, Kwara 2003, Supply 2001), gel electrophoresis (Mazars 2001), or non-denaturing high performance liquid chromatography (Evans 2004).

VNTR typing is considerably faster than IS*6110* RFLP typing, as it is applicable to crude low-concentration DNA extracts from early mycobacterial cultures. Furthermore, it has been adapted to high throughput format (Allix 2004, Kwara 2003, Supply 2001). Moreover, the results are expressed as numerical codes and are therefore easy to compare and exchange.

Currently, VNTR typing is often based on 12 Mycobacterial Interspersed Repetitive Units (MIRU) loci (Mazars 2001, Supply 2000) and has been integrated in TB control systems on a national scale in, for example, the USA (Cowan 2005). Based

on pilot studies with limited numbers of isolates, the discriminatory power of this 12 loci VNTR set approached that of IS*6110* RFLP typing to discriminate epidemiologically unrelated cases (Mazars 2001, Supply 2001), while VNTR types were stable among isolates from epidemiologically linked cases (Hawkey 2003, Kwara 2003, Savine 2002). A recent population-based study indicated that the use of this 12-loci method as a first-line screening in combination with spoligotyping provides adequate discrimination in most cases for large-scale, prospective genotyping of *M. tuberculosis* in the United States. However, IS*6110* fingerprinting is still required as an additional method to type the clustered isolates in a number of cases, when contact investigation, demographic or epidemiological data do not provide independent clues on the existence or the absence of links between patients (Cowan 2005).

Alternative sets of VNTR loci have been suggested to further improve the discrimination of unrelated isolates, as compared to that provided by this 12-loci system (Kam 2006, Kremer 2005b, Le Fleche 2002, Roring 2002, Roring 2004, Skuce 2002, Smittipat 2005, Surikova 2005). However, the collections of isolates studied were restricted to small samples of local origin and/or included only *M. bovis*, or representatives of only one or two of the defined *M. tuberculosis* lineages. The overall technical robustness and the clonal stability of the individual VNTR loci in the sets tested were not assessed. Furthermore, none of these studies were based on non-selected, population-based samples, and contact tracing data was not available, making it impossible to establish the predictive value of the various VNTR sets for studying ongoing *M. tuberculosis* transmission at a population-based level.

Recently, in an international collaboration, the resolution, stability and technical applicability of 29 VNTR loci was compared (Supply 2006). This study comprised the initial 12 loci and most of the other loci disclosed so far. The typing results of 824 *M. tuberculosis* isolates, including worldwide representatives of the main *M. tuberculosis* lineages, as well as multiple groups of epidemiologically linked or clonal isolates, revealed the 24 most optimal VNTR loci. Locus designations and PCR primer sequences for the 24-loci VNTR typing method are available in Table 9-2 at http://www.tuberculosistextbook.com/pdf/Table 9-2.pdf (Supply 2006).

Based on redundancy analysis, a highly discriminatory subset of 15 loci was selected for first-line epidemiological investigations. The use of these 15 VNTR loci was proposed as the new international standard for typing of *M. tuberculosis* complex isolates (Supply 2006). Extension to the use of 24 loci is especially useful in studying the phylogeny of strains.

As experienced after the standardization of IS*6110* RFLP typing in 1993 (van Embden 1993), it is expected that the international consensus on VNTR typing will facilitate the comparison of molecular epidemiological data from different geographical regions. The establishment of international VNTR databases and the meta-analysis of worldwide typing results will facilitate further study of the population structure of *M. tuberculosis*.

Many institutes in the world have large databases containing high numbers of IS*6110* RFLP patterns of *M. tuberculosis* isolates from extended periods, and are considering a switch from IS*6110* RFLP to VNTR typing. Because the epidemiology of TB demands the consideration of contacts with sources separated in time by years, the switch to VNTR typing cannot be done without any overlap of the use of the two typing methods. In order to trace transmission patterns retrospectively, it would be best to re-type all *M. tuberculosis* isolates from a number of years by VNTR typing. If this is too costly or time demanding, it could be considered to limit re-typing activities to strains from a more limited retrospective period; for instance three years. In addition, the typing of only one isolate from each IS*6110* RFLP cluster could reduce the re-typing workload significantly. If resistance issues play a role in the concerned setting, the re-typing could be restricted to resistant *M. tuberculosis* isolates. Alternatively, it could be considered to define an age limit for the re-typing activities, because active transmission mainly takes place through younger individuals (at least in low prevalence settings where this has been studied extensively) (Borgdorff 1999, van Soolingen 1999). However, these alternative approaches of re-typing, which do not include all isolates, will conceal a part of the VNTR polymorphism among the circulating isolates.

9.9. DNA fingerprinting to monitor eradication of tuberculosis

DNA fingerprinting may also be useful for studying the stage of the TB epidemic and to predict the future developments. In a recent study in the Netherlands, covering the period of 1993-2002, changes in TB transmission were determined using DNA fingerprinting to assess the progress towards TB elimination (Borgdorff 2005). Strains were defined as 'new' if their DNA fingerprint pattern had not been observed in any other patient during the previous two years. Other cases were defined as clustered and attributed to recent transmission. The incidence of TB cases involving new strains was stable among the non-Dutch and declined among Dutch nationals. However, the decline among the Dutch cases was restricted to those aged 65 years and over. It was concluded that the decline of TB in the Netherlands over the past decade is therefore mainly the result of a cohort effect: those with lower

infection prevalence replaced older birth cohorts with high infection prevalence. It is expected that TB will not be eliminated in the Netherlands in the near future, mainly because of the contact with high-burden countries through immigrants and international travel (Borgdorff 2005, Cobelens 2000).

9.10. Future prospects

Although the introduction of molecular markers for *M. tuberculosis* in the early '90s has greatly facilitated our understanding of the epidemiology of TB, even the latest most optimal typing, VNTR typing, will not be completely reliable. In fact, each genetic marker only reveals a minor part of the genomic information of a bacterium. Depending on the marker, different strains will exhibit identical genotyping profiles. Furthermore, in order to be able to follow the chains of transmission in a given area and to subdivide primary, secondary, etc. sources of infection, the turnover of genotyping profiles will never be in range with the pace of transmission.

To distinguish between even genetically related strains, and to be able to follow the spread of offspring of strains in the community, more detailed multiple-marker typing systems need to be developed. In fact, the most accurate typing would be whole genome sequence analysis of *M. tuberculosis* isolates. It is expected that with this information, the exact sequence in the evolutionary development of the offspring of a *M. tuberculosis* bacterium can be identified, without the interference of differences in time of incubation and confusion about the spread from primary and secondary, or even tertiary sources of infection in the same period. The current developments in DNA sequence techniques (Bennett 2005, Margulies 2005) provide possibilities to test these expectations and will provide more accurate predictions on transmission of TB. A largely unrecognized problem that has to be dealt with in due time is the occurrence of multiple (mixed) infections in high incidence settings (Shamputa 2004, Shamputa 2006, van Rie 2005, Warren 2004). This may hamper molecular studies on transmission severely.

Furthermore, the evolution of bacteria does not take place through whole population shifts in the genomic make up, but through mutation and multiplication of initially a single bacterium. By applying the current DNA amplification and sequence techniques, subtle genetic changes among bacterial strains are still difficult to visualize. However, creative future solutions may also deal with this phenomenon.

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Chapter 10: New Vaccines against Tuberculosis

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The current vaccine against tuberculosis (TB), bacille Calmette-Guérin (BCG), is a live vaccine derived from an attenuated strain of *Mycobacterium bovis*. BCG protects against severe childhood forms of the disease, but fails to protect against adult pulmonary TB in countries in which it is endemic. For more than 80 years, no new TB vaccine has successfully been developed. With TB eradication on the horizon, new vaccines with better protection than BCG are urgently needed.

The development of an effective TB vaccine seemed impossible until only a few years ago. In the last ten years of work with experimental laboratory models, many vaccine candidates have been developed. They include protein or DNA-subunit vaccines, modified BCG, and attenuated *Mycobacterium tuberculosis*. Some of these candidates are now being tested for safety and immunogenicity in human volunteers. For the first time, Phase I clinical trials of new TB vaccine candidates have started. Many of these new trials involve recombinant BCG or improved BCG immunity by boosting with vaccines consisting of subunits or attenuated Vaccinia virus expressing TB antigens. However, effective vaccination against TB presents diverse and complex challenges. For example, TB infection can become reactivated years later and infection does not guarantee resistance to a subsequent second infection. A truly effective TB vaccine may, therefore, have to elicit an immune response that is greater than that induced by natural infection. In addition, various different populations have to be protected: they include those vaccinated with BCG, and those infected with *M. tuberculosis* or with HIV.

The goal is a new generation of vaccines effective against the transmissible respiratory forms of TB. Good candidate vaccines able to boost BCG, thereby improving protection, could be a reality in the short term. The second step is to obtain a new generation of vaccines able to replace the currently used BCG and make the eradication of TB feasible. These new vaccines can be expected in the middle term, and live vaccines are reliable and promising candidates. Indeed, these ultimate goals may require safe live vaccines.

10.1. Introduction

M. tuberculosis, the causative agent of TB, is one of the most successful human pathogens. In some areas of the world, TB has reached alarming proportions with a growing number of cases and deaths associated to human immunodeficiency virus/

acquired immunodeficiency syndrome (HIV/AIDS) (World Health Organization 2005). The emergence of cases of multidrug resistant TB (MDR-TB), which sometimes cause outbreaks, is a serious public health problem for any attempt to control the disease (World Health Organization/ International Union against Tuberculosis and Lung Disease 2004). *M. tuberculosis* is responsible for more deaths than any other single infectious organism; there are more than 8 million new cases and 1.7 million deaths annually. Control strategies for TB rely heavily on case detection and treatment with at least three different drugs over long periods of time. Consequently, the development of multidrug resistance is a serious impediment to any attempt to control this disease (Espinal 2001). No new drug has been added to the first-line treatment regimen for TB for more than 30 years. In addition, the public health impact of *M. tuberculosis* has become increasingly severe, partly because of the HIV epidemic. There is a clear synergy between *M. tuberculosis* and HIV, and active TB increases HIV-related immunodeficiency and mortality (Toossi 2001). Indeed, TB remains the largest attributable cause of death in HIV-infected individuals, being responsible for 32 % of the deaths of HIV-infected individuals in Africa. In countries where the incidence of TB is highest, the populations most in need do not have access to treatment and, furthermore, in many cases anti-tuberculosis drugs are ineffective: the development of an effective TB vaccine is obviously now an urgent priority. Given the variable protective efficacy generated by the BCG vaccine against TB, there is a concerted effort worldwide to develop better vaccines that could be used to reduce the burden of TB.

10.2. Historical view

BCG is the only vaccine available for prevention of TB in humans. BCG is an attenuated live vaccine that was obtained after 230 successive passages in the laboratory between 1908 and 1921 from a pathogenic strain of *M. bovis*. It is an inexpensive vaccine that has been applied since the early ’20s and it has been given to more than 2.5 billion people since 1948. It has a long-established safety profile and an outstanding adjuvant activity, eliciting both humoral and cell-mediated immune responses. It can be given at birth or at any time thereafter, and a single dose can produce long-lasting immunity. Recent studies with long-term follow-up of American Indians demonstrated that a single dose in childhood maintains immunization for up to 50–60 years after vaccination (Aronson 2004). However, different studies in other parts of the world have shown that protection provided by BCG wanes over time, and the efficacy of BCG in adolescent and adult populations is reported to be highly variable among different geographical regions (Andersen 2005).

BCG protects against severe forms of childhood TB, including miliary TB and extrapulmonary localization, such as the often fatal tuberculous meningitis. This is why BCG continues to be recommended in the vaccination calendar of the WHO (http://www.who.int/vaccine_research/diseases/tb/en) in countries with a high TB prevalence and incidence. BCG vaccination is currently compulsory in at least 64 countries and administered in more than 167. Indeed, BCG remains the most widely used vaccine in the world (Fact Sheets – BCG Vaccine, http://www.cdc.gov/nchstp/tb/pubs/tbfactsheets/250120.htm). In addition, BCG confers protection against leprosy and it has also been licensed as a treatment for bladder cancer.

The level of protection conferred by BCG is very variable: it differs according to the form of pulmonary TB and can be affected in those cases in which TB is associated with AIDS. The efficacy of BCG vaccines against pulmonary TB varies between populations, showing no protection in India but 50-80 % protection in the United Kingdom. The lack of protection against pulmonary TB in endemic regions has enormous importance from the point of view of public health as regards eradication of TB (Fine 1995). The reasons for the failure of BCG have been widely debated, and remain the topic of active research. Natural exposure to environmental mycobacteria is thought to exert an important influence on the immune response, and this may mask or otherwise inhibit the effect of BCG vaccination in tropical countries. This theory has been supported by the fact that exposure to environmental mycobacteria is prevalent in those countries where BCG confers low protection, and by a number of studies showing that exposure to environmental mycobacteria has an impact on the protection afforded by BCG in animal models (Buddle 2002, de Lisle 2005, Lozes 1997, Brandt 2002). This phenomenon has been proposed as a plausible explanation for the North-South gradient in the effectiveness of BCG (Brandt 2002). Host-related differences, such as genetic and host immune status, use of different BCG preparations, diverse levels of nutrition, and socio-economic issues should also impact BCG efficacy in different populations. It has been recently demonstrated that cross reaction is due to antigens shared between BCG and environmental mycobacteria (Demangel 2005). New vaccines deprived of major antigens shared with environmental mycobacteria will overcome the problem of the antagonistic effect of BCG to previous environmental mycobacterial exposure.

In parallel, neonatal vaccination with BCG has been reported as effective in reducing the incidence of childhood TB in endemic areas. The risk of disseminated BCG among adult AIDS patients with childhood BCG immunization is very low, and in addition, childhood BCG immunization is associated with protection of

adults with advanced AIDS against bacteremia with *M. tuberculosis*. Studies in Zambia have shown that bacteremia due to BCG or *M. tuberculosis* is rare among children who have BCG immunization (even recent) and symptomatic HIV infection (Waddell 2001).

10.3. Genetic diversity between BCG vaccines

Since 1921, when BCG was used for the first time, different laboratories throughout the world have continued to sub-culture BCG, giving rise to the appearance of different variants, such as BCG Pasteur, BCG Moscow or BCG Brazil. These various BCG strains are different from each other and from their ancestors, such that it is prudent to refer to BCG vaccines in the plural because differences in protection and effectiveness could be due to variations between strains, and for this reason, the WHO has recommended lyophilization of BCG vaccine stocks and storage at -80°C (Behr 2002).

BCG vaccines have been classified into two major groups. BCG Tokyo, Moreau, Russia, and Sweden secrete large amounts of the MPB70 gene, have two copies of the insertion sequence IS*6110*, and contain methoxymycolate and MPB64 genes. In contrast, BCG Pasteur, Copenhagen, Glaxo and Tice secrete little MPB70, have a single copy of the insertion sequence IS*6110*, and do not contain the methoxymycolate and MPB64 genes (Ohara 2001).

Comparative genomic analysis has revealed the existence of several *M. tuberculosis*-specific regions that have been deleted from BCG with the loss of more than 100 genes (Behr 1997). These genomic comparisons have made it possible to determine the order of genetic events, including deletions and duplications, and changes in the IS*6110* copy number which occurred between its first use in 1921 and 1961 (Behr 1999). These complex genomic rearrangements in BCG strains have undoubtedly led to phenotypic and immunological differences and may contribute to the variability in vaccine efficacy. All these points reinforce the requirement for vaccines that are more effective than the currently used BCG vaccines against the respiratory forms and that are able to eradicate TB. Problems of substrain variability and protective efficacy of the current BCG vaccines could be overcome by new rationally-constructed live vaccines, for which the attenuation factor and immunity are known.

10.4. New vaccines: from the bench to clinical trials

Even if BCG has been demonstrated to be extremely useful and at the moment is the most utilized vaccine in the world (World Health Organization 1995), the development of new vaccines against pulmonary TB, which are able to replace the current BCG vaccine, is an important challenge (Kaufmann 2005). Since humans are the only reservoir of *M. tuberculosis,* the development of vaccines more effective than BCG could make TB eradication possible [see Development of New Vaccines for Tuberculosis Recommendations of the Advisory Council for the Elimination of Tuberculosis (ACET) on the Internet http://www.cdc.gov/mmwr/preview/mmwrhtml/00054407.htm].

The lung is the portal of entry of *M. tuberculosis* in most human infections and provides a suitable environment for this slowly replicating pathogen. Infection is established in alveolar macrophages of the distal alveoli before it is recognized by the adaptive immune response 5-6 weeks later. CD4+ and CD8+ T cells are recruited through the lung, inducing protective immunity.

Both CD4+ and CD8+ T cells are essential for protective immunity against *M. tuberculosis*. Resistance to *M. tuberculosis* involves the activation of mycobacterial-specific CD4+ and CD8+ T cells by dendritic cells, which migrate from the site of the infection in the alveoli to the draining lymph nodes. The development of interferon-gamma (INF-γ)-secreting CD4+ T cells is dependent on the secretion of IL-12 by infected dendritic cells. Subjects deficient in receptors for INF-γ and IL-12 are extremely susceptible to mycobacterial infections, confirming the absolute requirement for T helper 1 (Th1)-like T cells for host immunity (Flynn, 2004).

The nature of an effective immune response to TB is incompletely understood, but the most effective vaccination strategies in animal models are those that stimulate T-cell responses, both CD4+ and CD8+, to produce Th1-associated cytokines. Therefore, formulations that induce the production of enduring Th1 responses are desirable, and doubtless an essential element of a successful vaccine. Several adjuvants or live vaccines capable of inducing potent T-cell responses have been developed and some have entered clinical testing.

10.4.1 Challenges for tuberculosis vaccine development

There are a number of substantial underlying problems to be faced in developing vaccines with enhanced protective efficacy against TB (Table 10-1). In contrast to a classical vaccine-preventable disease such as smallpox, recovery from infection

with *M. tuberculosis* is not associated with sterilizing immunity against reinfection after clearance of the original infection with antibiotics. Studies of the molecular epidemiology of TB indicate that reinfection with new strains of TB is more frequent than previously believed (Caminero 2001). Therefore, vaccines need to be more effective than infection with *M. tuberculosis* itself (Van Rie 1999).

Table 10-1: Major challenges and concerns for TB vaccine development (modified from Martin 2005)

CHALLENGE	CONCERN
One third of human population infected with TB	New vaccines should be preventive and immunotherapeutic, too
Co-infection HIV/ TB	Safety: vaccines should be as attenuated as, or even more attenuated than BCG
Large percentage of population vaccinated with BCG	New vaccine candidates should be tested in a BCG-vaccinated population

A third of the population worldwide is estimated to be infected with *M. tuberculosis*. Therefore, any new TB vaccine should protect pre-exposure people from developing infection, as well as post-exposure, latently infected, healthy individuals from developing the disease, or should be used as an immunotherapeutic agent to act with antimicrobials to increase the rate of clearance of *M. tuberculosis*.

An additional challenge is that as a large percentage of the human population has already been immunized with BCG, and so any new generation vaccines against TB must also be able to protect the population that has already been vaccinated with BCG. Obviously, new vaccines must also be safe enough to be used in HIV-infected individuals (Vuola 2003).

Advances in the characterization of genes and antigens of *M. tuberculosis* and the technological development (Clark-Curtiss 2003), with the help of the genome sequences of different mycobacterial species (Cole 1998), have provided insights into the tubercle bacillus (see TubercuList Web Server on the internet http://genolist.pasteur.fr/TubercuList/. In addition, the current progress of mycobacterial genetics has made the inactivation of selected genes possible, allowing the rational attenuation of *M. tuberculosis* (Pelicic 1997). Finally, the improvement in comprehension of the basic immune mechanisms involved in TB has considerably contributed to the rational design of the next generation of vaccines. Remarkably, novel immunological concepts about the mechanisms underlying memory and regulation of the immune response against TB have been defined as relevant for the

rational design of new-generation vaccine candidates (Kaufmann 2005). Therefore, this progress in the different fields of TB research has placed us in a better position for the construction of new effective and safe vaccines against TB.

Many groups in numerous countries have embarked on the ambitious project of finding new vaccines that provide a greater level of protection than the present BCG (see EC TB VAC consortium on the internet http://ec.europa.eu/research/press/2004/pdf/pr2304-tb-vac.pdf) (Orme 2005, Martin 2005, Kaufmann 2000). As a result of this basic research, the enormous effort of the scientific community in the last 10 years has generated a great number of vaccine candidates against TB to be tested in different laboratory experiments, experimental animal models (Williams 2005, Orme 2006), and clinical trials in human populations (Skeiky 2006).

Broadly, two approaches have been used to improve the TB vaccine. The first involves subunit vaccines that can deliver immunodominant mycobacterial antigens. Both protein and DNA vaccines induce partial protection against experimental TB infection in mice but their efficacy has generally not been better than that of BCG (Huygen 1996). New antigen formulations, including multiple antigens or epitopes, are under investigation and it is hoped that they will afford better protection in humans. The second approach involves live vaccines. These may be BCG strains that have been genetically manipulated to express immunodominant antigens, or attenuated strains of *M. tuberculosis* produced by random mutagenesis and targeted deletion of virulence genes (Britton 2003).

10.4.2. Animal model for vaccine preclinical trials

The most commonly used animal model is the mouse, followed by the guinea pig. Primate models have also been developed and are being used as an important testing model prior to clinical trials (Langermans 2001).

The advantage of the mouse model comes from the amount of reagents and genetic information available, and its logistical and economical advantages, in comparison with other models such as the guinea pig. Mice have a certain tolerance to this infection; it triggers a moderate inflammatory reaction that allows the control of the bacillary concentration at a low level, without eradicating it. The commonest route of infection is intravenous, because this switches on acquired immunity very rapidly. The experimental model induced by aerogenesis, uses the most physiologically infectious route and at the same time is more aggressive for the host than intravenous administration. This happens because the induction of immunity is

quicker after intravenous inoculation than after aerosol. Both models have demonstrated that immunity against infection is based essentially on the stimulus of a Th1-type response, that is to say, in the stimulation of CD4+ T cells able to produce IFN-γ and to activate the infected macrophages (Orme 2001, Aguilar 2006). Testing the protection obtained from new vaccines using the guinea pig model has become a compulsory experiment because of the extreme sensitivity that this animal has demonstrated with *M. tuberculosis* inoculation, and the toxic response generated. This has allowed the comparison of different TB vaccine candidates (Williams 2005). On the other hand, the necessity to evaluate the protection of any new vaccine in an experimental model that is physiologically closer to humans, before carrying out human clinical trials, has led to the development of the primate model (Langermans 2001, Langermans 2005).

10.5. Subunit vaccine candidates

Due to safety reasons, non-viable sub-unit vaccines are the first to be considered for human trials. Subunit vaccines have been selected by various rational and experimental approaches (Table 10-2). Results with non-viable subunit vaccines are encouraging but their protective effects have to be at least equivalent to that of BCG before they can be considered for testing in humans.

Potential TB subunit vaccines have been obtained by using immunodominant TB antigens, for example ESAT-6 [6-kiloDalton (kDa) early secretory antigenic target], which confers some degree of protection against *M. tuberculosis* in mice (Olsen 2004) and recently in non-human primates (Langermans 2005).

A fusion protein based on ESAT-6 and antigen 85B administered to mice together with a potent adjuvant induced a strong dose-dependent immune response. This immune response was accompanied by protective immunity comparable to BCG-induced protection over a broad dose range. The vaccine induced efficient immunological memory, which remained stable at 30 weeks post vaccination. More recently, it has been documented that the synthetic adjuvant IC31 augmented the immune response and protective efficacy of the combination of Ag85B-ESAT-6 in the mouse aerosol challenge model of TB (Agger 2006).

Table 10-2: TB vaccine candidates tested in humans (modified from Martin 2006)

Vaccine type	Definition	Stage of development	Pharmaceutical company or research group
Sub-unit			
72f	Selected antigens identified from human response	Phase I trial ready for phase II BCG boosting strategy	GlaxoSmithKline (EU/TBVac/Aeras) (Irwin 2005)
85B-ESAT6	Recombinant major antigens	Phase I trial BCG boosting strategy	SSI (EU/TBVac) (Langermans 2005)
Viral vector			
MVA-85A	Recombinant modified vaccinia virus Ankara Ag85A	Phase I trial BCG boosting strategy	Oxford University, United Kingdom (EU/TBVac) (McShane 2004)
Live vaccines			
rBCG30	Recombinant BCG: over expression of Ag85B	Phase I trial	(UCLA/NIH /Aeras) (Horwitz 2003)

EU/TBVac: http://www.tb-vac.org
SSI: http://www.ssi.dk/sw1404.asp
UCLA: www.research.ucla.edu/tech

Key *M. tuberculosis* antigens have been identified by analysis of host responses in healthy individuals, and purification of proteins from positive donors. These selected antigens have been used for the development of subunit vaccines against TB, for example Mtb72F, which codes for a 72-kDa polyprotein (Mtb32(C)-Mtb39-Mtb32(N)). Immunization of mice with Mtb72F protein, formulated in the adjuvant AS01B, generated a comprehensive and robust immune response, eliciting strong IFN-γ and antibody responses for all three components of the polyprotein vaccine and a strong CD8+ response directed against the Mtb32(C) epitope. Mtb72F immunization resulted in the protection of C57BL/6 mice against aerosol challenge with a virulent strain of *M. tuberculosis*. Most importantly, immunization of guinea pigs with Mtb72F produced a prolonged survival (> 1 year) after aerosol challenge with virulent *M. tuberculosis,* comparable to BCG immunization. Mtb72F in the AS02A formulation is currently in phase I clinical trials, making it the first recombinant TB vaccine to be tested in humans (Skeiky 2004, Irwin 2005).

10.6. Subunit vaccines for boosting BCG

Since acellular vaccines have never been demonstrated to confer better protection than BCG in preclinical testing, they have been proposed to be used for boosting BCG. Heterologous prime-boost immunization strategies can evoke powerful T cell immune responses and may be of value in developing an improved TB vaccine. Importantly, this regimen of vaccination expands pre-existing memory T cells against antigenic epitopes shared by BCG and the booster vaccine. Experiments using protein subunits in animals previously vaccinated with BCG have given very good results (Brooks 2001). These experiments used Ag85A, because it was previously demonstrated that most CD4+ T cells accumulating in the lungs of memory-immune mice after challenge recognize this antigen. This vaccine strategy may have applications in the prevention of reactivation of TB in the elderly.

Enhanced immunogenicity and protective efficacy against *M. tuberculosis* has been demonstrated for BCG after boosting with a recombinant modified vaccinia virus called Ankara. The recombinant virus, expressing *M. tuberculosis* Ag85A, strongly boosts BCG-induced Ag85A-specific CD4+ and CD8+ T cell responses in mice. Protection correlated with the induction of Ag85A-specific, IFN-γ-secreting T cells in lymph nodes in the lung (Goonetilleke 2003). This vaccine was tested for the first time in humans (McShane 2004).

Similarly, a combination of vaccines has been shown to be more protective in preventing bovine TB in cattle than single vaccines. Tested in calves, prime-BCG boost strategies of vaccination were reported to induce cellular immune response (Vordermeier 2006) and high levels of protection against challenge with virulent *M. bovis* (Cai 2006, Skinner 2003).

10.7. Recombinant BCG vaccines

Recombinant BCG (rBCG) techniques may be useful for the development of a more effective mycobacterial vaccine than the parent BCG now in use. Various strategies have been used to develop recombinant BCG against mycobacterial diseases (Table 10-3). One is based on rBCG producing large amounts of autologous protective antigens; these supplementary antigens are designed to enhance immunity to other BCG antigens by increasing the expression of their genes, as is the case of the immunodominant TB antigens. rBCG vaccine (rBCG30), which expresses and secretes the 30 kDa major secreted protein of *M. tuberculosis,* also referred to as a-antigen and antigen Ag85B (Horwitz 2000), is associated with better host survival after challenge than parental BCG in the highly demanding

guinea pig model of pulmonary TB. Animals immunized with rBCG30 and then challenged with an aerosol of a highly virulent strain of *M. tuberculosis* survived significantly longer than animals immunized with conventional BCG (Horwitz 2003, Horwitz 2005).

Alternatively, BCG genes that have been lost by deletion from the parental *M. bovis* strain, and that are important antigens, can be restored. An example is the case of ESAT-6 deleted from region RD1 (region-of-difference 1) of BCG (see Unité Génétique Moléculaire Bactérienne on the internet http://www.pasteur.fr/recherche/unites/Lgmb/Deletion.html (Pym 2003). Both these approaches are attractive for improving or adding antigens to BCG and could be important for conferring immunity against TB (Table 10-3).

Table 10-3: Live tuberculosis vaccine candidates in advanced preclinical testing (modified from Martin 2006)

Vaccines	Definition	Research group
rBCG:RD1	Recombinant BCG RD-1 of *M. tuberculosis* introduced	Institut Pasteur Paris, France (EU/TBVac) (Pym 2003)
rBCG-Δure-hly	Recombinant BCG with BCG urease gene deleted and listeriolysin of *Listeria monocytogenes* introduced	Max-Planck Institute Berlin, Germany (EU/TBVac) (Grode 2005)
M. tuberculosis phoP mutant	Rational attenuation of clinically isolated *M. tuberculosis* by *deletion of* virulence gene	Zaragoza University, Spain Institut Pasteur (EU/TBVac) (Martin 2006)
M. tuberculosis auxotrophic mutant	Rational attenuated *M. tuberculosis H37Rv* by *lysA* and *panCD* deletion	Albert Einstein College of Medicine, New York, USA (NIH) (Sampson 2004)

A second strategy involves enhancement of the relatively low intrinsic ability of BCG to induce the CD8+ T cell response. This type of rBCG has the capacity to alter the permeability of the membranes of phagosomes in host cells and gain access to cytoplasm. Major histocompatibility complex (MHC) class I-restricted CD8+ T cells are believed to play a major role in protection against mycobacterial infection. As BCG persists within the phagosomal space of macrophages after infection, bacterial antigens should be released from phagosomal vacuoles into the cytoplasm of host cells leading to more pronounced presentation by MHC class I. Listeriolysin is a pore-forming sulfhydryl-activated cytolysin essential for the release of *Listeria monocytogenes* from phagosomal vacuoles into the cytoplasm of host cells, thereby facilitating presentation of antigens by MHC class I molecules. Hess and collaborators constructed an rBCG, which secreted biologically active

listeriolysin (hly^{+}-rBCG), shown to improve MHC class I-presentation of co-phagocytosed soluble protein (Hess 1998). Tested in mice, hly^{+}-rBCG elicited better protection against aerosol infection of *M. tuberculosis* than the parental BCG (Grode 2005). In addition, a version of a hly^{+}-rBCG, deficient in urease C, has been shown to significantly improve the level of protection against *M. tuberculosis* in mice and to increase apoptosis in infected macrophages. Urease deficiency enables acidification of the phagosome so that listeriolysin finds its optimum pH for perforation of the phagosomal membrane. The authors advocate that the high efficacy observed may be due to the presentation of extracellular antigens with the MHC class I molecules to CD8+ T cells (cross-priming) caused by apoptosis (Grode 2005).

In another approach, rBCG has been constructed to secrete diverse cytokines, including IL-2, IFN-γ, and others, in an attempt to enhance the immuno-stimulatory properties of BCG (Murray 1996).

Additionally, a major effort is being made to develop rBCG as a vaccine vehicle capable of simultaneously expressing antigens of numerous pathogens. The aim is the development of effective rBCG multivalent vaccines to control major infectious diseases. Promising rBCG vaccines against a variety of viral, bacterial and parasitic diseases have been shown to induce protective immune responses in murine and primate challenge models (Santangelo 2007, Ohara 2001, Winter 1995).

10.8. Live vaccines based on attenuated *M. tuberculosis*

Of the six immunodominant antigens of *M. bovis* (ESAT-6, CFP10, Ag85, MPB64, MPB70, MPB83), five are either deleted from or down regulated in some or all BCG strains. Moreover, RD1, a region of difference between *M. tuberculosis* and BCG (see the Annual report of Bacterial Molecular Genetics for 2002 on the internet http://www.pasteur.fr/recherche/RAR/RAR2002/Lgmb-en.html) includes two of the six immunodominant antigens ESAT-6 and CFP10, which have been shown to be important for protection against *M. tuberculosis* challenge in the guinea pig model (Pym 2003). The advantage of rational attenuated *M. tuberculosis* as a vaccine is that hundreds of genes deleted from BCG as a consequence of the progressive adaptation of BCG strains to laboratory conditions are still present in *M. tuberculosis*. The advances in TB research and the completion of the *M. tuberculosis* genome sequence (Cole 1998) have facilitated the analysis of the contribution of individual genes to the virulence of *M. tuberculosis* (Camacho 1999).

Several studies have described the development of attenuated strains of *M. tuberculosis*. A *M. tuberculosis phoP* mutant has been constructed by a single gene disruption (Perez 2001) and exhibits impaired multiplication *in vitro* within mouse-cultured macrophages; it is also attenuated in vivo in a mouse infection model. Thus, the PhoP gene might be involved in the regulation of complex mycobacterial lipids implicated in the virulence of *M. tuberculosis* (Gonzalo Asensio 2006). Results in an animal model make a *phoP* mutant a promising TB vaccine candidate (Martin 2006). Similarly, it was recently demonstrated that the lack of *mce* (mammalian cell entry) gene expression in *M. tuberculosis* decreases virulence and increases immunogenicity, providing better protection than BCG against TB in the mouse model (Aguilar 2006) (Table 10-3).

Auxotrophic mutants, which require the addition of nutrients for survival, maintain their infective ability, but have a limited replication in the host. These vaccines are attenuated to different degrees and have diverse potential as vaccine candidates, as assessed in animal models (Martin 2006, Smith 2001).

There are major issues associated with the use of live organisms, especially safety and regulatory hurdles, that need to be overcome, in particular with attenuated *M. tuberculosis*. The early use of BCG was marked by a tragic accident. In 1927, in Lubeck, Germany, more than 25 % of approximately 250 infants who received a batch of the vaccine developed TB. It was later recognized that this batch was accidentally contaminated with a virulent strain of *M. tuberculosis* (Kaufmann 2006).

In a world conference held in Geneva in 2005, a consensus document was elaborated on, aiming to promote the movement of the most promising vaccine candidates to the clinic and towards control of TB (Kamath 2005). A set of criteria were proposed to be considered during the vaccine development process. One of the criteria for a live candidate vaccine is the presence of at least two non-reverting independent mutations on the mycobacterial genome. In this regard, double auxotrophic mutants have recently been described (Sampson 2004, Sambandamurthy 2005, Sambandamurthy 2006). Some of these live vaccine candidates elicited protective immune responses similar to that of BCG in mice, and better than BCG in guinea pigs (Martin 2006, Williams 2005). These findings are encouraging, and further studies in non-human primates should be performed.

Live vaccines have been questioned because of the failure of the BCG vaccine due to pre-exposure to environmental mycobacteria, which was shown to block multiplication of BCG and induction of protective immunity in animal studies. Evidence was provided that sensitization with environmental mycobacteria may have a direct antagonistic effect on BCG vaccination (Flaherty 2006). Recently, it was experi-

mentally demonstrated (Demangel 2005) that cross-reaction is due to antigens shared between BCG and environmental mycobacteria, such as Ag85B, but not deleted antigens of BCG, such as ESAT-6 and CFP10. These results strongly suggest that prior exposure to live environmental mycobacteria primes the host immune system against mycobacterial antigens shared with BCG, and that recall of this immune response on vaccination results in accelerated clearance of BCG and hence decreased protection against TB. The authors demonstrated that the persistence of BCG in vivo could be markedly augmented by the stable insertion of RD1, which encompass *esat 6* and *cfp10* genes.

Rational attenuated *M. tuberculosis,* which includes regions deleted in BCG with major antigens not shared with environmental mycobacteria, will probably overcome the problem of the antagonistic effect of BCG to previous environmental mycobacterial immunization. *M. tuberculosis* mutant vaccine candidates have to induce long-term cellular immune responses, essential for effective protection against TB. New live vaccines should be stored lyophilized, and current technology allows monitoring of any possible variations of genomic composition by comparative hybridization experiments using DNA microarrays.

10.9. Conclusions

Although the efficacy of the BCG vaccine continues to be discussed, live attenuated BCG is still the only vaccine in use for the prevention of TB in humans because it is effective against the severe forms of TB and its use prevents a large number of deaths that would otherwise be caused by TB every year.

The choice of the BCG strain to be used for vaccination is a very important issue. It is currently difficult to determine which strain should be used, and further detailed analysis of the genomics and immunogenicity of BCG sub-strains may provide an answer to this important question. The World Health Organization (WHO) and the International Union Against Tuberculosis and Lung Disease (IUATLD) could then identify the BCG sub-strains that provide the best protection and recommend them for future vaccination worldwide (Corbel 2004).

For many years, the discovery of new TB vaccines effective against pulmonary TB has been considered an elusive quest, but the TB vaccine field has blossomed in the last decade. Research to develop improved TB vaccines seems to be at a decisive point in time. More than 200 vaccine candidates have been proposed as the result of work over recent years in experimental laboratory models, and some are now approaching clinical testing. The transition from laboratory to clinical trials has a

wide range of strategic and technical implications. In particular, facilities and funding need to be provided for the production of any successful vaccine appropriate for clinical use. After the Madrid Conference in March 1995 "Definition of a coordinated strategy towards a new TB vaccine" organized by the WHO and the IUATLD, a joint effort was established involving diverse governmental organizations in Europe (FP5 and FP6 Framework Programmes) and the USA by NIH, and recently by the AERAS Foundation.

For the first time, after 80 years of widespread use of BCG, evaluations of new candidates in humans are available including recombinant vaccine virus (Table 10-2). Nevertheless, the development of a new vaccine conferring better protection than BCG, and able to replace it, remains a challenge for the scientific community. If eradication of TB is to be possible and affordable, appropriate new vaccines must be found.

Subunit vaccines have potential advantages over live mycobacterial vaccines in terms of safety and quality control of the manufactured vaccine, and are good candidates to improve the effect of BCG. However, in order to confer the complex immunity required to protect against TB, it is possible that more than single antigens will be necessary. Progress to date with live attenuated *M. tuberculosis* vaccines indicates that it is possible to design strains that are highly attenuated, even for immunodeficient animals. These "classical" vaccine candidates have to mimic natural infection as closely as possible without causing disease (Young 2003).

The goal of evolving an effective licensed vaccine by the year 2015 has been proposed by Stop TB/WHO. It is estimated that at least 20 vaccine candidates should enter Phase I safety trials, with around half going forward for immunological evaluation in Phase II trials, leading to four Phase III efficacy trials (Young 2006). Vaccination is expected to make a major contribution to the goal of eliminating TB worldwide by 2050. Still, developing a new effective vaccine will require innovation in scientific research, a proactive approach to clinical trials of new vaccine candidates and application of vaccines as a part of an integrated approach to disease control (Young 2006).

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Chapter 11: Biosafety and Hospital Control

Maria Alice Telles and Afranio Kritski

11.1. Biosafety in the hospital

11.1.1. Introduction

Tuberculosis (TB) drug treatment can be carried out mainly at the ambulatory level, but the diagnosis of the disease is not always accomplished in the ambulatory setting. In big cities of developing countries, the diagnosis of TB is often made in the hospital before these patients are assisted at the local or regional outpatient centers. The reference treatment centers for hospitalizing TB patients are frequently the only health centers with specialized ambulatory facilities for assisting these patients, including those with co-morbidities. The percentage of patients diagnosed in hospitals may be 30 % or even higher. This kind of situation favors the exposure to TB infection in the nosocomial environment.

Around 25 to 50 % of the persons exposed to an intimate contact with active pulmonary TB will become latently infected with *Mycobacterium tuberculosis*. Exposure to the index case for 12 or more hours implies a high risk of infection, especially in closed environments without biosafety precautions. Immunosuppressed persons have an increased risk of infection and active disease compared with immunocompetent persons. Initially, the evaluation of the risk of transmission of TB within a health institution can be classified as follows:

- Low, if the institution admits less than six patients with active pulmonary TB per year or if it has more than 100 healthcare professionals per hospitalized pulmonary TB patient per year;
- High, if there are less than 10 healthcare professionals per hospitalized pulmonary TB patient per year (Menzies 1995, Hopewell 1986, Harries 1997).

In the last decade, high rates of drug-resistant TB have been described in prisons and hospitals. Thus, it is essential that health facilities are adequate to assist patients with active pulmonary TB or those suspected of having TB in order to reduce the risk of *M. tuberculosis* transmission to healthcare personnel and other sick people, mainly immunosupressed patients.

11.1.2. Healthcare Units

TB biosafety measures are often neglected. This increases the possibility of *M. tuberculosis* nosocomial transmission. During the '90s, transversal and longitudinal studies were accomplished on the risk of TB infection in general, as well as in reference and teaching hospitals in developed and developing countries. These studies identified a high rate of nosocomial transmission of TB to medicine, nursing, and physiotherapy students, as well as to healthcare personnel (Roth 2005, Alonso-Echanove 2001, Kruuner 2001, Harries 1997, Cuhadaroglu 2002, Do 1999, Tan 2002, Silva 2002, Resende 2004).

11.1.3. Tuberculosis infection control activities

Assuming the political commitment of the managers of public or private hospitals and the fulfillment of the international legislation suggested by the World Health Organization (WHO), TB transmission control measures in a health unit can be hierarchized into three levels: administrative, engineering, and individual protection (Jensen 2005, World Health Organization 1999, British Thoracic Society 2000).

Initially, the administrative measures can be deemed the most important. Besides being comprehensive, they are generally related to the permanent education and training of the healthcare personnel aimed at the implementation and appropriate fulfillment of the established norms.

The administrative measures include the evaluation of:

Number of pulmonary TB cases assisted annually in the health unit

- Number of annual pulmonary TB cases among the healthcare personnel
- Risk profile of the unit, by sector:
  - Low: < 6 pulmonary TB patients per year
  - Intermediate: ≥ 6 pulmonary TB patients per year and an annual average risk of TB infection (tuberculin skin test conversion) lower than 2 % among healthcare personnel
  - High: ≥ 6 patients with pulmonary TB and an annual risk of TB infection among healthcare personnel higher than 2 %

- Areas that potentially present a higher risk of transmission:
    - respiratory isolation rooms
    - ambulatory and phthisiology waiting rooms
    - thoracic radiology room
    - bronchoscopy and sputum induction rooms
    - pentamidine nebulization room
    - ventilatory assistance areas
    - day-hospital
    - emergency rooms
    - autopsy room
    - microbiology/mycobacteria laboratory

11.1.4. General practices

11.1.4.1. Management of hospitalized pulmonary tuberculosis patients

- The head nurse of the unit must have autonomy to isolate the patient if there is clinical suspicion of airborne TB.
- The patient in isolation must stay under the responsibility of the service that admitted him/her.
- The patients in isolation must be instructed to cover their mouth and nose when they cough or sneeze, even inside their room.
- The tests to be accomplished on the patients in isolation should be done as soon as possible, so that they spend a minimum time outside their room; the patient should not wait for the tests in the waiting rooms of the different services.
- When the patient needs to leave the room, a surgical mask must be used.
- Healthcare personnel must avoid unnecessary entry into the isolation rooms; in the same way, the number of visitors and attendants should be restricted to the smallest number possible. In this case, everyone should enter the isolation room using special masks (N95 or PFF2 respirators).

- In case of need, the priority in the isolation will be given to patients with acid fast bacilli (AFB) smear-positive microscopy results (bacilliferous) and shorter time of treatment.
- The patients with airborne TB (or suspicious cases) still in the infectious period should not be submitted to surgery unless in case of emergency.
- The hospital discharge of respiratory TB patients should be accomplished in the shortest possible time span.

Case searching

Instruct the healthcare personnel in the screening area, emergency department, at the admission and discharge area to suspect TB in:

- respiratory symptomatic patients (cough with expectoration for more than three weeks)
- contacts of active pulmonary TB cases for more than 12 hours
- pulmonary TB radiological suspects
- persons with predisposition to TB (immunosuppression, diabetes)

Diagnosis

- Ready request, accomplishment and release of the sputum AFB smear microscopy results in persons with presumptive TB diagnosis
- AFB smear microscopy result in 24 hours, at most
- Optimization of the diagnostic procedures, implementation of *M. tuberculosis* complex identification techniques and of anti-tuberculosis drug susceptibility testing

Every healthcare professional with signs or consistent symptoms of pulmonary TB should seek medical help and be submitted to laboratory tests (sputum AFB smear microscopy, when clinical specimen is available) and thoracic radiography. Until the pulmonary TB diagnosis is ruled out or the patient is considered non-infectious, healthcare workers with pulmonary disease should stay away from their activities. Healthcare personnel must be informed that patient care activities are not suitable for those harboring an immunosuppressive condition, such as human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS), malign neoplastic disease or deficient cellular immunity. HIV testing and counseling should be offered to all healthcare professionals.

Creation of a TB control committee responsible for:

- evaluating annual trends of *M. tuberculosis* drug resistance in the institution
- identifying transmission risk areas
- performing operational studies for the surveillance of compliance with biosafety norms
- accomplishing biosecurity educational activities for the healthcare personnel
- accomplishing periodic tuberculin skin testing in the healthcare personnel
- preventing recently infected persons from becoming ill; indicating chemoprophylaxis to the healthcare personnel with latent TB infection.

11.1.4.2. Guidelines for patient isolation

- Suspected or confirmed airborne TB cases
- HIV-positive respiratory symptomatic patient with any change in thoracic X-ray
- HIV-negative patient with a radiological image suggestive of pulmonary TB (hypotransparency in the superior lobes of the lung or in segment six, or with a diffuse micronodular infiltration, suggestive of miliary disease
- Patient with a request for sputum AFB microscopy examination and/or culture
- The head nurse of the unit must have autonomy to isolate the patient if there is clinical suspicion of respiratory TB

Respiratory isolation area

- Must be an individual room, with the door closed and windows that can be opened
- If absolutely necessary, two pulmonary TB patients can share an isolation room, provided they both have confirmed TB diagnosis and there is no epidemiological suspicion of drug-resistant TB (such as patients that have not received previous treatment and have not had contact with drug-resistant TB cases).

Time for discharge from respiratory isolation

- In confirmed TB cases under a treatment scheme containing rifampicin: after two weeks of treatment and with three sputum AFB smear-negative results or one induced sputum or bronchoalveolar lavage AFB smear-negative result
- In confirmed TB cases under a treatment scheme not containing rifampicin: after four weeks of treatment and with three sputum AFB smear negative results or one induced sputum or bronchoalveolar lavage AFB smear-negative result
- If one of the tests is positive, repeat the series after one week
- In suspicious cases: with three negative sputum-smear microscopy or one induced sputum or bronchoalveolar lavage with negative AFB smear microscopy

11.1.4.3. Ambulatory assistance under a standardized Reference Health System

- Signal the TB risk areas of the unit
- Avoid movements of confirmed or suspected TB patients inside the health unit in order to minimize contact with people awaiting assistance for other ambulatory specialties in waiting or examination rooms
- In outpatient units where ambulatory patients with pulmonary TB and conditions other than TB are assisted in the same places and/or by the same healthcare personnel, consultation appointments should be separated in different hours or days, in such a way to avoid TB exposure of uninfected persons, mainly those who are immunosuppressed
- Avoid crowding in the waiting rooms, assigning consultations to specific days for TB and setting a time for consultation, giving priority to the assistance of infectious patients and suspicious cases, thus avoiding the gathering of potentially infectious patients
- Avoid assisting immunosuppressed patients and children less than 5 years old in rooms contiguous to the ones assisting TB patients
- Offer surgical masks or tissue paper to infectious or suspected TB patients, mainly when circulating through the unit (specialist consultation, X-ray exams, sputum delivery to the laboratory, search for exam results, etc.)

- Instruct patients to cover their mouth and nose when they cough or sneeze.

11.1.4.4. Engineering measures

Engineering measures for TB control are architectural and technical devices aimed at the adaptation of the unit, or of a certain area, to provide care to pulmonary TB patients. The implementation of such measures contributes to decreasing the risk of TB transmission and should be directed by qualified personnel with special knowledge on the characteristics of TB transmission.

Objectives

Basically, the objective is the removal or dilution of infectious particles taking into account the following factors:

- Ventilation exhaustion: captures and removes contaminants suspended in the air near the source (patient)
- General ventilation: ventilation rate or number of air changes per hour; for example, a complete air exchange per hour of a certain area reduces the concentration of infectious particles by 63 %, while six complete exchanges are needed to reduce it by 99 %
- Direction of the air flow within the facilities: contains the contaminated air in a certain area of the facility and prevents spread into non-contaminated areas
- Negative pressure in the room with directional flow: contains the contaminated air in a certain area of the room and prevents spread to non-contaminated areas
- Adjustment of air flow pattern inside the room: prevents air stagnation short circuit
- Air filters and/or ultraviolet (UV) light: disinfect air

High efficiency particulate air (HEPA) filters

HEPA filters or absolute filters are those able to remove 99.97 % of particles with a diameter larger than 0.3 µm which pass through them. They can be placed in exhaustion ducts, in room ceilings or in movable filtration units.

The use of HEPA filters and/or UV light is strongly recommended for rooms where the following procedures take place: bronchoscopy, induced sputum, pentamidine nebulization, necropsy, and isolation. The combination of an adequate number of

air changes with negative pressure and a HEPA filter or UV light minimizes the risk of transmission in the environment in which the TB patient is assisted and in the area where the air is exhausted. The germicidal efficiency of the UV light is limited to its area of direct incidence and decreases with time.

HEPA filters are used:

- To purify the exhaustion of air of contaminated environments
- To recirculate the air inside the room or to other rooms facilitating the number of air changes per hour.

Basic engineering recommendations

In areas with a high risk of infection, the main engineering measure is to facilitate ventilation so that the particles suspended in the air are removed at the highest speed possible. The speed of air removal is calculated in air changes per hour and should be:

- six air changes per hour for the isolation, the ambulatory, the X-ray, the waiting and the emergency rooms, and the ventilatory assistance areas
- twelve air changes per hour for the bronchoscopy, the sputum induction, the pentamidine nebulization and the autopsy rooms and the mycobacteria laboratory

The use of negative pressure

Negative pressure prevents the dispersion of contaminated air into areas where people walk, mainly those in common use such as corridors. The exhaust air should never be directed towards these transit areas. If safe air exhaustion is not possible, the exhausted air should be filtered or sterilized.

Respiratory isolation room

The isolation room must:

- Be private and with suitable ventilation characteristics
- Be under negative pressure
- Be submitted to six or more air changes per hour
- Have air exhaustion to the open-air
- Have HEPA filters if the air is recirculated or exhausted to circulation areas

- Have anterooms (they increase isolation effectiveness, minimizing the escape risk)
- Have UV light (optional)
- Be submitted to six air changes between a patient's discharge and the following patient's admission

Outpatient clinic

In areas dedicated to ambulatory care, the minimum biosecurity conditions should include:

- Adequate (ventilated and sunny) site for sputum collection, preferentially outdoors
- Air flow adaptation of the waiting and consultation rooms, avoiding the use of ceiling fans; air conditioning is allowed only in combination with HEPA filters
- Suitable area for the waiting room, preferably outdoors, far away from any crowded area or other waiting rooms
- Within the assistance room, use of a standing fan either to direct the air flow towards the window (or door) or to produce an air "barrier" between the doctor and the patient
- The use of standing fans and exhaust fans in strategic points is a low-cost alternative to increase the number of air changes per hour
- Adaptation to the environment to which the air is being directed, avoiding other people being exposed to the risk of infection

11.1.4.5. Individual Protection

Measures for healthcare personnel at risk

- Masks: they can be of the N-95 type, with a National Institute for Occupational Safety and Health (NIOSH) certification of the United States (US) or of the PFF-2 type, with international standards certification; common surgical masks are not advisable: their effectiveness in preventing the inhalation of particles with diameters of 1 to 5 µm is less than 50 % (they were specifically designed to prevent the exhalation of particles)

- The protection masks should be supplied by the health service where the TB patients are assisted, preferably in various sizes and models
- Even if administrative and environmental control measures are in force, healthcare workers should wear appropriate respiratory protection devices (N95 or PFF2) at all times while they are in patients' rooms, during bronchoscopy, induced sputum, pentamidine nebulization, surgery or autopsy performed on suspected or confirmed TB cases
- Instruct the personnel on the correct use of the special masks, reminding male employees that they should have their faces shaved as beards and/or mustaches can prevent perfect adjustment of the mask to the face
- Special masks (N95 or PFF2 respirators) can be used for indeterminate periods of time, as long as they are kept dry, clean and intact (without any torn, frayed or crumpled areas); their storage in plastic bags after use must be avoided because bags retain humidity

For TB patients transiting through the institution

- Indicate the use of common surgical masks for the respiratory symptomatic patients as soon as they enter the unit (triage, emergency, ambulatory, when being admitted or when passing through). The surgical masks work as a barrier, capturing the damp particles (usually larger than 5 μm) and, therefore, do not work as filters.
- In the day-hospital sector, HIV-negative patients who have been coughing for more than three weeks should wear a common mask all the time whilst there; HIV/AIDS patients with any respiratory symptom should use a common mask all the time. When the engineering measures are not working in the room, the asymptomatic patient in the same setting should be instructed to use a special mask (N95 or PFF2), particularly if immunosuppressed.

11.1.5. Tuberculin skin test survey

The evaluation of the risk of infection from *M. tuberculosis* (through tuberculin investigation) should be performed on healthcare personnel in the following situations:

- Recently admitted personnel
- Personnel that report frequent contact with pulmonary TB patient

It is important that every health unit knows the prevalence of TB infection and TB disease among the healthcare personnel. In this sense, the healthcare worker that reports a past history of active TB or household contact with a pulmonary TB case in the last two years must be submitted to medical examination, tuberculin testing and a chest X-ray.

A tuberculin skin test (TST) should be applied and read by one of a limited-number of trained nurses tested for intra- and inter-reader variability. Tuberculin purified protein derivative (PPD) will be injected subcutaneously and the amount of induration should be measured at 48–72 hours. For healthcare workers with an induration < 10 mm, the tuberculin skin test should be repeated 7–10 days later. Those with a two-step tuberculin skin test < 10 mm should be asked to undergo a repeated tuberculin skin test 6–12 months later. Those with a tuberculin skin test ≥ 10 mm and those who experienced a tuberculin skin test conversion should undergo a medical evaluation to rule out TB disease.

Since 1995, bacille Calmette-Guérin (BCG) revaccination has not been recommended by the WHO. Few countries still maintain the use of BCG revaccination. Recently, in an open, randomized clinical trial performed in Brazilian children, it was found that a second BCG vaccination at school age has low effectiveness. Because of these results and those described in the international literature, BCG vaccination is no longer recommended for healthcare personnel in some countries, including Brazil (Rodrigues 2005).

According to a study performed in the US, tuberculin investigation every 12 months in areas with a high risk of TB infection would be more cost-effective than other measures for preventing TB. Chemoprophylaxis should be indicated to recent PPD converters (induration increase of 10 mm in relation to the last test) (Nettleman 1997).

11.1.6. Recommendations

Flaws related to biosecurity measures (administrative, environmental or of individual protection) are factors known to be associated with higher nosocomial TB transmission.

The primary tuberculous infection may manifest itself as a light respiratory condition with hardly any clinical or radiological signs. Consequently, it usually remains undiagnosed. During this process, *M. tuberculosis* spreads both lymphatically and hematogenously and the bacilli implanted in extrapulmonary organs or tissues are a potential source of subsequent reactivation. Generally, the tuberculin skin test is the sole indication that *M. tuberculosis* infection has occurred.

It is estimated that 10 % of individuals infected with *M. tuberculosis* will develop active TB at some time during their lifetime. The risk of becoming sick with TB is highest in the first two years after the infection, when about 5 % of infected individuals undergo a progression from latent to active disease. The other 5 % can develop active TB at any time in their lifetime if they do not receive the treatment recommended for latent infection.

Even in places where TB is endemic and BCG vaccination is universal, the result of the tuberculin skin test reflects, with reasonable accuracy, exposure to *M. tuberculosis*. In countries with a high prevalence of TB, in which 25 to 50 % of the population is considered to be infected by *M. tuberculosis*, the tuberculin skin test is highly specific and a positive result has a high probability of indicating tuberculous infection. The adequate establishment and fulfillment of TB biosafety measures are the tools needed to reach the goal of reducing the annual risk of infection in healthcare personnel to levels similar to those of the general population.

11.2. Biosafety in the laboratory

11.2.1. Introduction

Microbiology laboratories are unique and special work environments, where the handling of infectious organisms may pose risks of infection to the laboratory personnel or the surrounding community.

Several cases of infections acquired in the laboratory have been reported throughout the history of microbiology. By the end of the 19th century and the beginning of the 20th, reports had already been published describing laboratory-associated cases of typhoid, cholera, brucellosis, and tetanus. By the middle of the 20th century, a few publications reported cases of laboratory-related infections in the United

States. Some of these cases were attributed to carelessness or inappropriate techniques in the handling of infectious material (Meyer 1941, Sulkin 1949, Sulkin 1951).

A laboratory survey was updated in 1976 (Pike 1976) totaling 3,921 cases. Brucellosis, typhoid, tularemia, TB, hepatitis and Venezuelan equine encephalitis accounted for most of the infections. Not more than 20 % of these cases were associated with a documented accident. Exposure to infectious aerosols was considered to be a likely but unconfirmed source of infection in more than 80 % of the reported cases, in which the infected person had "worked with the agent". Pike, in 1979, concluded that "*the knowledge, the techniques and the equipment to prevent most laboratory infection were available*" (Pike 1979).

The actual risk of a laboratory-acquired infection is difficult to measure because there is no systematic reporting system. Besides, surveillance data on laboratory-associated infections are difficult to collect because the infections are often subclinical and have an atypical incubation period and route of infection. Another problem is that laboratory directors may not report incidents for fear of reprisal or embarrassment (Sewell 1995).

The risk of exposure to infectious agents tends to be lower for laboratory workers than other groups of healthcare workers. However, the risk of laboratory-associated infection in employees of clinical and research laboratories is greater than that of the general population, suggesting that unique risks are associated with the laboratory work environment (Kiley 1992).

The advent of the HIV/AIDS epidemic in the early '80s and the fact that the rate of new cases of TB began to rise in 1986 in developed countries (Tenover 1993), put laboratory safety and safety programs in the spotlight. The safety concerns led to the elaboration of guidelines and manuals (Centers for Disease Control 1987, Occupational Safety and Health Administration 1991). A decrease in the occupational risks associated with working in a clinic or laboratory was observed after these guidelines were adopted (Fahey 1991, Wong 1991).

The term "containment" is used when describing safe methods for managing infectious material in the laboratory environment where they are handled or stored. The purpose of containment is to reduce or eliminate exposure of laboratory workers, other people, and the outside environment to potentially hazardous agents.

Primary containment: protection of laboratory workers and the immediate laboratory environment from exposure to infectious agents is provided by both good

microbiological technique and the use of appropriate safety equipment. The use of vaccines may provide an increase in the level of personal protection.

Secondary containment: protection of the environment outside the laboratory from exposure to infectious materials is provided by a combination of facility design and operational practices.

Therefore, the three elements of containment include laboratory practice and technique, safety equipment, and facility design. The risk assessment of the work to be done with a specific agent will determine the appropriate combination of these elements (Blumberg 2000, Blumberg 2004, Centers for Disease Control and Prevention 1994, Centers for Disease Control and Prevention 2005).

The most important element of containment is the strict adherence to standard microbiological practices and techniques. People who work with infectious agents or potentially infected materials must be aware of potential hazards and must be trained and proficient in the practices required for the safe handling of these materials. The director of the laboratory is also responsible for providing or arranging the appropriate training of personnel.

Each laboratory should develop or adopt a biosafety manual or operations manual that identifies the hazards that are or may be found in the laboratory, and that specifies practices and specific procedures designed to minimize or eliminate the exposure to such hazards. Personnel should be informed about the special hazards and should follow the necessary practices and procedures.

A scientist trained and knowledgeable in appropriate laboratory techniques, safety procedures and hazards associated with the handling of infectious agents must be responsible for the conduct of work with any infectious agent or infected material.

M. tuberculosis is repeatedly ranked within the top-five most common laboratory-acquired infections (Collins 1998, Miller 1987, Sepkowitz 1994, Seidler 2005). Pike reported that laboratory and mortuary workers exposed to tubercle material have a TB incidence rate three times higher than that of the general population and indicated that only 18 % of infections could be traced back to a known event (Pike 1976). Despite the current knowledge and biosafety measures in place, a recent report in New York demonstrated rates from 2 to 6.6 % of TB conversion among healthcare workers (Garber 2003).

In addition, surveys suggest that the actual incidence of laboratory-acquired infections due to *M. tuberculosis* is greater than the number of reported cases. The documentation of a case of laboratory-acquired TB is difficult because the source of the infection is often unclear, as a result of the potential for exposure outside of

the workplace and the long incubation period before the development of symptomatic disease (Collins 1993, Pike 1979). The incidence of TB in laboratory personnel is estimated to be three to nine times that of individuals in other job environments (Harrington 1976, Reid 1957, Saint-Paul 1972).

Manipulation of specimens or cultures that generate aerosols is the most important risk factor for acquiring TB in the laboratory. Aerosolization occurs frequently during autopsies, preparation of frozen sections of infected tissues, and procedures involving liquid cultures (Centers for Disease Control and Prevention 1981, US Department of Health and Human Services 1993).

M. tuberculosis presents a low infective dose for humans of less than 10 bacilli (Riley 1957, Riley 1961), suggesting a high risk for laboratory-acquired infection.

Due to the nature of this organism, containment level 3 (CL3) laboratory operational and physical requirements have been recommended for manipulation of the live organism in North America (US Department of Health and Human Services 1995). Therefore, one would hypothesize that working in a CL3 with personal protective equipment, including a respirator, would be adequate to protect the worker. However, as tuberculin skin test conversion is still occurring (Blackwood, 2005), other practices and causes should be analyzed.

These recommended measures are implemented by healthcare facilities in high-income countries, but given their high cost, few facilities in low-income countries can afford to implement them (Pai 2006).

The WHO has proposed practical and low-cost interventions to reduce nosocomial transmissions in settings where resources are limited, and these are available on the internet at http://www.who.int/docstore/gtb/publications/healthcare/index.htm (World Health Organization 1999). Several simple interventions can ameliorate working conditions, such as training and supervising laboratory workers in good techniques and biosafety practices to provide the necessary organization (DeRiemer 2000, Joshi 2006).

11.2.2. Laboratory biosafety levels

Infectious microorganisms are classified by risk group. This type of classification is to be used for laboratory work purposes only.

- **Risk Group 1** (no or low individual and community risk)

A microorganism that is unlikely to cause human or animal disease.

- **Risk Group 2** (moderate individual risk, low community risk)

A pathogen that can cause human or animal disease, but is unlikely to be a serious hazard to laboratory workers, the community, livestock or the environment. Laboratory exposure may cause serious infection, but effective treatment and preventive measures are available and the risk of spread of infection is limited.

- **Risk Group 3** (high individual risk, low community risk)

A pathogen that usually causes serious human or animal disease but does not ordinarily spread from one infected individual to another. Effective treatment and preventive measures are available.

- **Risk Group 4** (high individual and community risk)

A pathogen that usually causes serious human or animal disease and that can be readily transmitted from one individual to another, directly or indirectly. Effective treatment and preventive measures are not usually available.

Biosafety Level designations: are based on a combination of the design features, construction, containment facilities, equipment, practices and operational procedures required for working with agents belonging to the various risk groups (World Health Organization 2004).

Laboratory facilities are designated as:

- Biosafety Level 1 – basic laboratory
- Biosafety Level 2 – basic laboratory
- Biosafety Level 3 – laboratory with containment conditions
- Biosafety Level 4 – laboratory with maximum containment

A national classification of microorganisms, by risk group, may be determined taking into account regional characteristics:

- Organism: pathogenicity, mode of transmission
- Host: immunity, density vectors, environment
- Preventive measures
- Treatment

11.2.3. Risk assessment

Any laboratory work should be done under appropriate biosafety conditions based on risk assessment. Such an assessment will take into considerations the agent risk group as well as other factors to establish the biosafety level (World Health Organization 2004).

Organism

Factors that should be considered concerning the organism include:

- Pathogenicity of the agent and infectious dose
- Potential outcome of exposure
- Natural route of infection
- Other routes of infection, resulting from laboratory manipulations (parenteral, airborne, ingestion)
- Stability of the agent in the environment
- Concentration of the agent to be manipulated
- Presence of a suitable host (human or animal)
- Information available from animal studies and reports of laboratory-acquired infections or clinical reports
- Laboratory activity planned (sonication, aerosolization, centrifugation, etc.)
- Any genetic manipulation of the organism that may extend the host range of the agent or alter the agent’s sensitivity to known, effective treatment regimens
- Local availability of effective prophylaxis or therapeutic interventions

On the basis of the information ascertained during the risk assessment, a biosafety level can be assigned to the planned work:

- appropriate personal protective equipment
- standard operating procedures (SOPs)
- other safety interventions developed to ensure the safest possible conduct of the work

11.2.4. General laboratory practices

There are different types of laboratory hazards, such as biological, chemical, radiation and physical, as well as electrical hazards, slips, trips, and falls.

Attitude, behavior and common sense are the key to prevent such accidents.

The main causes of laboratory accidents are: lack of training, knowledge or experience; excessive self-confidence, negligence, fatigue, taking shortcuts, work load, working too fast, deciding not to follow safe practices, and skepticism about biohazard.

Laboratory workers can be classified as “unsafe” and “safe”. Unsafe workers are those who have a low opinion of safety programs, take excessive risks, work too fast and are less aware of the risk of agents. Safe workers are those who adhere to safety guidelines, practice defensive work habits and recognize potentially hazardous situations (Phillips 1986, Harding 1995).

The laboratory has to be organized with responsibility levels. The manager needs to have an attitude of support towards the safety program, should provide adequate resources and training, should supply a safe work environment, monitor work practices, and assess and assign risk level for hazardous materials (biologicals, chemicals, animals).

The employees, on the other hand, have to comply with occupational safety and health standards, rules, regulations and orders, use personal protective equipment and safety equipment when needed, and report all work-related accidents and illness to the supervisor.

The types of regulations that support working safely in laboratories are government regulations, institutional regulations, and laboratory regulations or guidelines.

General safety guidelines

- Eating, drinking or smoking are not permitted in laboratories or offices
- Wear personal protective equipment when needed
- Practice good personal hygiene
- Children are not allowed into laboratory areas
- Good housekeeping
    - clean up after each manipulation
    - do not store materials on work surfaces
    - keep aisles clear
    - keep chemicals in storage cabinets
    - purge work areas of unnecessary or unused equipment, supplies or chemicals
- Minimize all exposure
- Never underestimate the risks
- Become familiar with:
    - emergency exits
    - emergency procedures
    - first-aid
    - fire responses
- Report all accidents and injuries
- Ask questions when in doubt

Personal safety guidelines

Take pride in your workplace and practice accident prevention by banning negative attitudes and bad habits such as:

- Overconfidence
- Showing off
- Stubbornness
- Laziness
- Carelessness
- Impatience
- Ignorance

Safety guidelines – slips, trips and falls

- Clean up spills
- Watch out for loose carpet, polished floors, or objects on floor
- Keep all chair legs on the floor
- Use step stools and ladders when reaching for top shelves
- Never lay cords across walkways
- Use as much light as possible
- Do not carry loads which block your vision

Safety guidelines – storage

- Avoid overloading file cabinets
- Close file cabinet drawers when finished
- Store heavy items on lower shelves
- Keep pointed and sharp objects in a box in your desk drawer

Safety guidelines – personal protective equipments

Appropriate personal protective equipment must be worn whenever working with hazardous materials.

- Lab coat
    - must be worn in the laboratory
    - should be buttoned at all times
    - should cover the arms, torso, back and legs above the knees
    - should be selected based upon the chemicals used
    - should be removed and replaced if soiled or contaminated
    - should not be worn outside of the laboratory
- Eye and face protection
    - eye glasses, face shield, or mask are worn for work with small quantities (< 1 L)
    - goggles are worn for work with large volumes (1-5 L)
    - chemical fume hood or other shield should be used for volumes > 5 L
    - should not be worn outside laboratory

- Gloves
  - should be selected based on the chemicals being used
  - should be inspected for tears or holes before use
  - should be replaced or discarded when dirty or contaminated
  - should not be removed from the laboratory
- Respiratory protection
  - must be worn in the laboratory where there is an inhalation hazard
  - should not be worn if not trained in their use
  - should be cleaned and inspected after each use and allowed to dry completely
  - should be selected based upon the chemicals used or agents handled
  - should not be taken out of the laboratory

Safety guidelines - controlling aerosols

- Avoid splattering and spilling solutions
- Use plastic-backed absorbent paper on work surfaces
- Place caps or tops on bottles and tubes
- Place balances in ventilated enclosures
- Use safety cups when centrifuging
- Work under containment
  - biological safety cabinet
  - chemical fume hood
  - ventilated enclosures
  - directional airflow

Risk assessment

- Identify all hazardous materials and processes
- Consider possible routes of entry
- Consult information resources
- Evaluate biosafety level or toxicity of material
- Evaluate quantitative information on toxicity

- Select procedures to minimize exposure
- Prepare for contingencies

Safety guidelines – Laboratory security

- Prevent unauthorized entry into laboratory areas
- Prevent unauthorized removal of hazardous materials
- Recognize that laboratory safety and security are different
- Control access
- Know who is in the laboratory
- Know what materials are being brought into the laboratory
- Know what materials are being removed from the laboratory
- Have an emergency plan
- Have a protocol for reporting incidents

11.2.5. Decontamination

Mycobacteria are generally more resistant to chemical disinfection than other vegetative bacteria (Russel 1986).

The subject of disinfectants, which are really effective against mycobacteria, is very controversial and can generate confusion.

Table 11-1 shows disinfectants active against mycobacteria. The most common disinfectants used in the mycobacteria laboratory are: phenol 5 %, ethanol 70 % and sodium hypochlorite 2%.

There are interesting studies showing the efficacy of disinfectants against mycobacteria and *M. tuberculosis* (Best 1988, Best 1990).

The WHO manual of laboratory biosafety also gives good information on disinfectants in general (http://www.who.int/csr/resources/publications/biosafety/WHO_CDS_CSR_LYO_2004_11/en/index.html).

Table 11-1: Disinfectants active against Mycobacteria

Disinfectant	Final concentration
Phenol	5 %
Sodium hypochlorite	10,000 ppm of Av Cl/mL
Sodium dichloroisocyanurate	6,000 ppm of Av Cl/mL
Ethanol	70 %
Glutaraldehyde-phenate	2 %

Av Cl = available chlorine

11.2.6. Handling of biological waste

It is strongly recommended that residues be segregated, packaged and properly labeled at the point of origin. They should then be immediately placed in distinctive containers according to their species and group, in order to reduce the amount of contaminated residues, as well as accidental risks, and to adopt the best conduction for the treatment of infectious or contaminant residues (Coelho 2000).

Potentially infectious: should be disposed of in plastic bags, made of polypropylene, resistant to autoclaving.

Bags should be filled up to 2/3 of their capacity and totally closed to prevent leaking of the content, even if turned upside down. Bags cannot be emptied or reused. In the laboratory, bags should be stored in garbage containers made of material that permits chemical or physical decontamination, identified with the label of hazardous biological waste having hinged-foot-activated mechanisms for opening and closing the lid, with rounded corners and edges.

Every biological residue generated in the laboratory should be previously treated before being disposed, even in the case of selective collection such as hospital or animal-house facility waste.

Sharp and cutting residues: should be disposed of in containers with rigid walls and lids resistant to sterilization.

The collecting containers for sharp and cutting material should be placed as near as possible to the area of use of such material. The containers should not be filled above 2/3 of their capacity.

After being filled, the collecting container should be closed and placed in plastic bags resistant to autoclaving. Such containers should be identified with self-adhesive labels, with the following information: “Do not reuse empty container”.

Decontamination: autoclaving sterilization is the safest method, for its penetration power is higher than dry heat. Microorganisms are destroyed by thermocoagulation of cytoplasmic proteins.

Before disposal, bags should be sterilized by autoclaving; sterilization occurs at a pressure of 1 atm at temperatures of up to 121°C (250°F) for 62 minutes, with a 7-minute interval pre-vacuum, 25 minutes heating, 25 minutes sterilization for surface material or 30 minutes sterilization for thick materials and 15 minutes of cooling.

If final disposal occurs after a 24-hour period, anatomical pieces, human and animal organs and carcasses that have undergone treatment, should be refrigerated or kept in formalin.

Leaking of biological residues: in case of disruption or leaking of bags containing biological residues that have not undergone prior treatment, the procedures below should be followed:

- Cover the spill and spill site with paper towels
- Pour disinfectant solution (for example sodium hypochlorite: a minimum of 10,000 ppm available chloride) on the paper towels for 30 minutes contact time
- Pick up paper towels and discard them into a plastic bag
- Reapply disinfectant and wait for 10 minutes
- Carry out the cleaning
- Decontaminate all materials that had direct contact with the spill
- The professional responsible for the cleaning of the spill must wear the necessary Individual Protection Equipment

Guidelines for internal collection and transport of residues

- Never pour the contents of the garbage bin into another container. The garbage bag should be picked up, closed, and placed inside the internal waste collection trolley
- Check if there is any leakage in the plastic bag prior to picking it up from the garbage bin. In case of leakage, the bag should be placed into another bag with the same specification and the garbage bin must be washed and disinfected
- Residue transport from the laboratory to the disposal room can be done by hand or by the internal waste collection trolley

11.2.7. Transport of infectious materials

Guidance on regulations for the transport of infectious substances can be found at: http://www.who.int/csr/resources/publications/biosafety/WHO_CDS_EPR_2007_2/en/index.html

11.2.8. Good Laboratory Practices (GLP)

Laboratories are complex and dynamic environments. Biomedical research and clinical laboratories must be able to adapt quickly to continuously increasing public health needs and pressures.

All biological research and clinical laboratories should be regularly certified to ensure that:

- Proper engineering controls are being used and are functioning adequately as designed
- Personal protective equipment is appropriate for the tasks being performed
- Decontamination of waste and materials has been adequately considered and proper waste management procedures are in place
- Proper procedures for general laboratory safety, including physical, electrical and chemical safety are in place

Laboratory certification is the systematic examination of all safety features and processes within the laboratory (engineering controls, personal protective equipment and administrative controls). Biosafety practices and procedures are also taken into account. Laboratory certification is an on-going quality and safety assurance activity that should take place on a regular basis.

Standardized practices can be found in the WHO Laboratory Biosafety Manual (http://www.who.int/csr/resources/publications/biosafety/WHO_CDS_CSR_LYO_2004_11/en/index.html). Some important topics are summarized below:

Safe handling of specimens in the laboratory

Improper collection, transport and handling of specimens in the laboratory carry a risk of infection to the personnel involved.

Specimen containers

Specimen containers may be of glass or preferably plastic. They should be robust and should not leak when the cap or stopper is correctly applied. No material should remain on the outside of the container. Containers should be correctly labeled to facilitate identification. Specimen request or specification forms should not be wrapped around the containers but placed in separate, preferably waterproof envelopes.

Transport of specimens within the facility

To avoid accidental leakage or spillage, secondary containers, such as boxes, should be used, fitted with racks so that the specimen containers remain upright. The secondary containers may be of metal or plastic, should be autoclavable or resistant to the action of chemical disinfectants, and the seal should preferably have a gasket. They should be regularly decontaminated.

Receipt of specimens

Laboratories that receive large numbers of specimens should designate a particular room or area for this purpose.

Opening packages

Personnel who receive and unpack specimens should be aware of the potential health hazards involved, and should be trained to adopt standard precautions, particularly when dealing with broken or leaking containers. Primary specimen containers should be opened in a biological safety cabinet. Disinfectants should be available.

Use of pipettes and pipetting aids

Mouth pipetting must be strictly forbidden. A pipetting aid must always be used for pipetting procedures. The most common hazards associated with pipetting procedures are the result of mouth suction. Oral aspiration and ingestion of hazardous materials have been responsible for many laboratory-associated infections.

Pathogens can also be transferred to the mouth if a contaminated finger is placed on the suction end of a pipette. A lesser known hazard of mouth pipetting is the inhalation of aerosols caused by suction. The use of pipetting aids prevents ingestion of pathogens.

Aerosols are generated when a liquid is dropped from a pipette on to a work surface, when cultures are mixed by alternate sucking and blowing, and when the last drop is blown out of a pipette. The inhalation of aerosols unavoidably generated

during pipetting operations can be prevented by working in a biological safety cabinet.

- All pipettes should have cotton plugs to reduce contamination of pipetting devices
- Air should never be blown through a liquid containing infectious agents
- Infectious materials should not be mixed by alternate suction and expulsion through a pipette
- Liquids should not be forcibly expelled from pipettes
- Mark-to-mark pipettes are preferable to other types, as they do not require expulsion of the last drop
- A discard container for pipettes should be placed within the biological safety cabinet, not outside it
- Syringes fitted with hypodermic needles must not be used for pipetting
- Devices should be used to open septum-capped bottles and to allow the introduction of pipettes, thus avoiding the use of hypodermic needles and syringes
- To avoid dispersion of infectious material dropped from a pipette, an absorbent material should be placed on the working surface; this should be disposed of as infectious waste after use

Avoiding the dispersal of infectious materials

Disposable transfer loops do not need to be resterilized and can, therefore, be used in biological safety cabinets. These loops should be placed in disinfectant after use and discarded as contaminated waste.

Discarded specimens and cultures for autoclaving should be placed in leak-proof containers, e.g. laboratory discard bags. Tops should be secured (e.g. with autoclave tape) prior to disposal into waste containers.

Working areas must be decontaminated with a suitable disinfectant at the end of each work period.

Special care should be taken when drying sputum samples, to avoid creating aerosols. Smears should be dried at room temperature inside the biological safety cabinet, or outside the biological safety cabinet on a temperature plate.

Use of biological safety cabinets

- The use and limitations of biological safety cabinets should be explained to all potential users, with reference to national standards and relevant literature. Written protocols or operation manuals should be provided to staff. In particular, it must be made clear that the cabinet will not protect the operator from spillage, breakage or poor technique
- The cabinet must not be used unless it is working properly
- The glass viewing panel must not be opened when the cabinet is in use
- Apparatus and materials in the cabinet must be kept to a minimum. Air circulation at the rear plenum must not be blocked
- Bunsen burners must not be used in the cabinet as the heat produced will distort the airflow and may damage the filters
- All work must be carried out in the middle or rear part of the work surface and be visible through the viewing panel
- Traffic behind the operator should be avoided
- The operator should not disturb the airflow by repeated removal and reintroduction of arms
- Air grills must not be blocked with notes, pipettes or other materials, as this will disrupt the airflow, causing potential contamination of the material and exposure of the operator
- The surface of the biological safety cabinet should be wiped using an appropriate disinfectant after work is completed and at the end of the day
- The UV-light should be switched on for 15 minutes before switching off the cabinet
- Paperwork should never be placed inside biological safety cabinets

The UV-light is very effective against mycobacteria when used for surface decontamination. For this reason we recommend its use in the cabinet to prevent cross-contamination of cultures (Ueki in press). In figure 11-1 the effect of UV light on a plate on which mycobacteria were inoculated is shown.

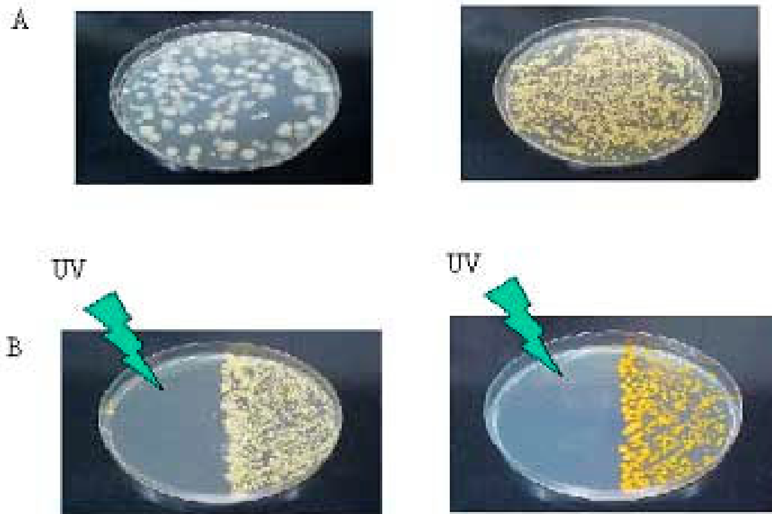

Figure 11-1: A) Two species of mycobacteria inoculated on Middlebrook 7H11 plates. The plates were then covered with aluminum foil and exposed to UV light inside the cabinet during 5 and 10 minutes. B) The same experiment, but only half of the plates were covered and the other half was exposed directly to the UV light.

Avoiding ingestion of infectious materials and contact with skin and eyes

- Large particles and droplets (> 5 µm in diameter) released during microbiological manipulations settle rapidly on bench surfaces and on the hands of the operator. For this reason, laboratory workers should wear disposable gloves and avoid touching their mouth, eyes or facial skin
- No materials should be placed in the mouth – pens, pencils, chewing gum – when in the laboratory
- Cosmetics should not be applied in the laboratory
- The face should be shielded or otherwise protected during any operation that may result in the splashing of potentially infectious materials

Avoiding injection of infectious materials

- Accidental inoculation resulting from injury with broken or chipped glassware can be avoided through careful practice and procedures; glassware should be replaced with plastic ware whenever possible, e.g. plastic Pasteur pipettes and tubes should replace those made of glass

- Needle-stick injuries can be reduced by: (a) minimizing the use of syringes and needles (e.g. simple devices are available for opening septum-stoppered bottles so that pipettes can be used instead of syringes and needles; or (b) using engineered sharp safety devices when syringes and needles are necessary
- Needles should never be recapped. Disposable articles should be discarded into puncture-proof containers fitted with covers

Use of centrifuges

- Satisfactory mechanical performance is a prerequisite of microbiological safety in the use of laboratory centrifuges
- Centrifuges should be operated according to the manufacturer's instructions
- Centrifuges should be placed at such a level that workers can see into the bowl to place trunnions and buckets correctly
- Centrifuge tubes and specimen containers for use in the centrifuge should be made of thick-walled glass or preferably of plastic and should be inspected for defects before use
- Tubes and specimen containers should always be securely capped (screw-capped if possible) for centrifugation
- The buckets must be loaded, equilibrated, sealed and opened in a biological safety cabinet
- When using angle-head centrifuge rotors, care must be taken to ensure that the tube is not overloaded as it might leak
- Use of homogenizers, shakers, blenders and sonicators
- Homogenizers used for Risk Group 3 microorganisms should always be loaded and re-opened in biological safety cabinets
- Sonicators may release aerosols. They should be operated in biological safety cabinets or covered with shields during use. The shields and the outside of sonicators should be decontaminated after each use
- Domestic (kitchen) homogenizers should not be used in laboratories as they may leak or release aerosols: laboratory blenders and stomachers are safer
- Caps and cups or bottles should be in good condition and free from flaws or distortion; caps should be well-fitting and gaskets should be in good condition

- Pressure builds up in the vessel during the operation of homogenizers, shakers and sonicators, as aerosols containing infectious materials may escape from between the cap and the vessel; plastic, in particular, polytetrafluoroethylene (PTFE) vessels are recommended because glass may break, releasing infectious material and possibly wounding the operator
- When in use, homogenizers, shakers and sonicators should be covered by a strong transparent plastic casing that should be disinfected after use; where possible, these machines should be operated under their plastic covers, in a biological safety cabinet
- At the end of the operation, the containers should be opened in a biological safety cabinet
- Hearing protection should be provided for people using sonicators

Use of tissue grinders

- Glass grinders should be held in absorbent material in a gloved hand; plastic (PTFE) grinders are safer
- Tissue grinders should be operated and opened in a biological safety cabinet

Care and use of refrigerators and freezers

- Refrigerators, deep-freezers and solid carbon dioxide (dry-ice) chests should be defrosted and cleaned periodically, and any ampoules, tubes, etc., that have broken during storage should be removed. Face protection and heavy-duty rubber gloves should be worn during cleaning; after cleaning, the inner surfaces of the cabinet should be disinfected
- All containers stored in refrigerators, etc., should be clearly labeled with the scientific name of the contents, the date stored and the name of the individual who stored them; unlabelled and obsolete materials should be autoclaved and discarded
- An inventory must be maintained of the freezer's contents
- Flammable solutions must not be stored in a refrigerator unless it is explosion proof; notices to this effect should be placed on refrigerator doors

Films and smears for microscopy

Fixing and staining of blood, sputum and fecal samples for microscopy do not necessarily kill all organisms or viruses on the smears. These items should be

handled with forceps, stored appropriately, and autoclaved before disposal. References about sputum smear microscopy and safe handling of cultures can be found in the literature (Giacomelli 2005, Chedore 2002, Schwebach 2001).

Decontamination

Hypochlorite and high-level disinfectants are recommended for decontamination. Freshly prepared hypochlorite solutions should contain available chlorine at 1 g/L for general use and 5 g/L for blood spillages. Glutaraldehyde may be used for decontaminating surfaces. More specific information on mycobactericidal agents can be found in Best 1988, Best 1990, and Rutala 1991.

DNA extraction

DNA extraction is better accomplished by heating for 10 minutes at 100°C. This procedure inactivates the bacilli (Zwadyk Jr 1994, Bemer-Melchior 1999).

For genotyping purposes, DNA extraction should be less drastic to avoid damaging the DNA. Heating for 20 minutes at 80°C is recommended in this case. There is controversy among some authors that heating at 80°C for 20 minutes might not inactivate the bacilli completely (Blackwood 2005, Doig 2002, Van Embden 1993, Warren 2006). Therefore, a sample submitted for such a procedure should be handled as infectious material and should not be removed from containment.

Opening of ampoules containing lyophilized infectious materials

Cultures of *M. tuberculosis* should not be lyophilized because of the high risk of aerosol production during ampoule preparation and opening.

Contingency plans and emergency procedures

Every laboratory that works with infectious microorganisms should institute safety precautions appropriate to the hazard of the organisms and the animals being handled.

A written contingency plan for dealing with laboratory and animal facility accidents is a requirement in any facility that works with or stores Risk Group 3 or 4 microorganisms (containment laboratory – Biosafety Level 3 and maximum containment laboratory – Biosafety Level 4). National and/or local health authorities should be involved in the development of the emergency contingency plan.

Contingency plan

The contingency plan should provide operational procedures for:

- Precautions against natural disasters, e.g. fire, flood, earthquake, and explosion
- Biohazard risk assessment
- Incident-exposure management and decontamination
- Emergency evacuation of people and animals from the premises
- Emergency medical treatment of exposed and injured persons
- Medical surveillance of exposed persons
- Clinical management of exposed persons
- Epidemiological investigation
- Post-incident continuation of operations

In the development of this plan, the following items should be considered for inclusion:

- Identification of high-risk organisms
- Location of high-risk areas, e.g. laboratories, storage areas, animal facilities
- Identification of personnel and populations at risk
- Identification of responsible personnel and their duties, e.g. biosafety officer, safety personnel, local health authority, clinicians, microbiologists, veterinarians, epidemiologists, and fire and police services
- Lists of treatment and isolation facilities that can receive exposed or infected persons
- Transport of exposed or infected persons
- Lists of sources of immune serum, vaccines, drugs, special equipment and supplies
- Provision of emergency equipment, e.g. protective clothing, disinfectants, chemical and biological spill kits, decontamination equipment and supplies

Emergency procedures for microbiological laboratories

Puncture wounds, cuts and abrasions

The affected individual should remove protective clothing, wash hands and any affected area(s), apply an appropriate skin disinfectant, and seek medical attention if necessary. The cause of the wound and the organisms involved should be reported, and appropriate and complete medical records kept.

Ingestion of potentially infectious material

Protective clothing should be removed and medical attention sought. Identification of the material ingested and circumstances of the incident should be reported, and appropriate and complete medical records kept.

Potentially infectious aerosol release (outside a biological safety cabinet)

All persons should immediately leave the affected area and any exposed persons should be referred to the appropriate center for medical advice. The laboratory supervisor and the biosafety officer should be informed at once. No one should enter the room for an appropriate period of time (e.g. 1 h), to allow aerosols to be carried away and heavier particles to settle. If the laboratory does not have a central air exhaust system, entrance should be delayed (e.g. for 24 h).

Signs should be posted indicating that entry is forbidden. After the appropriate time, decontamination should proceed, supervised by the biosafety officer. Appropriate protective clothing and respiratory protection should be worn.

Broken containers and spilled infectious substances

Broken containers contaminated with infectious substances and spilled infectious substances should be treated in the same way as biological residue leaks. Infectious substances spilled onto working areas should be covered with a cloth or paper towels. Disinfectant should then be poured over these and left for the appropriate amount of time. The cloth or paper towel and the broken material can then be cleared away; glass fragments should be handled with forceps. The contaminated area should then be swabbed with disinfectant. If dustpans are used to clear away the broken material, they should be autoclaved or placed in an effective disinfectant. Cloths, paper towels and swabs used for cleaning up should be placed in a contaminated-waste container. Gloves should be worn for all these procedures. If laboratory forms or other printed or written matter are contaminated, the information should be copied onto another form and the original discarded into the contaminated-waste container.

Breakage of tubes containing potentially infectious material in centrifuges without sealable buckets

If a breakage occurs or is suspected while the machine is running, the motor should be switched off and the machine left closed (e.g. for 30 min) to allow settling. If a breakage is discovered after the machine has stopped, the lid should be replaced immediately and left closed (e.g. for 30 min). In both instances, the biosafety officer should be informed. Strong (e.g. thick rubber) gloves, covered if necessary with suitable disposable gloves, should be worn for all subsequent operations. Forceps, or cotton held in the forceps, should be used to retrieve glass debris. All broken tubes, glass fragments, buckets, trunnions, and the rotor should be placed in a non-corrosive disinfectant known to be active against the organisms concerned. Unbroken, capped tubes may be placed in disinfectant in a separate container and recovered. The centrifuge bowl should be swabbed with the same disinfectant, at the appropriate dilution, and then swabbed again, washed with water and dried. All materials used in the clean-up should be treated as infectious waste.

Breakage of tubes inside sealable buckets (safety cups)

All sealed centrifuge buckets should be loaded and unloaded in a biological safety cabinet. If breakage is suspected within the safety cup, the safety cap should be loosened and the bucket autoclaved. Alternatively, the safety cup may be chemically disinfected.

Fire and natural disasters

Fire departments and other services should be involved in the development of emergency contingency plans. They should be told in advance which rooms contain potentially infectious materials. It is useful to arrange visits from these services to the laboratory to acquaint them with its layout and contents.

After a natural disaster, local or national emergency services should be warned of the potential hazards within and/or near laboratory buildings. They should enter only when accompanied by a trained laboratory worker. Infectious materials should be collected in leak-proof boxes or strong disposable bags. Salvage or final disposal should be determined by biosafety staff on the basis of local ordinances.

Emergency services: whom to contact

The telephone numbers and addresses of the following should be prominently displayed in the facility:

- The institution or laboratory itself (the address and location may not be known in detail by the caller or the services called)
- Director of the institution or laboratory
- Laboratory supervisor
- Biosafety officer
- Fire services
- Hospitals/ambulance services/medical staff (names of individual clinics, departments, and/or medical staff, if possible)
- Police
- Medical officer
- Responsible technician
- Water, gas and electricity services

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Chapter 12: Conventional Diagnostic Methods

Jacobus H. de Waard and Jaime Robledo

12.1. Introduction

Active tuberculosis (TB) is diagnosed by detecting *Mycobacterium tuberculosis* complex bacilli in specimens from the respiratory tract (pulmonary TB) or in specimens from other bodily sites (extrapulmonary TB). Although many new (molecular) diagnostic methods have been developed, acid fast bacilli (AFB) smear microscopy and culture on Löwenstein-Jensen medium are still the "gold standards" for the diagnosis of active TB and, especially in low-resource countries, the only methods available for confirming TB in patients with a clinical presumption of active disease. AFB smear microscopy is rapid and inexpensive and thus is a very useful method to identify highly contagious patients. Culture is used to detect cases with low mycobacterial loads and is also requested in cases at risk of drug-resistant TB for drug susceptibility testing, or in cases where disease due to another member of the *Mycobacterium* genus is suspected. AFB smear microscopy and culture can also be used to monitor the effectiveness of treatment and can help to determine when a patient is less likely to be infectious. Two manuals are recommended for the laboratory diagnosis of TB (Kent 1985, Master 1992). Good reviews on this issue are also available on the internet at http://www.phppo.cdc.gov/dls/ila/TB_Toolbox.aspx.

The authors of this chapter wish to emphasize that accurate case detection is the rate-limiting step in TB control (Perkins 2002). While as many as two thirds of sputum smear-positive cases probably remain undetected worldwide, the efforts to control the disease have focused more on curing TB cases than on detecting them. (Gupta 2004, Dye 2003). Even though the laboratory is essential for the diagnosis and control of TB, it does not receive enough attention in developing countries, where AFB sputum microscopy is often the only available method to diagnose TB. Sputum microscopy lacks sensitivity, especially in children and in people living with human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS). Its usefulness depends largely on the quality of the sputum specimen and the performance quality of the laboratory. Considerable efforts have been made to improve the sensitivity of sputum smear microscopy (Steingart 2006) and special emphasis will be given in this chapter to these efforts.

Culturing of *M. tuberculosis* has become increasingly important in the last decades, in particular because of the need for drug resistance testing. As most laboratories in

low-resource countries have no access to culturing mycobacteria, alternative simple culturing methods will be discussed, as well as the value of alternative culture media such as blood agar, which is more readily available in most laboratory settings than the traditional egg-based media used for mycobacterial isolation. Although no multicenter studies have been published to show their efficiency for cultivating mycobacteria, we think that these alternatives should be presented in this chapter, because they may be particularly useful in settings where standard procedures simply cannot be performed due to the absence of laboratory equipment or reagents. In addition, we will discuss the utility of adenosine deaminase activity for the diagnosis of extrapulmonary TB: tuberculous meningitis, tuberculous pleural effusions, and tuberculous peritonitis. These presentations of TB are difficult to diagnose by smear staining and culture, and for this reason, they probably remain underdiagnosed in resource-poor countries.

12.2. Specimen handling

12.2.1. Specimens

The successful isolation of the pathogen requires that the best specimen be properly collected, promptly transported and carefully processed. Many different types of clinical specimens may be obtained for the microbiological diagnosis. If pulmonary TB is suspected, specimens originating from the respiratory tract should be collected, i.e. sputum, induced sputum, bronchoalveolar lavage or a lung biopsy. For the diagnosis of pulmonary TB, three first-morning sputum specimens (not saliva) obtained after a deep, productive cough on non-consecutive days are usually recommended. Several studies have shown, however, that the value of the third sputum is negligible for the diagnosis of TB, as virtually all cases are identified from the first and/or the second specimen (Yassin 2003, Nelson 1998, Dorronsoro 2000, Finch 1997). Before processing, sputum specimens must be classified at the laboratory with regard to their quality, i.e. bloody, purulent, mucopurulent, saliva (see also section 12.2.2).

In patients who cannot produce it spontaneously, the sputum can be induced by inhalation of hypertonic saline solution. Otherwise, the specimen can be collected from bronchoscopy. This intervention usually provokes cough and post-bronchoscopy expectorated sputum specimens should be collected because they often provide satisfactory microorganism yields (Sarkar 1980, de Gracia 1988). Some studies suggest that a single induced sputum specimen is equally effective as bronchoscopy for diagnosing pulmonary TB (Anderson 1995, Conde 2000). A recent study demonstrated that the most cost-effective strategy is to perform three

induced sputum tests without bronchoscopy (MacWilliams 2002). On the contrary, transbronchial biopsy specimens contribute little to the bacteriological diagnosis of TB (Stenson 1983, Chan 1992). "Fasting" gastric aspirates is the specimen of choice in the case of young children who cannot cough up phlegm. Gastric lavage fluid must be neutralized with sodium carbonate immediately after collection (100 mg per 5-10 mL specimen).

Specimens to be collected for the diagnosis of extrapulmonary disease depend on the site of the disease. The most common specimens received in the laboratory are biopsies, aspirates, pus, urine, and normally sterile body fluids, including cerebrospinal fluid, synovial, pleural, pericardial, and peritoneal liquid. Stool can be collected when intestinal TB is suspected and also in the case of suspected *Mycobacterium avium* infection in AIDS patients. Whole blood and/or bone marrow specimens are collected only if disseminated TB is suspected, mainly in patients with an underlying severe immunosuppressive condition such as AIDS. Bone biopsies are the specimen of choice when skeletal TB is suspected. In general, AFB smear microscopy from body fluids is rarely positive and the whole sediment from concentrated specimens should rather be cultured. In tuberculous pleural effusions, the diagnostic value of the pleural biopsy is much higher than that of the fluid and, therefore, is the specimen of choice for the diagnosis (Escudero 1990, Valdez 1998). The diagnosis of peritoneal and pericardial TB is difficult and usually requires invasive procedures such as laparoscopy and biopsy.

Specimens should be collected in sterile, leak-proof containers and labeled with the patient's name and/or identification number before anti-tuberculosis chemotherapy is started. Induced sputum specimens should be labeled as such because they resemble saliva and may be disregarded at the laboratory. Specimens must be collected aseptically in order to minimize contamination with other bacteria.

Blood and other specimens prone to coagulate, including bone marrow, synovial, pleural, pericardial and peritoneal fluids, should be collected in tubes containing sulfated polysaccharides or heparin. Sulfated polysaccharides are the preferred anticoagulants as they enhance the growth of mycobacteria. Heparinized specimens are also satisfactory, but specimens collected in ethylenediaminetetraacetic acid (EDTA) are unacceptable as even trace amounts of this substance inhibit mycobacterial growth. Lymph nodes, skin lesion material, and tissue biopsy specimens should come without preservatives or fixatives and should not be immersed in saline or any fluid. Once in the laboratory, tissue specimens are homogenized in a sterile tissue grinder with a small amount of sterile saline solution before AFB smear staining or culture. Abscess contents or aspirated fluids can be collected in a syringe. If renal TB is suspected, the specimen of choice is the first-morning urine,

at least 50 mL, obtained by catheterization or from the midstream clean catch on three consecutive days. Urine specimens should be submitted to a decontamination step for mycobacteria prior to cultivation.

12.2.2. Specimen transport

Diagnostic specimens (sputum, blood, tissue) and mycobacterial cultures can be mailed, but transportation of dangerous or infectious goods is regulated by law in many countries. Specific shipping instructions can be found in the guide by Kent (Kent 1985). Shipments by private couriers are regulated by the International Air Transportation Association (IATA), and its shipping guidelines for infectious substances can be found on the internet at http://www.iata.org/training/courses/tcgp22.htm. The guidelines released by the WHO in 2007 are available at http://www.who.int/csr/resources/publications/biosafety/WHO_CDS_EPR_2007_2/en/index.html. Mailing instructions for specimens and cultures are also available at the National Jewish Hospital site: http://www.njc.org/pdf/2005 shipping instructions.doc.

Specimens should be transported rapidly to the laboratory to avoid overgrowth by other microorganisms. This is particularly true for specimens from non-sterile sites, such as sputum. When the transport or the processing is delayed, specimens should be stored for not more than five days at 4°C until transported or presented for bacteriological processing. The cetylpyridinium chloride (CPC) method is widely used for the transport of sputum specimens (Smithwick 1975). CPC eliminates the associated flora in sputum specimens and treated specimens should not be submitted to further decontamination prior to cultivation. The detection of AFB with Ziehl-Neelsen staining can be significantly reduced in specimens preserved by this method (Selvakumar 2004, Selvakumar 2006). Re-staining seems to increase the detection in sputum specimens transported in CPC solution (Selvakumar 2005). In addition, CPC inhibits mycobacterial growth, especially when inoculated in culture media including Middlebrook 7H9 and 7H10, which have an insufficient neutralizing activity for this quaternary ammonium compound. Therefore, specimens treated with CPC should be preferentially inoculated in egg-based media (Smithwick 1975). In a comparative study, sodium carbonate was found to be a better preservative of sputum specimens for AFB smear microscopy as well as culture (Bobadilla 2003). However, no comparative study has been undertaken to confirm this observation.

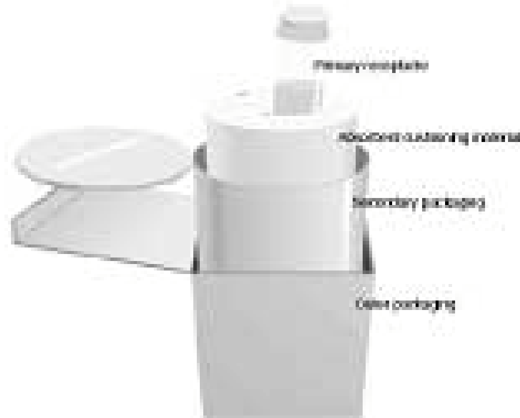

Figure 12-1: Example of packaging of infectious specimens.

12.2.3. Biosafety

This subject is thoroughly discussed in Chapter 11. Thus, in this chapter, laboratory safety will only be briefly addressed. Laboratory diagnosis of TB involves a risk of infection for laboratory personnel. Specimens with high mycobacterial loads, such as sputum or cultures, are often manipulated with limited biosafety measures, especially in low-resource countries. Although only limited information can be found in the literature on the risk of developing TB in laboratory personnel (Reid 1957, Kim 2007), studies in healthcare workers in contact with TB patients clearly show that TB can be considered as an occupational disease (Joshi 2006, Seidler 2005, Kilinc 2002).

Biosafety cabinets are seldom available in developing countries, and safety facilities for working with infectious specimens are limited. The only measure often taught to give any protection against infection with *M. tuberculosis* is processing infectious specimens behind the flame of a Bunsen burner, that is, the specimens are handled and the smear is prepared with the flame of the Bunsen burner interposed between the operator and the specimen. As far as we know, however, this measure has never been evaluated. Good laboratory practice is required for the protection of laboratory staff from infectious airborne bacilli, i.e. good ventilation, use of laboratory coats, surgical gloves and face masks, hand washing and regular disinfection of the laboratory floor and surfaces, especially benches, with a disinfectant that is active against mycobacteria. This disinfectant may be 70 % ethanol or sodium hypochlorite (house bleach) at a concentration of 0.2-0.5 % (see Chapter

11). Ultraviolet light, emitting rays of wavelength 254 nm, is very effective in killing the tubercle bacillus and other mycobacteria; it is also an additional measure for decontaminating work surfaces and killing airborne microorganisms (David 1973, Riley 1989). Good laboratory practice for handling TB specimens can be found in the guidelines by Kent (Kent 1985) and on the web page of the Centers for Disease Control and Prevention http://www.cdc.gov/od/ohs/tb/tbdoc2.htm; this web page also offers an inquiry for assessing safety in your laboratory at http://www.phppo.cdc.gov/mpep/pdf/mtb/tb-ayl.pdf.

12.3. Smear staining

12.3.1. AFB smear staining

AFB smear microscopy plays an important role in the early diagnosis of mycobacterial infections because most mycobacteria grow slowly and culture results become available only after weeks of incubation. In addition, AFB smear microscopy is often the only available diagnostic method in developing countries. Smear staining is based on the high lipid content of the cell wall of mycobacteria which makes them resistant to decolorization by acid-alcohol after the primary staining (see Chapter 3). To determine that a clinical specimen contains AFB, the specimen is spread onto a microscope slide, heat-fixed, stained with a primary staining, decolorized with acid-alcohol solution and counterstained with a contrasting dye in order to obtain better differentiation between the microorganism and the background. The slide is observed under the microscope for the detection of AFB. Several methods can be used for determining the acid-fast nature of an organism.

Two methods, Ziehl-Neelsen and Kinyoun, utilize basic fuchsin in ethanol for primary staining. In both cases, AFB appear red after decolorization with acid-alcohol. Ziehl-Neelsen is a hot acid-fast stain because the slide has to be heated during incubation with fuchsin. In contrast, Kinyoun staining is a cold acid-fast staining procedure and therefore does not require heating. Kinyoun's cold carbol fuchsin method is inferior to the Ziehl-Neelsen staining (Somoskovi 2001, Van Deun 2005). Details for the preparation of smear staining with the Ziehl-Neelsen method can be found in the following guidelines available on the internet: The WHO Laboratory Services in TB Control Part II Microscopy http://www.phppo.cdc.gov/dls/ila/documents/lstc2.pdf), the CDC Acid-Fast Direct Smear Microscopy Participant Manual (http://www.phppo.cdc.gov/dls/ila/acidfasttraining/participants.aspx), and the IUATLD technical guide (http://www.iuatld.org/pdf/en/guides_publications/microscopy_guide.pdf).

In the fluorochrome procedure, primary staining is done with auramine O. The AFB fluoresce yellow against a counterstain of potassium permanganate when observed with a fluorescence microscope. While the reading of fuchsin-stained smears requires 1000x magnification, fluorochrome-stained smears are examined at 250x or 450x. The lower magnification used in this staining method allows the microscopist to observe a much larger area of the smear during the same period of time and thus, fewer fields must be read. This makes the method faster and reduces laboratorist fatigue. Allegedly, fluorescent staining is more sensitive than Ziehl-Neelsen staining (Steingart 2006). However, it has been claimed that both methods have comparable sensitivity, provided procedural standards are followed, and a minimum of 300 fields are read with the Ziehl-Neelsen staining (Somoskovi 2001). Because of the rapidity of the fluorochrome method, laboratories processing large numbers of specimens should adopt this technique. A real disadvantage of the fluorochrome method is that fluorescence fades with time. For this reason, the slides must be read within 24 hours. This staining method is not often available in developing countries due to the high cost of the fluorescence microscope and, especially, that of its maintenance.

The results of the smear microscopy should be reported according to an internationally agreed quantitation scale.

Table 12-1: Quantitation scale recommended by the World Health Organization and the International Union Against Tuberculosis and Lung Disease

Count on Ziehl-Neelsen /Kinyoun stain (1000x)	Count on Auramine/rhodamine (450x)	Report
0	0	Non AFB observed
1-9/100 fields		Exact count
10-99/100 fields	Observed count divided by 4	1+
1-10/field		2+
> 10/field		3+

12.3.2. Quality control of AFB smear microscopy

Patients with infectious TB may remain undetected due to unreliable laboratory diagnosis. In addition, errors in AFB smear microscopy reading can result in patients being put on treatment without having the disease. Therefore, quality assurance of AFB sputum smear microscopy is essential, and the quality of laboratory

services should be considered a high priority of the National TB Control Programmes.

More details for quality assessment for AFB smear microscopy can be fond in the following guidelines available on the internet: Quality Assurance of Sputum Microscopy
(http://www.phppo.cdc.gov/dls/ila/acidfasttraining/participants/pdf/ParticipantModule10.pdf) and Quality Assurance of Sputum Microscopy in the DOTS Programmes (http://www.wpro.who.int/NR/rdonlyres/769B76D6-270F-4438-A4BE-D6B9FAC3902E/0/Quality_assurance_for_sputum_WP.pdf). These quality assurance programs are based on systematic monitoring of working practices, technical procedures, equipment and materials, including quality of stains, site evaluation of laboratory/quality improvement and also training, when needed.

An aspect usually overlooked in TB case finding and laboratory quality assessment is the quality of the sputum specimen itself. When the patient is thoroughly instructed on sputum sampling, the microscopic diagnosis of TB improves substantially (Alisjahbana 2005). Poor quality of the sputum specimen often results in AFB smear microscopy negative results (Hirooka 2004). Registering the quality of the sputum specimens received at the laboratory could help to improve sputum sampling. Satisfactory quality implies the presence of mucoid or mucopurulent material and a volume of 3-5 ml, although smaller volumes are acceptable if the consistency is adequate. If a relatively high percentage of the specimens received are saliva, the laboratory should report this to the medical staff, and instructions should be given to nurses and physicians on how to improve the quality of sputum sampling.

12.3.3. Concentrated sputum smears

Sputum is the most common specimen received for TB diagnosis. The minimum number of bacilli needed to detect their presence in stained smears has been estimated to be 5,000-10,000 per mL of sputum. For diagnosis, the sensitivity of AFB smear staining relative to culture has been estimated to vary from 50 % to over 80 %. Several studies have been published on improving smear microscopy performance using methods that concentrate the bacilli present in the sputum specimen. The methods consist of submitting the specimen to a liquefaction step prior to concentrating it by sedimentation or centrifugation. The smears are then performed from the sediment and stained for microscopic examination. The chemical method used for the liquefaction depends on the next step following concentration; smear staining only or smear staining followed by culturing.

The best known concentration procedure is the 'bleach microscopy method', in which the sputum is liquefied with sodium hypochlorite (NaOCl or household bleach), and concentrated by centrifugation before AFB staining. This technique is inexpensive and easy to perform. In addition, NaOCl is a potent disinfectant that also kills mycobacteria, thus reducing the risk of laboratory-acquired infection but, at the same time, rendering the method unsuitable for culturing. A significant improvement in the proportion of positive AFB smear results has been reported, ranging from 7 % to 253 % (Angeby 2004).

Other concentration methods should be used if the specimen is to be cultured. The sediment of a sputum specimen liquefied and decontaminated with sodium hydroxide-N-acetyl-L-cysteine method (see decontamination procedures below) and concentrated by centrifugation can also be examined by AFB smear staining. An advantage of this method is that the same sediment can be cultured, in contrast to those liquefied with the NaOCl method described above. Other methods involving sputum liquefaction with different substances, and concentration either by sedimentation or centrifugation, have been proposed. The methods using dithiothreitol (Murray 2003), chitin (Farnia 2004) and C(18)-carboxypropylbetaine (Scott 2002) have been evaluated favorably for the preparation of concentrated smears. Sputum concentration methods were recently reviewed (Steingart 2006) and compared with direct smears. The authors concluded that concentration by centrifugation and sedimentation with any of several chemical methods (including bleach) is more sensitive than direct AFB sputum smear examination.

12.4. Adenosine deaminase activity

Adenosine deaminase, also known as ADA, is an enzyme involved in the metabolism of purines. Its presence is needed for the breakdown of adenosine from food and for the turnover of nucleic acids in tissues. Many articles and reviews have reported the utility of ADA determination in body fluids (spinal, pleural, ascitic, pericardial) for the diagnosis of tuberculous meningitis (Kashyap 2006, Lopez-Cortes 1995), tuberculous pleurisy (Banales 1991, Goto 2003, Perez-Rodriguez 2000), peritoneal TB (Aston 1997, Gilroy 2006) and pericardial TB (Tuon 2006, Reuter 2006). AFB may be difficult to isolate from these specimens because they are often diluted in large fluid volumes. Biopsy specimens have a better yield, but biopsy is not always available in low-resource settings. ADA determination is simple and cheap and also has a high positive predictive value, especially in high endemic countries. The routine use of this method is justified in exudates of pleural, peritoneal and pericardial fluids. The specificity is very high in fluids with a

lymphocyte-to-neutrophil ratio higher than 0.75 (Burgess 1996, Diacon 2003). The suggested laboratory cut-off of ADA activity is 40 U/L for pleural, peritoneal and pericardial fluids and 10 U/L for cerebrospinal fluid. However different laboratories have established different cut-offs varying between 33 and 50 U/L.

As the description of the ADA assay is not readily available in the literature, it is detailed here. ADA is determined colorimetrically (Giusti 1974). A 25 µL specimen is incubated for 60 min at 37°C with 500 µL of 21 mM adenosine in 50 mM phosphate buffer pH 7.0. The reaction is interrupted by incubation with 1.5 mL of phenol nitroprusside at 37°C for 30 min (106 mM phenol, 0.17 mM sodium nitroprusside) in the presence of 1.5 mL of sodium hypochlorite (11 mM NaOCl plus 125 mM NaOH). The amount of ammonium ion released by the ADA reaction is determined as absorbance (optical density, OD) at 628 nm wavelength in a spectrophotometer (blue color). To control for the ammonium present in the patient's specimen before addition of exogenous adenosine, specimens without substrate are run in parallel (specimen blank). A standard (15 mM ammonium sulfate stock solution) and a reagent blank (50 mM phosphate buffer pH 7.0) are also included in the assay. The activity in the patient's specimen is calculated with the formula:

Activity in specimen = (OD specimen – OD specimen blank) / (OD standard – OD reagent blank) x 50 with the result expressed in Units/L.

12.5. Culture

12.5.1. Sterile or contaminated specimens

Acid-fast microscopy is easy and quick, but it does not confirm TB diagnosis because mycobacteria other than *M. tuberculosis* are also AFB in the smear microscopic examination. In addition, a high bacterial load is needed in the specimen to render an AFB microscopy result positive. Culture techniques have been estimated to detect as many as 10–1,000 viable mycobacteria per mL of specimen. Therefore, although most TB control programs do not support its widespread use due to enhanced laboratory complexity, biohazard and cost, in our view, all specimens should be submitted to culture, regardless of AFB smear microscopy results. A positive culture for *M. tuberculosis* confirms the diagnosis of active disease.

For culturing of mycobacteria, two types of clinical specimens are considered: contaminated specimens and specimens collected aseptically from normally sterile sites. Sterile specimens can be inoculated directly onto the culture medium. Specimens from non-sterile bodily sites are considered contaminated and therefore require processing before culturing in order to eliminate the associated flora. If not

properly eliminated, this flora will overgrow the culture medium long before mycobacteria have the chance to develop visible colonies.

12.5.2 Decontamination procedures

Several methods have been used to minimize culture contamination when specimens from non-sterile body sites are processed. Most of these methods include the digestion of mucus or organic debris and treatment to eliminate micro-organisms from the normal flora. Both steps are done to maximize the probability of isolating mycobacteria in culture. No single decontamination method is applicable to all circumstances, laboratories and clinical specimens; therefore, a laboratory should use the best suited method that keeps the contamination rate between 3 % and 5 %. A contamination rate lower than 3 % may indicate that the procedure used is too harsh and may be killing the mycobacteria (Della Latta 2004).

The decontamination methods most commonly used are (Table 12-2):

- **Sodium hydroxide.** This method uses sodium hydroxide at concentrations ranging between 2 % and 4 % to digest and, at the same time, decontaminate the specimen. Each laboratory should determine the lowest concentration for optimal digestion and decontamination (Della Latta 2004).
- **N-acetylcysteine-sodium hydroxide, sodium chloride and sodium hydroxide.** This method, one of the most used worldwide, uses N-acetylcysteine for mucus digestion and sodium hydroxide as the decontaminant (Della Latta 2004).
- **Oxalic acid.** This method is recommended for decontamination of clinical specimens that may have *Pseudomonas aeruginosa* as a contaminant, usually urine specimens and pulmonary specimens from cystic fibrosis patients (Della Latta 2004, Cooper 1930).
- **Ogawa-Kudoh.** This is a very simple and practical decontamination method that obviates the use of specimen centrifugation prior to culturing. The procedure was described by Kudoh using sodium hydroxide as the digestant-decontaminant and inoculation in a modified Ogawa media (Kudoh 1974). It does not require laboratory facilities and can be performed in the field, as described below:

- With the aid of a sterile cotton swab, obtain a significant portion of the specimen
- Submerge the applicator in a tube containing 3 mL of 4 % sodium hydroxide solution
- Incubate at room temperature for two minutes
- Remove the applicator from the sodium hydroxide solution
- Inoculate a tube containing modified Ogawa culture medium
- With the same applicator, make a smear for AFB microscopic examination

Table 12-2: Decontamination methods

Method	Use	Advantage	Disadvantage
Sodium hydroxide	Laboratories using concentration by centrifugation	- Digestion / decontamination at the same time when used at a final concentration of 2 % - Low cost	- Precise timing needed to avoid killing mycobacteria - may kill some mycobacteria even at 2 % concentration
N-acetyl-cysteine - sodium chloride - sodium hydroxide	- Most used in developed countries - Used in combination with centrifugation	- Good mucolytic action - Use of NaCl as mucolytic reduces NaOH concentration and its potential deleterious action on mycobacteria	- Short shelf-life of prepared reagents (24 h) - Higher cost
Oxalic acid	- Recommended to eliminate P. aeruginosa contamination (e.g. in urine, specimens from cystic fibrosis patients)	- Effective in inhibiting overgrowth by Pseudomonas	- Use restricted to inhibit Pseudomonas
Ogawa-Kudoh	- Ideal method for low-resource settings	- Centrifugation or concentration is not necessary - Low cost - Can be used in the field	- May have higher contamination rates
Cetyl pyridinium - sodium chloride	- For preservation and digestion / decontamination while in transport to the laboratory	- Avoids overgrowth of contaminants for up to eight days	- Egg-based media required since compound remains active in agar and may be deleterious to mycobacteria

- **Cethyl pyridinium and sodium chloride.** This method is useful to preserve specimens from contaminant flora overgrowth while in transit to the laboratory and also fulfills the decontamination step required prior to culture. It has demonstrated viability of mycobacteria exposed for eight days to CPC (Smithwick 1975).

12.5.3. Concentration of mycobacteria

With the exception of the Ogawa-Kudoh method, specimens are inoculated after a concentration step, which is done by spontaneous sedimentation or, more frequently, by centrifugation. Many laboratories report centrifugation speeds in revolutions per minute (rpm). However, this speed measure is related to each particular centrifuge. The measure of sedimentation efficiency is the relative centrifugal force (RCF), which considers the radius from the centre of the rotating head to the bottom of the centrifuge tube (R max) and the centrifuge speed (rpm). The RCF may be calculated from the formula:

RCF = 1.12 x R max (in mm) x (rpm/1,000) x 2

The optimum RCF and centrifugation time combination have been determined to be 4,000 x g for 15 min. (Perera 1999) but an RCF of 3,000 x g applied for 15 min, or an RCF of about 2,000 to 2,500 x g applied for 20 min, is still considered adequate to concentrate mycobacteria in clinical specimens. Lower RCF and/or centrifugation times may lead to a considerable loss of mycobacteria. The sediment of the concentration step will be inoculated directly onto the culture medium, or after decontamination in the case of non-sterile specimens that did not undergo decontamination before the concentration step.

12.5.4. Culture media

Different culture media are in use for the isolation of mycobacteria. The most common are based on egg and also contain high concentrations of malachite green to overcome contamination with other bacteria. Detailed guidelines for the preparation of the most widely used egg-based media, Löwenstein-Jensen and Ogawa, are freely accessible on the internet (Laboratory Services in Tuberculosis Control Part III: Culture: http://www.phppo.cdc.gov/dls/ila/documents/lstc3.pdf). In general, after the centrifugation step, sediments are inoculated onto two Löwenstein-Jensen slants. In areas with a high incidence of bovine TB, a tube with Stonebrink

medium should be added. *M. bovis* and other species of the *M. tuberculosis* complex (*M. microti* and *M. africanum*) are unable to use glycerol as a carbon source due to the lack of a functioning pyruvate kinase. Thus, these organisms will often fail to grow on Löwenstein-Jensen medium, which contains glycerol as the only available carbon source (Keating 2005, see Chapter 3). Stonebrink medium has the same composition as Löwenstein-Jensen, with the exception that glycerol is replaced by 0.5 % sodium pyruvate. Many diagnostic laboratories that employ egg-based medium for the isolation of mycobacteria, omit the use of Stonebrink medium. This probably leads to an underestimation of the actual weight of *M. bovis* as a human TB agent, especially in developing countries (see Chapter 8).

The Ogawa medium is another egg-based medium, which is comparable in its composition with Löwenstein-Jensen. It is more economic because it replaces asparagine by sodium glutamate, an amino acid more readily available and much cheaper. Modified Ogawa medium (pH 6.4) is the same egg-based Ogawa medium that has been acidified in such a way as to allow the direct inoculation of specimens decontaminated by the Kudoh method. This combination is very suitable for culturing sputum specimens in rural settings.

Middlebrook 7H10 and 7H11 are agar-based media. Their basic ingredients are commercially available: powder base, agar and Middlebrook OADC enrichment. Middlebrook 7H9 is a liquid medium and may be prepared from commercially available powder base supplemented with Middlebrook ADC enrichment after sterilization. Incubation in a 5 % to 10 % CO_2 atmosphere is recommended. Middlebrook media have been shown to achieve slightly higher isolation yields than egg-based media, but are considerably more expensive.

Details for the preparation of the above mentioned media can be found in the annual on Laboratory Services in Tuberculosis Control Part III: Culture, which is freely available on the internet at http://www.phppo.cdc.gov/dls/ila/documents/lstc3.pdf.

The BACTEC TB-460 system was the first semi-automated system to appear on the market for mycobacteria culturing, and still serves as the bench-mark for quality, reliability and performance. In the last decade, several new commercial culture media have been introduced, such as the Mycobacteria Growth Indicator Tube (MGIT), Bract/Alert, ESP Mice, MB Redox and KRD "Niche B", biphasic Septic-Check AFB and Mice-Acid, and BACTEC MGIT960 systems. Like BACTEC TB-460, these newer systems are based on liquid media. Liquid culture media has been proven to be significantly more sensitive than egg-based solid media for the isolation of mycobacteria from clinical specimens (Hines 2006). However, one disad-

vantage is the much higher price, which is often too high for cost-effective TB diagnosis in resource-limited countries. These methods are described in detail in Chapter 14.

Blood agar is an alternative culture medium for isolation of mycobacteria. Historically, microbiologists and medical students have been taught for decades that isolation of mycobacteria requires a defined, egg-based medium such as Löwenstein-Jensen. In fact, the tubercle bacillus does not have special growth requirements (see Chapter 3), and blood agar is at least as efficient as the widely recommended egg-based media. *M. tuberculosis* grows within one to two weeks on blood agar plates, and it has been reported that the average number of colonies isolated from clinical specimens on blood agar is significantly higher than the number of colonies on the egg-based medium (Dracut 2003). Preliminary studies suggested that blood agar can also be used as an alternative medium for susceptibility testing of *M. tuberculosis* against isoniazid, rifampicin, streptomycin and ethambutol. Reportedly, results are obtained much earlier with blood agar (2 weeks) than with 7H10 agar (3 weeks) (Coban 2005, Coban 2006). Desiccation is prevented by sealing the plates with tape or by using tubes instead of plates. Blood agar plates are readily available in most laboratories dedicated to general bacteriology, and thus, in the absence of more specific media, they could be used for the culture of mycobacteria, especially in resource-limited countries.

12.5.5. Reading of results

Conventional culture media such as those based on egg and agar should be examined for growth twice a week for the first four weeks starting on day 3 to 5 post-inoculation, and thereafter, once a week until the eighth week. All specimens showing growth in culture should be confirmed as AFB by smear microscopy of the colonies and reported immediately as "culture positive for mycobacteria pending identification". All cultures reported positive for mycobacteria should be identified to the level of species using either biochemical or molecular methods.

M. tuberculosis bacilli are slow-growing mycobacteria. This means that, in primary isolation, they hardly show any visible growth during the first week of culture. On egg-based media they produce characteristic non-pigmented colonies, with a general rough and dry appearance simulating breadcrumbs (Figure 12-2). On agar-based media, the colonies appear flat, dry and rough with irregular edges.

Figure 12-2: Colonies of *Mycobacterium tuberculosis* in Löwenstein Jensen medium (*Courtesy of A. Martin*).

Cross-contamination can produce false positive results. The causes of false-positive cultures include contamination of clinical devices (i.e. bronchoscopes), clerical errors, and laboratory cross-contamination. In a recent review, false-positive cultures were identified in 13 of the 14 studies that evaluated 100 or more patients; the median false-positive rate was 3.1 % (interquartile range, 2.2 % - 10.5 %). Patients having only one positive culture when two tubes were inoculated and patients with only a few colonies in the culture should be further evaluated for the possibility of a false-positive result (Burman 2000).

12.6. Identification

12.6.1. Biochemical procedures

The final species identification of *M. tuberculosis* is based on characteristics such as slow growth, colony morphology, and biochemical tests. From a practical point of view, most isolates from human disease belong to the species *M. tuberculosis.* However, depending on geographical and epidemiological circumstances, it may be necessary to differentiate species within the *M. tuberculosis* complex (see Chapter 8).

An initial identification as *M. tuberculosis* is defined on AFB bacilli from slow-growing, non-pigmented colonies that are niacin positive, are inhibited by p-nitrobenzoic acid and display nitratase activity. Additional tests that confirm an

isolate as *M. tuberculosis* are susceptibility to pyrazinamide, growth on thiophene carboxylic acid hydrazide, absence of catalase production at 68°C and absence of iron uptake. Table 12-3 summarizes the differential characteristics of species in the *M. tuberculosis* complex.

Table 12-3: Colony morphological and biochemical characteristics of species in the *M. tuberculosis* complex

Test	*M. tuberculosis*	*M. bovis*	*M. bovis BCG*	*M. africanum*	*M. microti*	*"M. canettii"*
Morphology	rough	rough	rough	rough	rough	smooth
Pyruvate rather than glycerol as carbon source	-	+	+	-	-	-
Pyrazimidase	+	-	-	+	+	+
Niacin	+	-	-	+/-	+	-
Nitratase	+	-	-	+/-	-	+
Urease	+/-	-	+	+/-	+/-	+
Susceptibility to TCH	R	S	S	S	S	R
O_2 requirement	aerobic	Micro-aerophilic	aerobic	Micro-aerophilic	Micro-aero-philic	Unknown

R= resistant, S= susceptible, TCH= Thiophene-2-carboxylic acid hydrazide

12.6.2. Main biochemical tests to identify *M. tuberculosis*

- **Niacin accumulation test.** Nicotinic acid or niacin is produced by all mycobacteria, but some species, such as *M. tuberculosis*, *Mycobacterium simiae* and *M. bovis* BCG, excrete it due to a blockade in their scavenging pathway. The excreted niacin accumulates in the culture medium and is evidenced in the presence of cyanogen halide with a primary amine (Figure 12-3). Niacin-negative *M. tuberculosis* strains are extremely rare.

- **Growth in the presence of p-nitrobenzoic acid.** This compound inhibits the growth of several species in the *M. tuberculosis* complex: *M. tuberculosis*, *M. bovis*, *M. africanum* and *M. microti* (Tsukamura 1984, Leão 2004).

- **Nitrate reduction test.** This test is particularly useful for differentiating *M. tuberculosis*, which gives a positive reaction, from *M. bovis,* which is negative (Tsukamura 1984, Vincent 2003).

- **Catalase test.** Catalase is an intracellular enzyme that transforms hydrogen peroxide to oxygen and water. The 68°C catalase is a heat-tolerance test measuring the catalase activity at high temperature. Characteristically, *M. tuberculosis* gives negative results, as do other species in the *M. tuberculosis* complex. (Vincent 2003).
- **Pyrazinamidase test.** Pyrazinamidase is an enzyme that hydrolyzes pyrazinamide to ammonia and pyrazinoic acid. The test is useful to differentiate *M. tuberculosis* (positive) from the other species of the *M. tuberculosis* complex (negative), with the exception of "*M. canettii",* which is also positive. Some strains of *M. tuberculosis* may acquire resistance to pyrazinamide due to selective pressure induced by treatment with this drug. These strains give a negative pyrazimidase test (unable to transform pyrazinamide to pyrazinoic acid, the active form of the drug) (Vincent 2003).
- **Growth in the presence of thiophen-2-carboxylic acid hydrazide.** This test is useful to distinguish *M. tuberculosis,* which grows in the presence of this compound, from other members of the *M. tuberculosis* complex. "*M. canettii"* and most non-tuberculous mycobacterial species are also positive to this test (Vincent 2003, Leão 2004).

Figure 12-3: Niacin test (*Courtesy of A. Martin*).

12.6.3. Isolation of non-tuberculous mycobacteria

Non-tuberculous mycobacteria (NTM) are ubiquitous organisms that are frequently isolated from environmental sources, including surface water, tap water, and soil. Specimens from the respiratory tract often grow NTM. The isolation of NTM species from a respiratory specimen is not enough evidence for the presence of NTM lung disease, the diagnosis of which must rely on clinical, radiographic, and bacteriologic criteria. Both the American Thoracic Society (http://www.thoracic.org/sections/publications/statements/pages/mtpi/nontuberc1-27.html) and the British Thoracic Society (http://www.brit-thoracic.org.uk/c2/uploads/OppMyco.pdf) have issued guidelines regarding the diagnosis of lung disease due to NTM. Both guidelines highlight the difficulty inherent in differentiating patients with clinical lung disease caused by NTM from those in whom the isolation of clinical specimens raises a suspicion of disease. At least three respiratory specimens should be evaluated from each patient to establish the clinical significance of the infection. The NTM species most frequently associated with pulmonary disease are *M. avium, Mycobacterium kansasii* and *Mycobacterium abscessus.*

Wound infections, prosthetic valve endocarditis, infections complicating mammary augmentation surgery, and other cutaneous/subcutaneous infections have been attributed to rapidly growing mycobacteria. *Mycobacterium fortuitum*, *M. abscessus*, and *Mycobacterium chelonae* are the most common mycobacteria implicated in these infections, which are thought to be caused by local environmental strains or contaminated commercial surgical materials, devices or solutions for injection. Rapidly growing mycobacteria often grow on classical bacterial culture media, especially on blood agar plates. However, due to their delay in forming visible colonies (up to 10 days), they are usually not detected in the routine bacteriology laboratory. They can also be isolated on most media available for the isolation of mycobacteria. Although the optimum temperature for most species is 30-32°C, they also grow at 36°C to 37°C, the standard temperature for isolation of the tubercle bacillus. Mycobacteria should be identified at the species level before starting treatment, because different species display different antibiotic resistance patterns. An extensive review on rapidly-growing NTM has been recently published (Brown-Elliott 2002).

Certain NTM are fastidious and special culture conditions or growth requirements should be observed for their isolation. Because of this, the organisms are not be isolated using routine mycobacterial culture techniques. If disease by one of these

mycobacteria is suspected, the bacteriologist must be notified so that appropriate cultivation conditions can be implemented. *Mycobacterium marinum* causes localized cutaneous lesions in patients with a history of a penetrating cutaneous injury and prolonged or repeated aquatic exposure. This microorganism grows at 30°C and its growth is inhibited at 37°C. Two other mycobacterial pathogens require special conditions for laboratory culture: *Mycobacterium haemophilum*, which causes cutaneous, joint, or pulmonary infection in immunocompromised patients and lymphadenitis in children, grows preferentially at 30°C to 32°C, and requires iron-supplemented media i.e. egg-based medium supplemented with iron complexes or blood agar medium; and *Mycobacterium genavense,* which produces disseminated infections in HIV/AIDS patients, grows in Middlebrook 7H11 agar supplemented with mycobactin J, and requires prolonged incubation periods (Coley 1992).

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Chapter 13: Immunological Diagnosis

Mahavir Singh and Clara Espitia

13.1. Historical Overview

The gold standard for tuberculosis (TB) diagnosis is the demonstration of mycobacteria in various body fluids. This is often not possible, due to the paucibacillary nature of the illness in some cases, for example in children. On the other hand, microscopic identification and culture of mycobacteria in sputum are the most common methods for diagnosis of pulmonary disease, but the detection of extrapulmonary TB is often more difficult. In the search for rapid and cost-effective diagnostic methods for TB, immunodiagnosis is considered an attractive option. Basically, it uses the specific humoral and cellular immune responses of the host to infer the presence of infection or disease. The tuberculin skin test (TST) (Huebner 1993) and, more recently, the antigen-specific *ex vivo* induction of interferon-gamma (IFN-γ) production have been used to detect infection with *Mycobacterium tuberculosis* (Pai 2004). At the same time, a wide variety of serological tests for the detection of antibodies in individuals suspected to have TB have also been evaluated to detect active disease (Gennaro 2000, Chan 2000). Serology has additional advantages in situations when:

- the patient is unable to produce adequate sputum
- sputum smear results are negative
- TB is extrapulmonary

13.1.1. Serological diagnosis

Historically speaking, serology for the diagnosis of TB has been explored since 1898, when crude cell preparations containing carbohydrates, lipids, and proteins from *M. tuberculosis* or *M. bovis* BCG were used as antigen preparations showing high sensitivity but low specificity (Arloing 1898). Modern developments in the purification of antigens, generation of monoclonal antibodies and chromatographic techniques, have led to a considerable improvement in specificity. During the last three decades, a large number of purified (native and recombinant) antigens have been assessed, showing substantial progress in the serodiagnosis of TB (Jackett 1988).

13.1.2. The century-old skin test for detection of latent tuberculosis

In 1882, about eight years after the discovery of the tubercle bacillus, Robert Koch announced a cure for TB. He obtained a heat-inactivated filtrate from cultures of *M. tuberculosis*, and found that this material would protect guinea pigs from experimental TB. This product, known as “Koch´s Old Tuberculin”, was then administered to patients with TB, and Koch claimed that this treatment resulted in the cure of the disease (Kaufmann 2000, Gradmann 2001, Gradmann 2006). However, TB patients who received tuberculin had generalized systemic reactions, including fever, muscle aches, and abdominal discomfort with nausea and vomiting, in contrast to people without TB, who did not develop this violent reaction. These observations were the basis for the proposal of the use of tuberculin as a diagnostic test, despite its failure as a therapeutic substance. The intradermal injection of tuberculin was described by Mantoux, and his method became widespread because of the reproducibility of the results. After local application of the product injected intradermally, a hallmark response is elicited within 24 to 72 hours, which includes induration, swelling and monocytic infiltration into the site of the injection (Figure 13-1). The skin reaction, classified as delayed type hypersensitivity (DTH), has been used since then to test if prior exposure to an antigen has occurred. Koch's tuberculin was an impure extract of boiled cultured tubercle bacilli. In 1934, Siebert made a simple protein precipitate of the old tuberculin and named it purified protein derivative (PPD) (Figure 13-2).

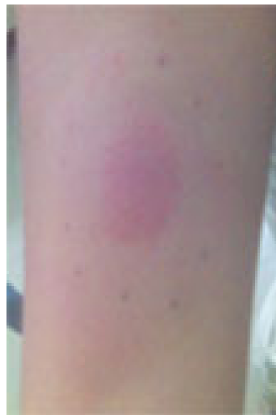


Figure 13-1. Tuberculin skin test.

Figure 13-2. Florence Seibert.

13.2. Current methods of tuberculosis diagnosis

An overview of the current general methods of TB diagnosis is given in Table 13-1.

Table 13-1: Current methods of TB diagnosis (© LIONEX, Braunschweig)

Method	Advantages	Disadvantages
Clinical signs	Rapid diagnosis	Not specific, not conclusive Not always present
X-Ray	Readily available	Not specific or conclusive
Microscopy (smear for acid-fast bacilli)	Low cost Rapid diagnosis	Low sensitivity (up to 2/3 of pulmonary TB cases are negative) Difficult sample collection
Culture	Specific	Time consuming (up to 4-8 weeks) Not always possible
PCR	Relatively quick Very specific	Relatively expensive High level of training required Expensive instrumentation Can detect latent disease

Method	Advantages	Disadvantages
BACTEC	Specific	Slow, 2-3 weeks Expensive Not possible in all cases
Tuberculosis ELISA test kits	Quick (procedure time: 110 min) Reproducible Minimal training	Some equipment required
Rapid tests	Very quick (procedure time: only 15 min) Minimal training No special equipment required	Lower sensitivity compared to ELISA test kits No quantitative results

13.3. Basis of immunological diagnosis

There is strong evidence both *in vivo* and *in vitro* of cellular immune reaction in TB patients and in those infected with *M. tuberculosis. In vivo,* this reaction can be measured by DTH response to PPD; and in vitro, by the proliferation of lymphocytes to different compounds of the bacteria. (Lalvani 1998, Pai 2004). In the last decade, extensive studies have shown that immunodominant antigens, such as the 6-kDa early secretory antigenic target (ESAT-6) and its homologues, are highly suitable for detecting infection. There is no cross-reaction with the BCG vaccine, since these antigens are absent in the BCG vaccine strains.

On the other hand, there have been several studies showing humoral response to antigens in patients suffering from active disease (Bothamley 1994, Wilkinson 1997). But the general view in the scientific community has been that the specificity and the magnitude of the humoral immune response are inadequate from the point of view of TB diagnosis. The majority of studies have concentrated on smear-positive TB with very little emphasis on smear-negative TB disease, which may account for 30-60 % of the cases depending on the prevalence of TB (Jackett 1988). Also, smear-negative TB disease is the future target for serodiagnosis, because smear-positive TB cases are easily diagnosed by microscopy.

13.3.1. Humoral immune response

Early studies utilizing crude antigen preparations of *M. tuberculosis* showed seroreactive antibodies in TB patients. However, cross-reactions occurred in healthy individuals, elicited by commensal bacteria, environmental mycobacteria and BCG vaccination (Bardana 1973, Laal 1997). During the last two decades, the antibody

response to purified antigen preparations of 38 kDa (PhoS), 30/31 kDa (antigen 85, 19 kDa lipoprotein, 14 kDa, 16 kDa (ACR) and lipoarabinomanan (LAM) was also tested. The 38 kDa antigen has shown the highest sensitivity and specificity (Wilkinson 1997, Espitia 1989). It is interesting to note that anti-38 kDa antibodies seem to be restricted to advanced TB (Jackett 1988, Davidow 2005), which is the main cause of TB transmission. There are also reports on antigens (16 kDa and 88 kDa) to which antibodies are generated in the early stages of the disease (Laal 1997).

There seems to be some association of TB and TB-specific antibody levels with human leukocyte antigens (HLA) (Bothamley 1989), which may be responsible for a heterogeneous humoral immune response to TB antigens in human populations. While augmenting the humoral immune response, some genes suppress the spontaneous and antigen-induced lymphocyte response in DR2-positive patients with active disease (Selvaraj 1998).

Weldingh *et al*. (Weldingh 2005) performed a detailed study on 35 antigens of *M. tuberculosis* proteins that are absent in BCG. The authors reported the identification of additional antigens useful for improving the sensitivity of serodiagnosis in African populations. But further studies are needed to confirm these results.

A number of studies with purified native or recombinant antigens have agreed that in order to produce a useful serological tool for TB diagnosis, several antigens must be combined as a cocktail mixture. Several decades of published literature on a series of expression systems and applications also show that fusion proteins can be produced incorporating several antigens using the standard recombinant DNA technology. There is nothing novel in this kind of approach.

One must note that all published work on individual antigens or mixtures of antigens has been produced by individual research groups and there has been no confirmation of these results by other groups, except those concerning antigens such as 38 kDa and 16 kDa. Even in these cases, wrong interpretations have been stated, most probably due to the insufficient information available on the size, solubility and chemical confirmation of a given antigen. In our view, this aspect of a protein antigen candidate for studying serological response is of utmost importance. Another serious flaw in published studies is the almost complete omission of the influence of human populations (TB and healthy) from different geographic regions on the immune response to a particular antigen.

13.3.2. Cellular immune response and delayed type hypersensitivity

In 1964, Mackaness established unambiguously that immunity to *M. tuberculosis* and to certain other facultative intracellular bacterial pathogens was cell-mediated (Mackaness 1964). The cellular immune response comprises the activation of T helper lymphocytes (CD4+) and cytolytic T lymphocytes (CTL) or killer T lymphocytes (usually CD8+). These two types of effector T lymphocytes play a critical role in eliminating or controlling chronic microbial infections. Activated CD4+ T cells can differentiate into either T helper 1 (Th1) or T helper 2 (Th2) cells that secrete specific subsets of cytokines. In general, Th1 cells secrete the cytokines IFN-γ and tumor necrosis factor alpha (TNF-α), whereas Th2 lymphocytes secrete the cytokines IL-4, IL-5, IL-10, and IL-13. The Th1 immune response is associated with a strong, cell-mediated CTL response whereas the Th2 response is characterized by a humoral or antibody-mediated immune response (Esser 2003). In addition to providing cytokines for the development and maintenance of a strong CTL response, the IFN-γ and TNF-α secreted by the Th1 cells can have direct lytic effects on intracellular parasites such as *M. tuberculosis.* The induction of a Th1 immune response is dependent on another cytokine, IL-12, which is produced by macrophages and dendritic cells (Flynn 2001).

DTH was also previously found to be a reaction mediated by the cellular arm of the immune system (Landsteiner 1942). For a long time, the role of DTH in cell mediated immunity has been the subject of contentious debate. This reaction has been shown to be absolutely dependent on the presence of memory T cells. Both the CD4+ and the CD8+ fractions of T cells have been shown to modulate a response. The contemporary debate regarding the reaction focuses on the role of the Th1 and Th2 cells. It has been postulated that the Th1 cell is the "inducer" of the DTH response, since it secretes IFN-γ, a potent stimulator of macrophages, while the Th2 cell is either not involved or else acts as a downregulator of the cell mediated immune response. Despite the early experimental success of this theory, further studies have shown that Th2 cells may be involved in certain types of proinflammatory cell mediated immunity (Black 1999). A positive DTH reaction to PPD reflects the triggering of a repertoire of T-cell clones different from those involved in a protective immune response. Indeed, Pais *et al*. have demonstrated that T cells involved in protection to a challenge with live TB bacilli recognized predominantly low-mass culture filtrate antigens below 15,000 MW, while cells recruited in the DTH to PPD were directed to molecular mass fractions between 15,000 and 31,000 MW (Pais 1998).

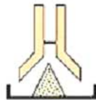
13.4. Serological assays

13.4.1. Enzyme Linked Immunosorbent Assay (ELISA)

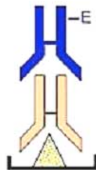
In TB patients, the serological response to mycobacterial antigens has been primarily evaluated using standard ELISA with *in house* methodologies and protocols which certainly differ from laboratory to laboratory. A general outline of the ELISA procedure is shown in figure 13-3.

Few commercial tests based on the detection of specific antigens, such as the 38 kDa protein, have been developed and have been in use, primarily in developing countries (Wilkinson 1997, Lionex available at http://www.lionex.de/content/inhalt06.htm). There is still a need to improve the sensitivity or specificity of commercial serological tests.

96 well microtiter plate with **immobilized antigen cocktail**



TB-specific I antibodies from the sample bind to the immobilized antigen.



Peroxidase-conjugated anti-human antibody binds to the antibodies.

TMB-substrate: blue colour development
Stop-solution: colour turns from blue to yellow.
OD at 450 nm (optionally 620 nm reference wave length)

Figure 13-3: General outline of the ELISA procedure.

13.4.2 Rapid tests

Immunochromatographic assays, also called lateral-flow tests or simply strip tests are a logical extension of the technology used in latex agglutination tests. The benefits of immunochromatographic tests include:

- User-friendly format
- Very short time to test result
- Long-term stability over a wide range of climates
- Relatively inexpensive to make

These features make strip tests ideal for applications such as home testing, rapid point-of-care testing, and testing in the field. In addition, they provide reliable testing that might not otherwise be available to low-resource countries.

An example of test procedure (Figure 13-4)

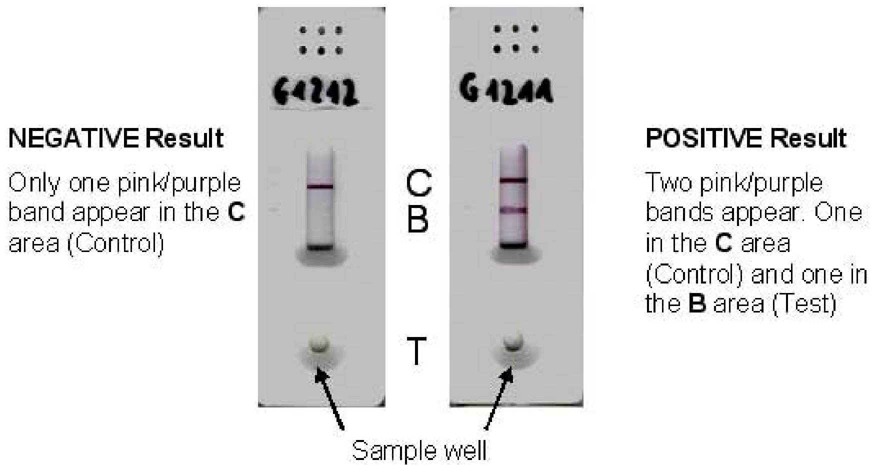


Figure 13-4: Rapid test procedure.

- Remove as many test cards from the pouches as needed. Lay on a clean flat surface.
- Add 40 µl of serum or plasma sample to the T (Test) area of the test card using a measuring pipette or add 1 drop of sample to the T (Test) area by using the provided pipette (included in each test pack). Sample volume for whole blood or EDTA blood: 20 µL.
- Follow sample addition with 2 drops of the diluent provided in the dropper bottle by holding the bottle vertically over the T (Test) Area.
- Results are then read in as little as 20 minutes.

13.5. T cell based assays

One third of the total world's population - two billion people - is believed to be latently infected with *M. tuberculosis.* Latently infected individuals have a 10 % lifetime risk of developing the disease, and this huge global reservoir of infection and disease serves as a continuous source of transmission. Since *M. tuberculosis* is sometimes difficult to culture from patients with active TB, and impossible to culture from latently infected healthy people, it is therefore vital to have efficient tools for diagnosis of active TB and screening for latent *M. tuberculosis* infection. The only widely used test is the century-old TST, based on the intradermal injection of PPD, a crude mixture of *M. tuberculosis* proteins widely shared among *M. tuberculosis*, *Mycobacterium bovis* bacille Calmette-Guérin (BCG), and most environmental mycobacteria (Andersen 2000). Hence, false-positive results are common in people exposed to environmental mycobacterial and/or previously vaccinated with BCG. Thus, there has been an intensive search for specific *M. tuberculosis* antigens that are not cross-reactive with BCG (Mori 2004, Agaard 2004).

By screening eluted fractions of antigens from *M. tuberculosis* and *M. bovis* culture filtrates for recognition by T cells from infected humans and cattle, respectively, Andersen and co-workers identified several low-molecular mass antigens that are major targets of cellular mediated immune responses (Sorensen 1995). Subtractive DNA hybridization of pathogenic *M. bovis* and BCG (Mahairas 1996) and comparative genome-wide DNA microarray analysis of *M. tuberculosis* H37Rv and BCG (Behr 1999) identified several regions of difference, designated RD1 to RD16, between *M. tuberculosis* and *M. bovis* (see Chapters 2 and 8). All represent segments that have been deleted from the *M. bovis* genome. RD1 was lost early during the process of *M. bovis* BCG attenuation and is therefore missing in all the

daughter strains known today (Mahairas 1996). This region has been the subject of detailed studies and a number of antigens have recently been characterized as candidate antigens for diagnostic and vaccine development (van Pinxteren 2000, Andersen 2000, Brusasca 2001). Antigens, such as early secreted antigen target (ESAT–6) and culture filtrate protein (CFP–10), are located in this region and have already shown great potential for TB diagnosis (Ulrichs 1998, Ravn 1999, Arend 2000, Brock 2001).

13.5.1. Tuberculin skin test

TST has been used to identify patients actively infected with TB, to measure the prevalence of infection in a community, and to select susceptible or high-risk patients for BCG vaccination. The test has been in existence for more than 100 years and has remained more or less unchanged for the last 60 years (Huebner 1993, Curley 2003).

TST works by intradermally injecting 0.1 mL of 5 TU PPD on the forearm. On examination, after 48-72 hours, a positive reaction is indicated by erythema and induration of > 10 mm in size. Erythema (redness) alone is not taken as a positive reaction. All persons with prior infection with tubercle bacilli will mount an immune response to bacilli proteins (Curley 2003).

As the active ingredient used in the skin test contains a whole series of proteins that are shared with the BCG vaccine and other mycobacteria common in the environment, the skin test is often falsely positive. It is currently estimated that almost one third of people positive to TST do not actually have TB infection. The sensitivity of the skin test is estimated to be around just 70 % in known active TB cases; so the test misses up to 30 % of people who are infected. This sensitivity decreases to as low as 30 % in immunocompromised people.

TST is difficult to administer correctly, as small variations in the way it is performed vary the amount of PPD delivered into the skin and thus, the resulting size of the reaction. Furthermore, the measurement of the reaction is highly subjective; the variations in diagnosis based on different clinicians reading the same bump in different ways is well documented.

A common problem in those people who are regularly screened for TB infection using the skin test (e.g. healthcare workers) is that they start to become immunized to PPD by its repeated administrations. This is called 'boosting' and results in a false positive reaction to the skin test (a detailed description of the PPD test is

available on the internet at the Medline Plus Medical Encyclopedia http://www.nlm.nih.gov/medlineplus/ency/article/003839.htm).

13.5.2. Interferon-gamma determination

One of the most significant developments in the diagnostic armamentarium for TB in the last hundred years seems to be the assays based on IFN-γ determination. The assays stem from the principle that T cells of sensitized individuals produce IFN-γ when they re-encounter the antigens of *M. tuberculosis* (Tufariello 2003). Recent evaluations showed that IFN-γ assays that use *M. tuberculosis* RD1 antigens, such as ESAT6 and CFP10, may have advantages over tuberculin skin testing (Arend 2000, Brock 2001, Lalvani 2001).

IFN-γ assays that are now commercially available are: the enzyme-linked immunospot (ELISPOT) T SPOT-TB assay (Oxford Immunotec, Oxford, United Kingdom), the original QuantiFERON-TB, and its enhanced version QuantiFERON-TB Gold assay (Cellestis International, Carnegie, Australia).

13.5.2.1. Enzyme-linked immunospot for interferon-gamma

The ELISPOT assay for diagnosis of *M. tuberculosis* infection is based on the rapid detection of T cells specific for *M. tuberculosis* antigens. IFN-γ released *ex vivo* from these cells can be detected by the extremely sensitive ELISPOT (Lalvani 1998). Each such T cell gives rise to a dark spot and the readout is the number of spots. The T cells enumerated by the ELISPOT assay are effector cells that have recently encountered antigen in vivo and can rapidly release IFN-γ when re-exposed to the antigen (Kaech 2002). In contrast, the long-life memory T cells, which persist long after clearance of the pathogen, are relatively quiescent and less likely to release IFN-γ during the short period of exposure to antigen in the *ex vivo* ELISPOT assay (Lalvani 1998).

Lalvani *et al*. developed the first generation of new ELISPOT tests for latent TB by using the ESAT-6 peptide to stimulate single blood samples. This test detects as few as one in 60,000 IFN-γ producing cells. In a preliminary trial, this test was positive in 96 % of 47 TB patients and in 85 % of 26 persons presumed to have latent TB. The ELISPOT test was negative in 26 BCG-vaccinated control subjects, and this specificity implies a major advantage over TST (Lalvani 2001). The assay has been evaluated by different groups (Lalvani 2001a) and the results have shown that ELISPOT offers a more accurate approach than TST for the identification of individuals who have latent TB infection. These tests could improve TB control by more precise targeting of the preventive treatment.

A commercial ELISPOT test, T SPOT-TB® (Oxford Immunotec, Oxford, United Kingdom) is now available. Related information can be found on the internet at http://www.finddiagnostics.org/news/presentations/ltbi_mar_2006/lalvani.pdf.

13.5.2.2. Quantiferon-TB test

QuantiFERON-TB® and Bovigam® are two registered products which measure the release of interferon-gamma in whole blood from human subjects and cattle infected with *M. tuberculosis* and *M. bovis* respectively, in response to stimulation by PPD. The IFN-γ secreted by T-cells into the plasma is measured by ELISA to indicate the likelihood of TB infection. Different studies demonstrated that the QuantiFERON-TB test was comparable to TST in its ability to detect latent TB infection. These studies also showed that the QuantiFERON-TB test was less affected by BCG vaccination, discriminated responses due to non-tuberculous mycobacteria, and also avoided the variability and subjectivity associated with administering and reading the skin test (Pottumarthy 1999).

QuantiFERON-TB® was approved by the Food and Drug Administration (FDA) of the United States (US) in 2001. In 2003, the US Centers for Disease Control and Prevention released guidelines for using the QuantiFERON®-TB Test in the diagnosis of latent *M. tuberculosis* infection, which can be found on the internet at http://www.cdc.gov/mmwr/preview/mmwrhtml/rr5202a2.htm. The manufacturer's Quick Reference Guide to the procedure can be found on the internet at http://www.cellestis.com/IRM/Company/ShowPage.aspx?CPID=1161.

More recently, an evaluation of the whole blood IFN-γ test for TB diagnosis, based on the specific antigens ESAT-6 and CFP-10, showed that the recombinant antigens could increase the specificity of the whole blood test and enhance the discriminative power of the test between TB infection, atypical mycobacterial reactivity, and reactivity due to BCG vaccination (van Pinxteren 2000).

New, potential T-cell antigens and mixtures of antigens are being evaluated in both human and bovine TB, thus, IFN-γ assays based on these antigens appear to be promising (Aagaard 2004, Aagaard 2006, Leyten 2006). A new product, QuantiFERON®-TB Gold, which includes the TB-specific antigens ESAT-6 and CFP-10 that are only present in *M. tuberculosis* and are absent from all strains of *M. bovis* (BCG) and most environmental mycobacteria, has been introduced. QuantiFERON®-TB and QuantiFERON-TB Gold assays are manufactured by Cellestis International (Carnegie, Australia, http://www.cellestis.com/). The antigens used in the Gold version are provided by the Statens Serum Institute in Denmark (http://www.ssi.dk/sw162.asp).

13.6. Conclusions and Perspectives

Despite a large number of studies published over the past several years, serology has found little place in the routine diagnosis of TB, even though it is rapid and does not require specimens from the site of disease. Sensitivity and specificity depend on the antigen used, the gold standard used for the diagnosis of TB, and the type of TB disease. Though most of these tests have high specificity, their sensitivity is poor. In addition, these tests may be influenced by factors such as age, prior BCG vaccination and exposure to non-tuberculous mycobacteria strains.

In contrast, while the initial results of IFN-γ determination for the detection of latent infected individuals appear promising, it remains to be seen whether this will translate into practically useful results in the field (Sharma 2006). Indeed, IFN-γ assays are expensive tests and their higher cost appears to limit their wider applicability, especially in resource-limited settings and developing countries, where TB is highly rampant. The ELISPOT test is not yet suitable for widespread use, because it is costly and requires isolation of mononuclear cells, a procedure that is not performed in clinical laboratories.

Because most mycobacterial epitopes are recognized in the context of specific HLA antigens, the IFN-γ based assays should be evaluated at multiple geographic locations, among patients of different ethnicities. Although BCG vaccination does not yield false-positive results in IFN-γ assays using selected antigens, the specificity of the test should be studied in persons exposed to environmental mycobacteria, such as members of the *M. avium* complex. Studies with larger numbers of TB patients are needed to address this issue. The diagnosis of latent TB represents a major advance in the quest for better tests. The explosion of microbial genomics, proteomics, and transcriptomics will yield more *M. tuberculosis* specific genes and antigens; and IFN-γ assays, using peptides from multiple antigens, should be more sensitive than the ones using only ESAT-6 or/and CFP10.

Until we find a reliable diagnostic test for detecting active disease, "TB or not TB?" shall remain a question.

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Chapter 14: New Diagnostic Methods

Enrico Tortoli and Juan Carlos Palomino

14.1. Introduction

The diagnosis of mycobacterial infections remained practically unchanged for many decades and probably would have not progressed at all without the unexpected resurgence of tuberculosis (TB) which characterized the last twenty years of the 20th century.

With microscopy lacking wide margins for improvement, the areas which most benefited from the renewed interest in TB were culture and identification, while a completely new approach emerged, aimed towards the direct detection of mycobacterial nucleic acids in clinical specimens.

14.2. Automated culture methods

Although known for decades, liquid media for cultivation of mycobacteria had never attracted the attention of mycobacteriologists. In fact, the ability of a liquid medium to support a faster growth was heavily hampered by its susceptibility to contamination. The use of antimicrobial combinations suitable of inhibiting the growth of the whole spectrum of potential contaminants (Gram-positive and Gram-negative bacteria as well as fungi) represented a turning point.

During the same period, automation was taking its first steps in microbiology, with blood cultures leading the field. The apparently banal idea of exporting such technology to mycobacterial cultures evolved into selective liquid media, which were a[0] breakthrough for diagnostic mycobacteriology.

14.2.1. BACTEC TB-460

The BACTEC TB-460 system (Becton Dickinson, Sparks, MD) was the first, and for many years the only, automated approach in mycobacteriology. It makes use of a radiometric instrumentation developed for blood cultures with the broth bottles replaced by vials containing a medium specific for mycobacteria.

The principle

The medium: A modified Middlebrook 7H9 medium is used, in which one of the components, palmitic acid, is radiolabeled with ^{14}C. Contamination is controlled by the addition, prior to use, of a mixture of polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin (PANTA) reconstituted with a poly-oxyethylene solution. The use of such a combination of antibiotics does not eliminate the decontamination step, which needs to be performed before inoculation of the samples. The vials containing the medium remain sealed through the whole culture process and the specimen is inoculated by puncturing the rubber septum with a needle (Figure 14-1).

Figure 14-1: Inoculation of a BACTEC vial

The instrumentation: Once the paired needles have perforated the rubber septum of the vial, the gaseous phase is aspirated and replaced with air containing 5 % CO_2. The aspirated gas is analyzed by a β-counter to quantify the eventual presence of radiolabeled CO_2 (Figure 14-2).

The rationale: When viable mycobacteria are present in the culture vial, the radiolabeled palmitic acid is metabolized and radioactive CO_2 is liberated into the gaseous phase.

The performance

The BACTEC TB-460 was first commercialized in 1980 and soon became popular worldwide. It is not a fully automated system, as the vials, which are held in an

external incubator, must be loaded into the instrument for reading. The reading is usually performed twice a week during the first 15 days of incubation, and weekly thereafter, until the 42nd day. BACTEC TB-460 is still used in many laboratories worldwide but its glorious course is starting to wane. The increasing cost of radioactive waste disposal and the interest of the manufacturer to promote newly-developed alternative systems are slowly prevailing over its still excellent performance.

Figure 14-2: The BACTEC TB-460 instrument

From the first evaluations, BACTEC TB-460 revealed a clear superiority over solid media systems in terms of sensitivity and time to detection of positive culture (Middlebrook 1977, Damato 1983, Morgan 1983, Takahashi 1983, Park 1984, Siddiqi 1984). Many years later, despite the release of new systems, the BACTEC radiometric method is still competitive (Alcaide 2000, Brunello 1999, Laverdiere 2000, Piersimoni 2001, Badak 1996, Ganeswrie 2004, Scarparo 2002, Tortoli 1998, Tortoli 1999). The BACTEC TB-460 system is also suitable for mycobacterial blood culture using designated bottles.

14.2.2. BACTEC MGIT960

The BACTEC MGIT960 system (Becton Dickinson, Sparks MD) uses the technology of the previously developed blood culture instrument. The original system (BACTEC 9000) was first adjusted to support mycobacterial cultures but was sub-

sequently completely redesigned to process tubes, which are much less cumbersome to handle than the original bottles.

The principle

The medium: Mycobacteria Growth Indicator Tube (MGIT) is a modified Middlebrook 7H9 medium in which a supplement is added at the moment of use. The supplement is a mixture of oleic acid, albumin, dextrose, and catalase (OADC) enrichment and the same PANTA antibiotic mixture used in the radiometric system. The presence of PANTA does not do away with the decontamination step, which needs to be done before inoculation. As the tubes containing the medium are screw-capped, no needle is needed for inoculation. A silicon film embedded with a ruthenium salt is present at the bottom of the tube as a fluorescence indicator (Figure 14-3).

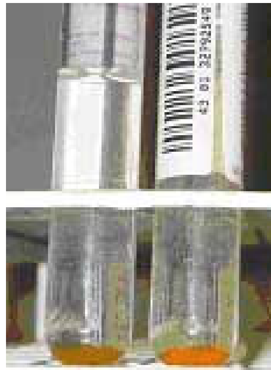


Figure 14-3: MGIT tube

The instrumentation: Incubator and reader are combined in a single cabinet (Figure 14-4). The bottom of each tube, stimulated by ultraviolet light, is monitored by a fluorescence reader. Fluorescence-emitting tubes are reported as positive. It is also possible to use the MGIT tubes without instrumentation, by holding the tubes in a normal incubator and observing the fluorescence under a Wood's lamp.

The rationale: The oxygen normally present in the medium quenches the natural fluorescence of the ruthenium salt. If viable mycobacteria are present in the tube, oxygen is consumed due to their metabolism, the quenching effect lowers accordingly, and the bottom of the tube fluoresces when exposed to ultraviolet light.

Figure 14-4: The MGIT960 instrument

The performance

The BACTEC MGIT960 is a typical walk-away instrumentation which monitors the tubes at one-hour intervals, alerts when they become positive and signals the end of the incubation period.

Many studies have evaluated the BACTEC MGIT960 in comparison with similar competitor systems and also with solid media. BACTEC MGIT960 turned out to be clearly faster and more sensitive than solid media, while the comparison with other automated and semi-automated systems revealed substantially overlapping performance (Alcaide 2000, Badak 1996, Scarparo 2002, Tortoli 1999, Casal 1997, Pfyffer 1997, Tortoli 1997). Mycobacterial blood cultures cannot be performed with this system.

14.2.3. VersaTREK

The VersaTREK (previously known as the ESP system II) uses the technology of a previously developed blood culture system and is commercialized by Trek Diagnostic Systems.

The principle

The medium: It uses a modified Middlebrook 7H9 medium to which the OADC enrichment must be added. Two different antimicrobial mixtures are available. The first one, also known as AS, includes polymyxin B, azlocillin, fosfomycin, nalidixic acid, and amphotericin B. The second contains polymyxin B, vancomycin, nalidixic acid, and amphotericin B (PVNA). Usually, AS is used for specimens originating from sterile samples or with a low risk of contamination, while PVNA is used for heavily-contaminated samples. The presence of such antimicrobial mixtures for contamination control does not eliminate the decontamination step, which needs to be performed before inoculating the sample. The bottles of medium (Figure 14-5) hold a cellulose sponge whose large surface area allegedly improves growth. Bottles are inoculated through a rubber septum by means of a syringe.

Figure 14-5: VersaTREK bottle (*Courtesy of TREK Diagnostic Systems*)

The instrumentation: Incubator and reader are combined in a single cabinet (Figure 14-6), which also shakes the bottles during the incubation. The pressure within each bottle is monitored by a manometer through a proper connector. Cultures presenting a decreased headspace pressure are reported as positive.

The rationale: If viable mycobacteria are present in the bottle, the oxygen consumption due to their metabolism reduces the internal pressure.

The performance

VersaTREK is a typical walk-away instrumentation which continuously monitors the bottles, alerts when they become positive and signals the end of the incubation period.

Several studies have evaluated VersaTREK in comparison with solid media and similar automated and semi-automated competitor systems. VersaTREK clearly performs better than solid media but shows no substantial advantage over other systems (Tortoli 1998, Williams-Bouyer 2000, Woods 1997).

Figure 14-6: The VersaTREK instrument (*Courtesy of TREK Diagnostic Systems*)

Mycobacterial blood cultures can also be performed with the VersaTREK system. However, whole blood cannot be used and a previous treatment is required to obtain sediment for inoculation. Either a buffy-coat or sediment obtained with the lysis-centrifugation method is suitable to inoculate the bottles. The lysis-centrifugation method (Isolator, Oxoid, United Kingdom) consists of saponin-containing tubes to lyse blood cells, a proper centrifugation procedure, and special pipettes for elimination of supernatant and collection of the sediment.

14.2.4. BacT/Alert 3D

BacT/Alert 3D (previously known as MB/BacT) is commercialized by bioMérieux and uses the technology of a previously developed blood culture system.

The principle

The medium: A modified Middlebrook 7H9 medium is used in which a supplement, a mixture of OADC enrichment and polymyxin B, amphotericin B, nalidixic acid, trimethoprim, vancomycin and azlocillin, is added at the moment of use. The presence of such contamination-controlling antibiotics does not eliminate the decontamination step needed before inoculation. The bottles of medium have a CO_2 sensor at the bottom and are inoculated through a rubber septum by means of a syringe.

The instrumentation: Incubator and reader are combined in a single machine (Figure 14-7) which does not shake the bottles during incubation.

Figure 14-7: The BacT/Alert system

The CO_2 sensor is impacted by a light whose reflected ray is monitored by a photodiode (Figure 14-8). Bottles producing specific changes in the intensity of the reflected light are reported as positive.

The rationale: If viable mycobacteria are present in the bottle, the CO_2 produced by their metabolism causes a change in the color of the sensor, from green to yellow, which alters the intensity of the reflected light ray (Figure 14-9).

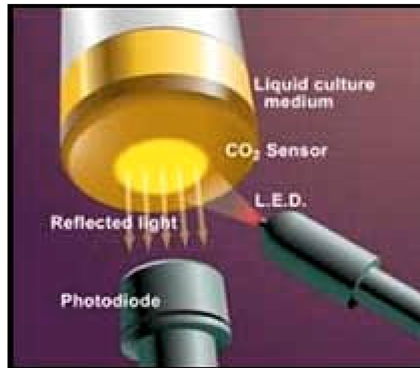


Figure 14-8: The BacT/Alert technology

Figure 14-9: Negative and positive bottles

The performance

BacT/ALERT 3D is a typical walk-away instrumentation which monitors the bottles at 10-min intervals, alerts when they become positive, and signals the end of the incubation period.

Many studies have evaluated the BacT/ALERT 3D. The system turned out to be clearly faster and more sensitive than conventional media, while the comparison with other automated and semi-automated systems did not reveal significant differences (Alcaide 2000, Brunello 1999, Laverdiere 2000, Nogales 1999, Piersimoni 2001, Roggenkamp 2000, Rohner 1997, Saito 2000, Yan 2000).

The system is also suitable for mycobacterial blood cultures, provided proper bottles are used; no previous treatment of the blood is required.

14.3. Nucleic acid amplification methods

When the polymerase chain reaction (PCR) methodology took its first steps into diagnostic microbiology, a restricted number of micro-organisms appeared to have the potential to benefit from the novel technique. *M. tuberculosis* was among them, and the dream of the rapid diagnosis of TB appeared to be about to come true.

14.3.1. *In house* methods for diagnosis of tuberculosis

One of the first findings on the way to developing a PCR technique (Figure 14-10) aimed at *M. tuberculosis* detection was that, although different targets were investigated, none of them were suitable for differentiating *M. tuberculosis* from other species belonging to the *M. tuberculosis* complex. Such limitation, due to the extremely high genome similarity (close to 100 %) among the members of the *M. tuberculosis* complex, did not, however, cool the enthusiasm. In fact, the differentiation of such species is of very limited relevance from the clinical and therapeutic point of view.

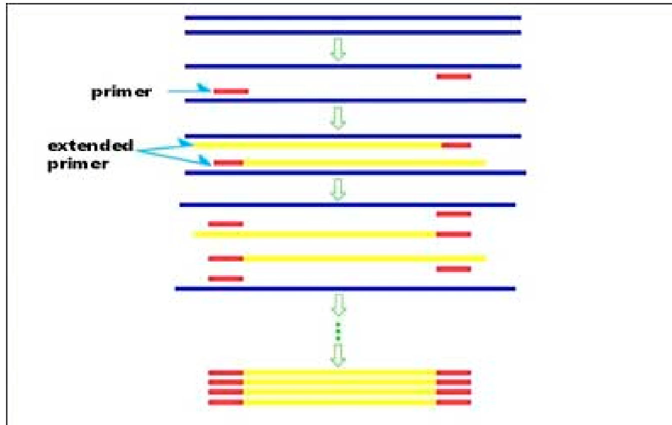


Figure 14-10: PCR

Among the first proposed genomic targets for diagnostic PCR was the newly detected insertion element IS*6110* which, being present in multiple copies (from four to 20 in more than 95 % of *M. tuberculosis* strains), appeared to have the potential for an enhanced sensitivity (Figure 14-11). Other successfully used desoxyribonucleic acid (DNA) regions include the 65 kiloDalton (kDa) heat-shock protein gene, the gene encoding the 126 kDa fusion protein, and the gene encoding the β-subunit of ribonucleic acid (RNA) polymerase; all of them are present in single copies in *M. tuberculosis* complex genomes.

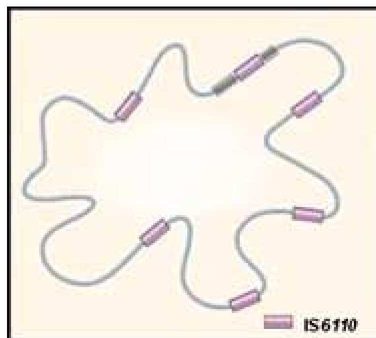


Figure 14-11: *M. tuberculosis* genome with six copies of IS*6110*

In the ’90s, many laboratories developed various *in house* PCR protocols and soon the aspiration of an increased sensitivity led to the adoption of “nested” PCR. In this procedure, the amplification of a large region of DNA is followed by a second amplification targeted to a shorter internal stretch. Most of such *in house* PCR procedures achieved a sensitivity never matched by commercial systems but were often burdened by the high incidence of false positive results due to amplicon cross-contamination of specimens.

In subsequent years, with the purpose of avoiding the PCR patent, alternative amplification methods were developed. Most successful were the reverse transcriptase PCR, the ligase chain reaction, and the strand displacement amplification.

14.3.2. Commercial methods

In the last few years, several amplification methods have been commercialized; only four methods have gained worldwide diffusion and been widely validated by international studies, although one of them (LCx, Abbott) is no longer on the market.

Amplified MTD

Amplified *Mycobacterium tuberculosis* Direct Test (AMTD), developed by Gen-Probe (San Diego, CA, USA), is an isothermal (42°C) transcriptase-mediated amplification system.

The principle

A *M. tuberculosis* complex-specific region of the 16S ribosomal RNA gene produces double-stranded ribosomal DNA, due to the combined action of reverse-transcriptase and ribonuclease. In turn, RNA polymerase catalyzes the synthesis of multiple stretches of ribosomal RNA from the ribosomal DNA synthesized before. A new cycle starts when the newly produced ribosomal RNA undergoes further transcription by reverse transcriptase (Figure 14-12).

The sensitivity of the method is increased by the presence, in each bacterium, of a high number of 16S ribosomal RNA target molecules (about 2,000) compared to only one copy of 16S ribosomal DNA. Another advantage of the amplification from RNA relies on the low stability of such a molecule; this minimizes both the risk of contamination and the incidence of false-positive results due to the persistency of stable nucleic acids (DNA) in the host organism, even after the complete eradication of the infection.

The detection of amplification products relies on hybridization with a specific, single-strand DNA probe labeled with a chemiluminescent molecule (Hybridization Protection Assay).

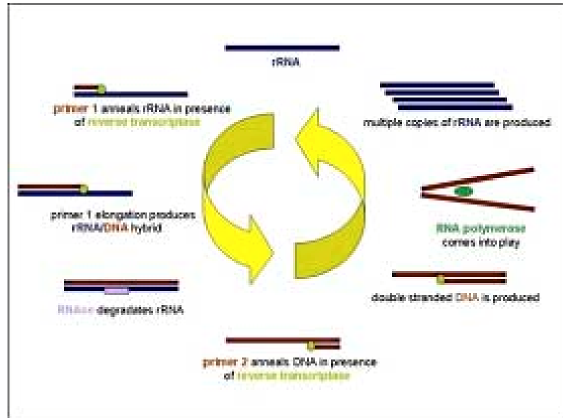


Figure 14-12: The cycle of the transcriptase-mediated amplification

The features

The whole process is performed manually, starting with the extraction by means of sonication, continuing with the addition of different reagents until the final reading with the luminometer (Figure 14-13). Thermal-cyclers are not needed and the whole amplification step is carried out on a heating block at 42°C. The turnaround time is 2.5 hours. No internal control is provided in the kit to monitor the presence of inhibitors.

The method is approved by the Food and Drug Administration of the United States of America (US FDA) for testing smear-positive and smear-negative respiratory samples.

Figure 14-13: The luminometer

The performance

From a review of the huge amount of literature available, sensitivity ranging from 91.7 % to 100 % in smear-positive samples and from 65.5 % to 92.9 % in acid fast bacilli (AFB) smear-negative samples has been reported (Alcala 2001, Chedore 1999, Chedore 2002, Gamboa 1998, O'Sullivan 2002, Piersimoni 2003, Woods 2001). To reduce the prevalence of false-positive results, an equivocal zone in the interpretation of results has been recently introduced with the recommendation of retesting samples scoring within this range (Kerleguer 2003, Middleton 2002).

Amplicor MTB Test

The Amplicor MTB Test (Roche Molecular Systems, Basel, Switzerland) relies on standard PCR.

The principle

A 584 bp fragment of the 16S ribosomal RNA gene, comprising a species-specific region flanked by genus-specific sequences, is amplified using biotinylated primers. In the master mix, an unusual combination of nucleotides is present – as an adjunct to adenine, guanidine and cytosine, uracil is used in place of thymine. As a consequence, the amplification product differs from the target DNA in that it contains uracil instead of thymine. This device is part of a contamination-control system based on the use of uracil-N-glycosylase, an enzyme that fragments DNA wherever uracil is present. The enzyme, added to the samples before amplification,

destroys any amplicon resulting from previous amplifications without damaging the uracil-free target DNA. Because of the genus-specific nature of the annealing regions, 16S ribosomal DNA belonging to any mycobacterial species is amplified by this PCR. The use, in the revealing phase, of magnetic beads coated with *M. tuberculosis* complex-specific probes allows the removal, by washing, of any other DNA. The detection of the specific amplification product is performed by adding an avidin-enzyme conjugate and a chromogenic substrate.

The features

The amplification and detection steps are carried out automatically by the Cobas Amplicor instrument (Figure 14-14). Once the sample extraction has been performed by heating (95°C), the tube is placed in the thermal cycler integrated in the Cobas instrument. Without further handling, the amplification product will be automatically transferred into the detection station where the chromogenic reaction is developed and read. The turnaround time is 6-7 hours. The method is approved by the US FDA for testing smear-positive respiratory samples. It includes an internal control, composed of synthetic DNA characterized by identical annealing sequences as the mycobacterial target; when this is not amplified, it signals the presence of inhibitors. The detection of *M. tuberculosis* complex DNA can also be carried out without the Cobas instrument, using a manual kit that, however, does not include an internal control.

Other Amplicor kits are available for detection of *Mycobacterium avium* and *Mycobacterium intracellulare* DNA in clinical samples.

Figure 14-14: The Cobas Amplicor instrumentation

The performance

From the literature review, specificity is close to 100 % while sensitivity ranges from 90 % to 100 % in smear-positive samples and from 50 % to 95.9 % in smear-negative ones (Bogard 2001, Eing 1998, Gamboa 1997, Gomez-Pastrana 2000, Mitarai 2000, Piersimoni 2003, Rajalahti 1998, Reischl 1998, Shah 1998).

BD ProbeTec ET

The BD ProbeTec ET (Becton Dickinson, Sparks, MD) uses DNA polymerase and isothermal strand displacement amplification to produce multiple copies of IS*6110*, an insertion element unique to *M. tuberculosis* complex.

The principle

The rationale of strand displacement amplification is extremely complex; what is presented here is an extreme simplification. In the initial phase (target amplification), amplification is started by two pairs of primers complementary to contiguous sequences delimiting the target. The elongation of the upstream primer, also named bumper, determines the displacement of the simultaneously elongating downstream primer and finally releases the produced amplicon. A restriction site, present in the downstream primer, will also be present in the released amplicon (Figure 14-15A). In the exponential amplification phase, a new primer anneals to the amplicon and, following digestion by the restriction enzyme, the upstream fragment acts as bumper and displaces the downstream fragment (Figure 14-15B).

Real-time detection is based on the energy transfer technology. A hair-pin-shaped probe, complementary to IS*6110*, is marked by two fluorescent molecules, one of which, the donor, is quenched by the other, the acceptor; furthermore, it presents a restriction site in the sequence between the two markers (Figure 14-16A). Once its free end has hybridized with the amplification product, the probe undergoes elongation (Figure 14-16BC) before being displaced by a primer annealed upstream to the same amplicon (Figure 14-16D). The elongation makes the probe able to bind a new primer (Figure 14-16E) which, while elongating, stretches out the “hair-pin” and moves the acceptor away from the donor (Figure 14-16FGH). The nicking of the restriction site by a proper enzyme further separates donor and acceptor and allows the first to free a fluorescence signal (Figure 14-16I).

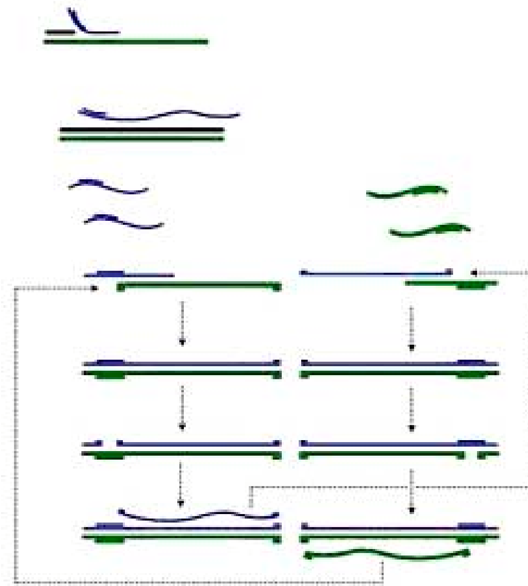


Figure 14-15: ProbeTec ET amplification cycles

The features

Some manipulation is required before introduction of the sample into the automatic instrument (Figure 14-17); each sample is first inactivated at 105°C, and then sonicated to extract the DNA, transferred into a priming well at 72.5°C, and subsequently into an amplification well at 54°C. In the BD ProbeTec ET instrument, the microplate containing the samples and the amplification reagents is incubated at 52.5°C and the fluorescence emitted is continuously monitored. A thermal cycler is not required. The turnaround time is 3.5 to 4 hours.

An internal control is present, characterized by the same annealing sequences as the mycobacterial target. In case of amplification failure, this control alerts for the presence of inhibitors.

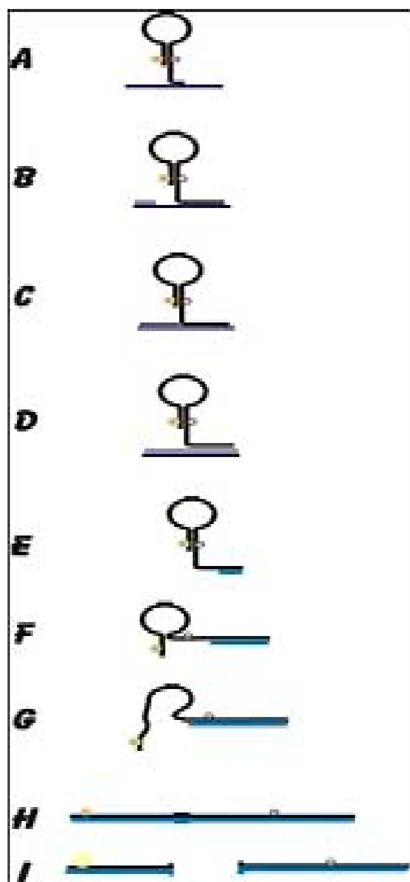


Figure 14-16: The detection step of ProbeTec ET

The system is not yet approved by the US FDA.

Kits are also available for the amplification of nucleic acids of *M. avium*, *M. intracellulare* and *Mycobacterium kansasii*.

The performance

The literature reports a rate of sensitivity ranging from 98.5 % to 100 % for smear-positive samples and very variable (0.33 %-100 %) for smear-negative ones (Barrett 2002, Bergmann 1998, Bergmann 2000, Johansen 2002, Maugein 2002, Mazzarelli 2003, Pfyffer 1998, Piersimoni 2002).

Figure 14-17: The BD ProbeTec ET instrument

14.3.3. Comments on amplification methods

Although direct amplification methods are used worldwide, they are far from having revolutionized clinical mycobacteriology. Culture, supported by microscopy, still remains the gold standard, and molecular methods only represent a useful support in some cases, to speed up the diagnosis of TB.

The unsatisfactory sensitivity is the major limitation of amplification-based methods. It is now evident that paucibacillary specimens have little chance of being detected by molecular amplification. Factors that contribute to the reduction of the sensitivity are the uneven distribution of bacilli in the sample, the suboptimal extraction of nucleic acids, and sometimes the presence of inhibitors. The phenol-chloroform extraction unquestionably provides the best yield but, being cumbersome and time consuming, also raises the risk of contamination. To minimize this risk and to make the technique user friendly, the commercial systems have probably oversimplified this step by reducing it to sonication or heat treatment only. The sediment of a number of samples contains substances inhibiting the amplification process. The reason for their presence is unknown and at present there is no known method for neutralizing them. The use of an internal control represents a major feature to be taken into account at the moment of choosing an amplification method.

Although the specificity of amplification methods is substantially good, the possibility of false-positive results should be borne in mind by both microbiologists and clinicians. A therapy like the one against TB, which is long and not exempt from side-effects, should not be undertaken only on the basis of a positive result of a single amplification test. The major reason for false-positive nucleic acid amplification results is the contamination of samples, possibly in the pre-analytic, but mostly in the analytic phase. The application of dedicated procedures, such as the one employing uracil-N-glycosylase or the adoption of sealed amplification chambers, is useful. More important still are general precautions such as the frequent decontamination of the work environment with 10 % bleach and the exposure of pipettes, tips and bench surfaces to ultraviolet light when not in use. Finally, a major role is played by the training and the expertise of the operator.

A particular category of false-positive results is that concerning samples obtained from patients under treatment. In these patients, the detectability of mycobacterial DNA over a long period of time, despite the effectiveness of the treatment, is well known and clearly makes DNA amplification useless for treatment monitoring.

The above limitations and recommendations have also been stressed by the US Centers for Disease Control and Prevention (CDC, detailed information on the internet at http://www.cdc.gov/mmwr/preview/mmwrhtml/mm4926a3.htm) (Figure 14-18). The observance of such recommendations is extremely important. However, the CDC's advice against the use of commercial PCR methods with non-respiratory samples may appear outdated nowadays. In recent years, a number of articles have been published showing that the amplification methods can be of use for extrapulmonary specimens too, although impaired by lower sensitivity. Still, their limitations should be kept in mind and a system with internal control should be used due to the high frequency of inhibitors (Alcala 2001, Chedore 1999, Chedore 2002, Eing 1998, Gamboa 1998, Johansen 2002, Maugein 2002, Mazzarelli 2003, O'Sullivan 2002, Reischl 1998, Rimek 2002, Woods 2001). An obvious, but often disregarded, point is that the lower sensitivity is not, in the large majority of cases, due to the extrapulmonary origin but to the lower bacterial load inherent to such samples.

Among the impressive amount of publications assessing different amplification methods and the few studies concerning direct comparisons (Della-Latta 1998, Piersimoni 2002, Scarparo 2000), none convincingly demonstrates the superiority of one over the others. All are characterized by equally good specificity and insufficient sensitivity. The substantially similar performance of all systems makes it difficult to understand the different rating given by the FDA to the systems available on the market.

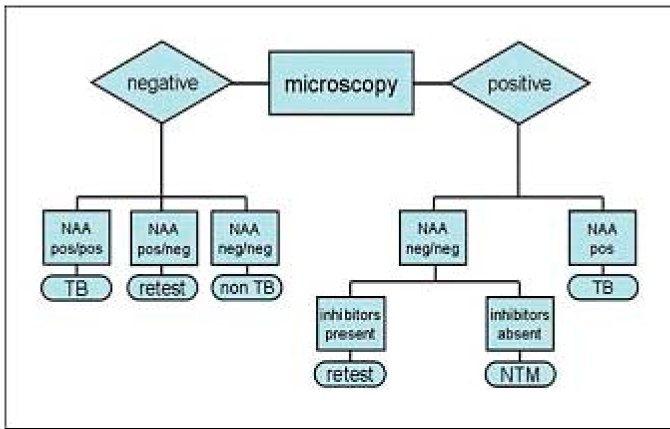


Figure 14-18: CDC recommendations for interpretation of nucleic acid amplification tests

14.3.4. Future prospects

Real-time PCR now seems to be on the point of being adapted for the diagnosis of TB. The evident delay at this step, in contrast with its well established use in other fields of diagnostic microbiology, suggests the emergence of some problems. Major expectations are concerned with the increase in sensitivity, while the availability of quantitative results may represent the first step towards its use for treatment monitoring.

Nevertheless, despite its evident usefulness and potential improvements, it seems unlikely that nucleic acid amplification can replace culture for diagnosis of TB in the short term. Culturing is still essential for monitoring the response to therapy and testing antimicrobial susceptibility.

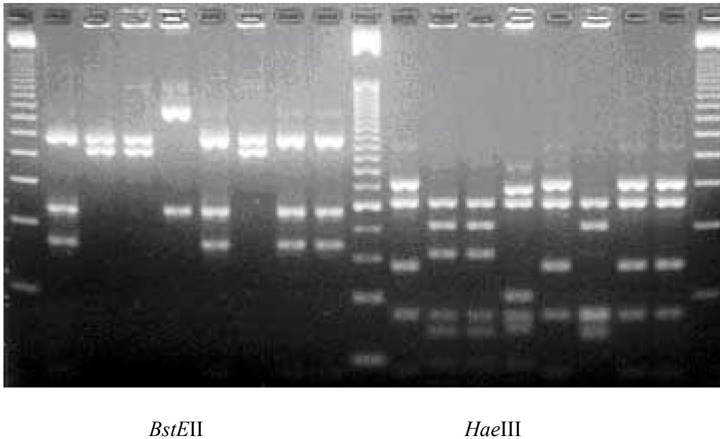
14.4. Genetic identification methods

Following the extraordinary development of molecular methods, the identification of mycobacteria, previously based on phenotypic investigations, suddenly started to rely on genotypic methods. Different genetic approaches developed in research laboratories became rapidly popular in diagnostic laboratories and some of them were transformed into commercial diagnostic kits.

14.4.1. PCR restriction-enzyme analysis

The principle

The PCR restriction-enzyme analysis (PRA) method is based on the amplification of a 441-bp fragment of the *hsp65* gene by PCR, followed by the digestion of the amplified product with two restriction enzymes *BstE*II and *Hae*III according to the procedure first described by Telenti (Telenti 1993). The products of the digestion reaction are then separated and visualized by agarose gel electrophoresis (Figure 14-19). The restriction pattern thus obtained is compared to an algorithm present in the PRASITE available on the internet at http://app.chuv.ch/prasite/index.html. This database comprises 74 PRA patterns corresponding to 38 defined species of mycobacteria. In recent years, several novel PRA patterns from newly characterized species have been described. The PRA method has also been used with other genomic regions for the identification of mycobacteria such as the *rpoB* and *gyrB* genes with good results (Lee 2000, Goh 2006).



*BstE*II *Hae*III

Figure 14-19: PRA patterns of different species of mycobacteria (*Courtesy S. Leão*)

The features

The PRA method can be applied on heat-inactivated and washed bacterial suspensions obtained from mycobacteria grown either on solid or in liquid medium. There is only one report on the direct application of PRA to clinical samples (Magalhaes

2002). Being an *in house* method, and when properly standardized, it is a convenient alternative to more costly commercial identification methods.

The performance

There are many studies on the application of the PRA-*hsp65* method for the rapid identification of mycobacteria. In general, the method has proved to be practical, cost-effective, and highly accurate for mycobacterial identification. In a recent multicenter evaluation, performed in eight laboratories in Latin America that received a set of coded strains for identification, the PRA-*hsp65* method proved to be highly accurate and easy to perform. The accuracy of the identification can be further improved when combined with minimal microbiological characteristics such as growth rate and pigmentation. Nevertheless, attention should be paid to a few technical details such as gel preparation and running, and some training is needed in the interpretation of patterns (Leão 2005).

14.4.2. DNA probes

The DNA-probe technology for identification of fastidious organisms is still one of the most successful molecular diagnostic procedures worldwide. In this sense, the role played by commercial DNA probes in the quality improvement of mycobacterial identification in clinical laboratories cannot be disregarded.

AccuProbe

The precursor system, AccuProbe (Gen-Probe, San Diego, CA), was developed almost 20 years ago. It is still very popular, mainly due to its extremely simple procedure. Indeed, it is the only DNA-probe system not requiring previous amplification of the target.

The principle

The probe is a single-stranded DNA oligonucleotide, complementary to a short, species-specific sequence within a hypervariable region of the 16S ribosomal DNA. It is labeled with an acridinium ester, a chemiluminescent molecule, which gives light when properly excited. Once the mycobacterial cell has been lysed by sonication, the extract is mixed with the probe under stringent conditions, allowing their hybridization only in case they are 100 % complementary.

As the chemiluminescent marker, easily accessible in the native probe, turns out to be protected in the double-stranded hybrid (Hybridization Protection Assay), the

addition of a hydrolyzing agent makes the first undetectable without affecting the second. Any hybridization is accompanied by light emission, which is detected with a luminometer, thus simplifying the identification of the test strain.

The features

Different AccuProbe kits are available for identifying mycobacteria belonging to the *M. tuberculosis* complex, the *M. avium* complex and the species *M. kansasii*, *Mycobacterium gordonae*, *M. avium* and *M. intracellulare.* They can be used on cultures grown either on solid or in liquid medium. The only equipment required is a sonicator for cell lysis and a luminometer (Figure 14-13) for the final reading.

The performance

The sensitivity and specificity of AccuProbe are widely acknowledged in the literature (Bull 1992, Drake 1987, Enns 1987, Gonzales 1987, Kiehn 1987, Lebrun 1992, Musial 1988, Saito 1989, Tortoli 1994, Tortoli 1996). Only a few unspecific reactions have been reported, mainly for the *M. avium* complex probe, which also hybridizes with the recently described species *M. palustre* (Torkko 2002), *M. parascrofulaceum*, and *M. saskatchewanense* (Turenne 2004).

Line probe assays

The line-probe assay uses the reverse hybridization technology with differently-specific DNA-probes immobilized in parallel lines on a paper strip. The target DNA, previously extracted by boiling, is PCR-amplified using biotinylated primers and finally incubated with the strip. Once the hybridization has been carried out under highly stringent conditions and the unbound amplicons have been washed out, the hybridized probe is revealed as a colored band, developed following the addition of a streptavidin-labeled enzyme and a chromogenic substrate (Figure 14-20). The specificity of the hybridized line-probe is inferred by the position of the colored band on the strip. Three commercial methods are available, INNO-LiPA MYCOBACTERIA (Innogenetics, Ghent, Belgium), GenoType Mycobacterium (Hain, Germany), and GenoType MTBC (Hain, Germany).

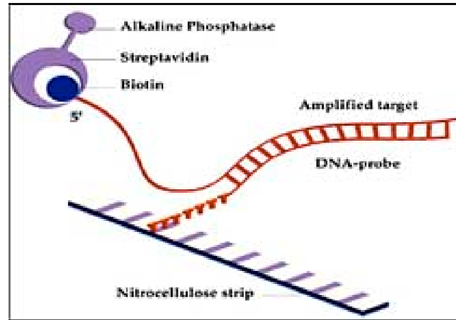


Figure 14-20: Line Probe assay (*Courtesy of Innogenetics N.V.*)

INNO LiPA Mycobacteria

The line probes of INNO LiPA Mycobacteria are species-specific fragments of the internal transcribed spacer (ITS) region interposed between 16S and 23S ribosomal RNA genes. The system includes a genus *Mycobacterium*-specific probe, two complex-specific probes (*M. tuberculosis* complex and *M. avium* complex) and 23 other probes suitable for identifying 18 species and several intra-specific variants (within the *Mycobacterium chelonae-abscessus* group and in species *M. kansasii*) (Figure 14-21). A thermal cycler and a shaking water bath are needed; an automated instrumentation is also available to carry out the hybridization step (Figure 14-22).

Figure 14-21: Inno-LiPA interpretation

Figure 14-22: Auto-LiPA instrument

GenoType Mycobacterium

The line probes of GenoType Mycobacterium are fragments of the 23S ribosomal RNA gene mostly shared by more than one species. In this case, the identification is not based on the specificity of a single line but on the different combinations of multiple bands characterizing each species (Figure 14-23). Different patterns are suitable for ascertaining if the test strain belongs to a group of related genera characterized by high guanosine plus cytosine content, to the genus *Mycobacterium*, to the *M. tuberculosis* complex or to any of 35 mycobacterial species. Furthermore, it distinguishes two intra-specific variants within the species *M. fortuitum*. The system is available as two kits which are sold separately. One of them, GenoType CM, identifies the more frequently detected mycobacterial species with 17 line-probes, while the other, GenoType AS, includes 18 probes aimed at the less common species.

A thermal cycler and a shaking water bath are needed; an automated instrumentation is also available to carry out the hybridization step.

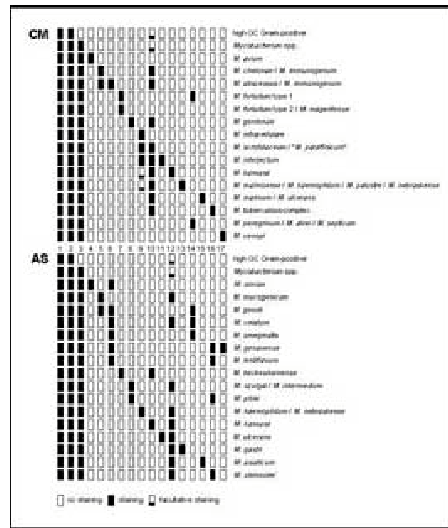


Figure 14-23: GenoType Mycobacterium interpretation chart

GenoType MTBC

The newly developed GenoType MTBC is a reverse hybridization system devoted to the identification of the species belonging to the *M. tuberculosis* complex which cannot be differentiated by the analysis of any of the most frequently investigated conserved regions (16S ribosomal DNA, ITS, 23S ribosomal DNA). In this kit, multiple genetic regions are targeted in a multiplex PCR assay. Eleven probes are present on the strip: one is aimed at the 23S ribosomal DNA, nine at four regions of the *gyrB* gene, and one at the flanking regions of RD1. The 23S ribosomal DNA-specific probe is used to confirm the isolate as belonging to the *M. tuberculosis* complex.

The hybridization patterns of the nine probes aimed at different regions of the *gyrB* gene, in which single-nucleotide mutations may be present, differentiate *M. tuberculosis*, *Mycobacterium africanum* type I, *Mycobacterium bovis*, *Mycobacterium caprae,* and *Mycobacterium microti*; they cannot, however, distinguish *M. tuberculosis* from *M. africanum* type II and from "*Mycobacterium canettii*". The differentiation of *M. bovis* from *M. bovis* BCG, which is not feasible on the basis of *gyrB* mutations, is obtained with the last probe suited to detect the deletion of RD1 characterizing *M. bovis* BCG. This probe, which is complementary to the two genetic regions delimiting RD1, can, in fact, only hybridize if RD1 is missing, as is the

case in BCG (Figure 14-24). A thermal-cycler and a shaking water bath are required; automated instrumentation is also available to carry out the hybridization step.

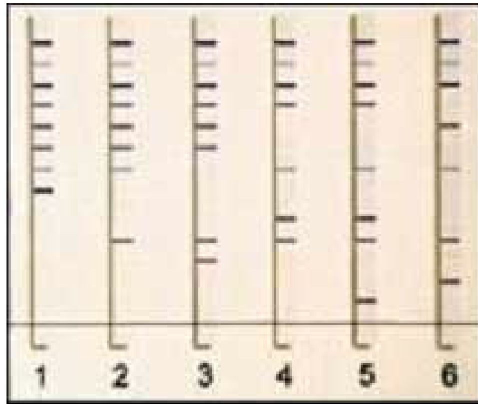


Figure 14-24: GenoType MTBC interpretation: 1, *M. tuberculosis*; 2, *M. africanum* type I; 3, *M. microti*; 4, *M. bovis*; 5, *M. bovis* BCG; 6, *M. caprae (Courtesy of HAIN Lifescience)*

The performance

Line-probe assays represent an important progress in DNA-probe technology since they allow the simultaneous testing of the organism with a number of probes. Specificity and sensitivity are high (70-80 %) The only cross reactions reported so far concern rarely encountered rapidly growing mycobacteria (Tortoli 2001, Tortoli 2003) or species not previously described at the moment at which the probes were developed (Tortoli 2005, Tortoli 2006). A limitation of GenoType is the presence of a number of equivocal hybridization patterns that are shared by two or more species due to a moderate variability of the 23S ribosomal DNA (Figure 14-23).

14.4.3. Genetic sequencing

The targets

Every genetic region which is highly conserved and, at the same time, includes moderately variable sequences is a potential target for identification. A number of such regions are known in the genome of living organisms; among them, the best

known include several genes: 16S ribosomal DNA, 23S ribosomal DNA, *hsp65,* and the non-encoding stretch ITS.

The 16S ribosomal DNA, which is about 1,500 bp long, is by far the most popular target for sequencing, and the one for which the largest database is available. In this gene, universal sequences shared by practically every living organism co-exist with genus-specific sequences common to the organisms belonging to the same genus (e.g. the genus *Mycobacterium*) and with species-specific sequences that differentiate between species. With regard to the mycobacteria, almost all the sequences characterized by species-specific variability are concentrated in the first third of the gene (Figure 14-25), namely in two stretches called hypervariable region A and hypervariable region B. The first covers nucleotides between positions 130 and 210, and the second includes nucleotides from position 430 to position 500 (such numbers indicate the corresponding positions within the *Escherichia coli* 16S ribosomal RNA gene) (Rogall 1990a, Rogall 1990b). A practical consequence is that the determination of the nucleotide sequence of the first 500 bp of the gene allows the differentiation of almost all mycobacterial species known at present.

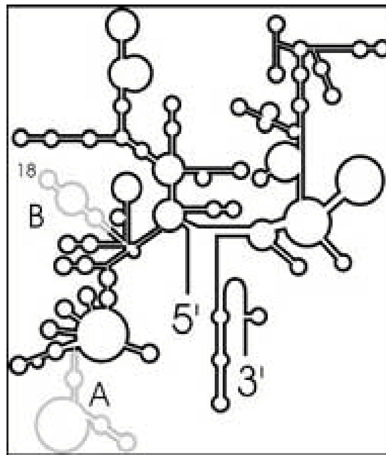


Figure 14-25: The 16S ribosomal RNA

The principle

Genetic sequencing is nowadays performed using automatic sequencers that render it highly reproducible (Figure 14-26). Initially, the target region is PCR-amplified

using proper primers and standard nucleotides. Then, the amplification product, once denatured, undergoes a second amplification in which the 3’ and the 5’ primers are used in separate tubes. In this step, in addition to standard nucleotides, a lower proportion of special nucleotides terminating the chain elongation are present. The random incorporation of terminator nucleotides during the amplification procedure (the four bases are marked with a different fluorochrome) produces strands whose lengths range from that of the primer elongated by a single nucleotide to that of the whole target. The amplicons, each marked with the fluorochrome specific for the nucleotide with which it ends, are put in order of length by means of electrophoresis. The emerging patterns of fluorescent markers identify the last bases of a continuous series of stretches, each being one nucleotide longer than the previous one, and consequently determine the genetic sequence of the region.

It is very important to perform the sequencing of both 3’ (forward) and 5’ (reverse) strands, to carefully resolve the discrepancies.

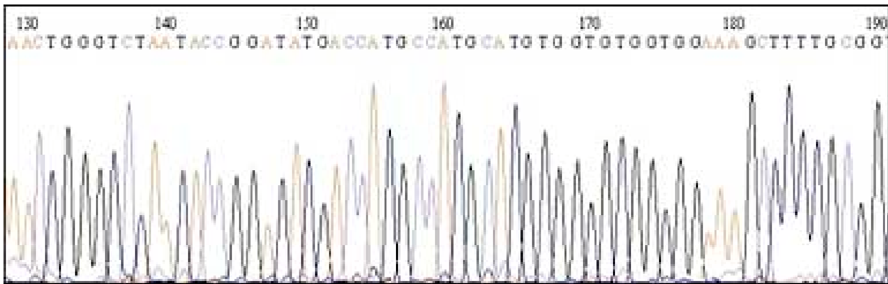


Figure 14-26: Automatic sequencing: the electropherogram

The databases

Once a sequence has been determined, its comparison with known sequences is required. Several databases are available on the Internet; fortunately, the most popular exchange new submissions made to any of them. These databases are GenBank (http://www.ncbi.nlm.nih.gov/Genbank/index.html), EMBL Nucleotide Sequence Database (http://www.ebi.ac.uk/embl/) and the DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp/). The available sequences in such databases are continuously updated by new submissions from the users. This feature is at the same time a strength and a weakness. In fact, the control of submissions is very soft and the presence of short fragments (sequences as long as 50 bp are accepted), or of

sequences determined in the years in which the sequencing technique was still dawning, turn out to be misleading (Turenne 2001).

Ribosomal Differentiation of Medical Microorganisms web-server is a public-domain database (RIDOM: http://www.ridom-rdna.de/) limited to sequences of the 16S ribosomal DNA and the 16S-23S ITS region. Different from the previous ones, it is strictly controlled and does not allow submissions by users. Unfortunately, the value of RIDOM, which indeed represents an ambitious innovation, has been hindered by the total lack of updating in the last years.

The performance

Unquestionably, genetic sequencing is nowadays the reference identification method, not only for mycobacteria but for all microorganisms, and the 16S ribosomal DNA is still the most important target sequence. Sequencing of the 5' end (about 500 bp) provides final results for the vast majority of members of the genus *Mycobacterium*. The determination of the full gene is needed to distinguish *M. peregrinum* from *M. septicum* (Schinsky 2000, Tortoli 2003), *M. murale* from *M. tokaiense*, *M. marinum* from *M. ulcerans* (Kirschner 1993), and *M. novocastrense* from *M. flavescens* sqv. ii. The only species that cannot be distinguished from each other on the basis of 16S ribosomal DNA are *M. kansasii* from *M. gastri* (Böddinghaus 1990), *M. mucogenicum* from *M. phocaicum* (Adékambi 2006), *M. fluoranthenivorans* from *M. hackensackense* (Tortoli 2006), and lastly, *M. abscessus*, *M. massiliense* (Aékambi 2004) and *M. bollettii* from each other (Adékambi 2006).

Due to its wider variability (its length ranges from 270 to 400 bp), ITS (Roth 1998) can be usefully sequenced to differentiate the rapidly growing species, which are more closely related to each other than the slow growers. Rapid growers have two copies of the ribosomal operon (except for *M. chelonae* and *M. abscessus* which have one) and a single organism may possess two different ITS copies. This may make the interpretation of the electropherograms problematic because of the presence of overlapping peaks. To obviate this problem, cloning of the genetic region is required before sequencing.

Another increasingly used genetic target for identification purposes is a 440 bp sequence of the 65 kDa heat shock protein gene, whose length is 1,623 bp. The corresponding database has almost been completed in the last few years (McNabb 2004). As is the case with the ITS region, this gene shows much higher variability than the 16S ribosomal DNA (McNabb 2004, Ringuet 1999).

Among other sequence targets for mycobacterial differentiation, the most important include the genes *recA* (Blackwood 2000), involved in DNA repair, *sodA* (Bull

1995) encoding for superoxide dismutase, and *rpoB* encoding for the beta-subunit of RNA-polymerase. The latter, which includes highly variable regions and is present in a single copy in all mycobacteria, has been recently proposed as the gold standard for the differentiation of rapidly growing mycobacteria (Adékambi 2003).

Although the minimal standards concerning genetic analysis have not been defined, it is universally agreed that at least the sequence of the full 16S ribosomal DNA gene must be determined for the description of a sp. nov.

14.5. Non-conventional phenotypic diagnostic methods

In addition to the so-called conventional methods for TB diagnosis and besides the automated and molecular diagnostic methods described above, some new technologies have been proposed, such as phage-based assays and rapid detection of growth by microscopic observation of microcolonies in solid or liquid media.

14.5.1. Phage-based assays

The phage-based assay relies on the ability of *M. tuberculosis* to support the growth of an infecting mycobacteriophage. The number of endogenous phages, representing the original number of viable bacilli, is then determined in a lawn of a rapidly growing mycobacterium such as *M. smegmatis* (McNerney 2001). Several studies have been performed to assess the *FASTPlaque TB* assay, a commercial test based on this technology, for the early detection of *M. tuberculosis* (Albert 2002). In a comparative study with auramine smear microscopy and culture in Löwenstein-Jensen medium in 1,692 sputum specimens, it was found that the *FASTPlaque TB* test detected TB in 75 % of culture-confirmed cases and in 70 % of cases with a clinical diagnosis of TB with a specificity of 98.7 % and 99.0 %, respectively. On the other hand, the concentrated auramine smear microscopy had a sensitivity of 63.4 % and 61.3 % and a specificity of 97.4 % and 97.3 % in culture-confirmed and clinically-confirmed cases, respectively. In another study done in Pakistan, the *FASTPlaque TB* compared to acid-fast smear microscopy and culture in Löwenstein-Jensen medium had a sensitivity and specificity of 87.4 % and 88.2 %, respectively, in smear-positive specimens, and a sensitivity and specificity of 67.1 % and 98.4 %, respectively, in smear-negative samples (Muzaffar 2002). As a conclusion of these studies, the *FASTPlaque TB* was able to detect mycobacteria in 50-65 % of smear-negative specimens with a specificity of 98 %, and a combination of the test with smear microscopy confirmed the presence of *M. tuberculosis* in 80-90 % of culture-positive specimens. However, *FASTPlaque TB* failed to detect

about 13 % of the smear-positive specimens and 8 % to 19 % of the smear-negative samples gave a false-positive result (Takiff 2002).

An interesting study that compared the original in-house method with the *FAST-Plaque TB* found that neither method was able to outperform direct microscopy in sputum samples while contamination rates of 40 % were obtained with the *FAST-Plaque TB* test (Mbulo 2004). Recent modifications to this commercial system include incorporation of an antibiotic mixture to decrease the high rate of contamination.

Some other phage-based technologies using reporter mycobacteriophages have also been proposed for the rapid detection and identification of *M. tuberculosis*; however, they have not been thoroughly evaluated in clinical settings of highly-endemic countries (Carriere 1997, Banaiee 2001).

14.5.2. The micro-colony method

The micro-colony method or thin-layer agar technique is an old method for culturing and identifying mycobacteria; it allows both rapid detection and presumptive identification of isolates based on the characteristic morphology of mycobacteria in culture, and has been proposed as an inexpensive alternative method for the rapid detection and culture of mycobacteria (Welch 1993). A few years ago, Mejia *et al.* described a procedure based on this method for the rapid detection of *M. tuberculosis* microcolonies isolated from clinical samples and observed under a standard microscope. The Thin Layer 7H11 agar (TL7H11) allowed the detection of more than 60 % of the culture-positive samples within the first ten days and more than 80 % after two weeks of incubation compared to 10 % on Löwenstein-Jensen medium (Mejía 1999). In a report comprising more than 1,800 clinical samples, the same authors showed a sensitivity of 72 % for TL7H11 as compared to standard cultivation in Löwenstein-Jensen medium and concluded that the simultaneous use of both media increased the sensitivity of detection (Mejía 2004).

In a further validation of the method in different settings, the TL7H11 was evaluated in a phase II prospective multicenter study performed in six laboratories in different countries in Latin America (Robledo 2006). A total of 1,118 sputum and extrapulmonary specimens were studied. All smear-positive samples yielded positive cultures, while smear-negative samples yielded *M. tuberculosis* in 3.2 % of Löwenstein-Jensen medium cultures compared to 4.4 % by TL7H11. Sensitivity was 92.6 % (95 % CI 87.9-95.9) for TL7H11 and 84.7 % (95 % CI 78.8-89.0) for Löwenstein-Jensen medium with a median time to detection of 11.5 days (95 % CI

9.3-15.0) for TL7H11 and 30.5 days (95 % CI 26.9-39.0) for Löwenstein-Jensen medium. The reported contamination rate was 5.1 % for TL7H11 and 3.0 % for Löwenstein-Jensen medium. Taking into consideration the different characteristics and implementation conditions of the participating laboratories, the TL7H11 proved to be robust enough to enter into further evaluations and cost-effectiveness studies. Figure 14-27 below shows the typical microcolony morphology of *M. tuberculosis* after several days of incubation.

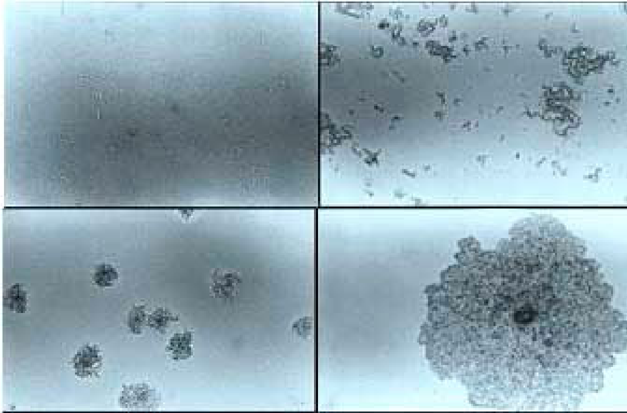


Figure 14-27: Microcolonies of *M. tuberculosis* after, 4, 6, 8, and 15 days of culture (*Courtesy J. Robledo*)

14.5.3. Microscopic observation broth-drug susceptibility assay (MODS)

MODS has been described for the early detection of *M. tuberculosis* growth in liquid medium, allowing a more timely diagnosis and drug susceptibility testing. The method is based on the observation of the characteristic cord formation of *M. tuberculosis* visualized microscopically in liquid medium with the use of an inverted microscope (Caviedes 2000). In this study, sputum samples were analyzed by staining, cultivation, and PCR. Sensitivity of MODS (92 %) compared favorably with the most sensitive of the other culture methods (93 %) with a median turnaround time of nine days. The method has been proposed as a rapid, inexpensive, sensitive, and specific method for *M. tuberculosis* detection and susceptibility testing, appropriate for use in developing countries.

In a recent operational study conducted in Peru, the performance of the MODS assay was investigated for the rapid diagnosis of TB (Moore 2006). The assay was compared with an automated mycobacterial culture system and culture on Löwenstein-Jensen medium. The sensitivity for the detection of *M. tuberculosis* was 97.8 % compared to 89.0 % for the automated mycobacterial culture, and 84.0 % for Löwenstein-Jensen medium ($P < 0.001$); the median turnaround time was 7, 13, and 26 days for MODS, the automated culture system, and Löwenstein-Jensen medium, respectively ($P < 0.001$). One limitation of the MODS assay is the requirement for an inverted microscope, which is necessary to observe the cord formation in liquid medium.

14.5.4. Analysis of cell wall mycolic acids

Mycobacteria have an unusually high lipid content in their cell wall. Such lipids include mycolic acids and other saturated and unsaturated fatty acids. Mycolic acids are branched, long-chain fatty acids present in the cell wall of a limited number of genera; they exhibit the maximum length in the genus *Mycobacterium* (Table 14-1).

Seven types of mycolic acids, differing mainly in the presence of functional groups, are variously combined in the cell wall of different species of the genus *Mycobacterium*. These types are: alpha-, alpha'-, methoxy-, keto-, epoxy, wax esters, and omega' methoxy-mycolates.

Table 14-1: Mycolic acid-containing genera

Genus	Chain length (carbon atoms)
Corynebacterium	22-38
Rhodococcus	34-52
Nocardia	44-60
Gordonia	48-66
Tsukamurella	67-78
Mycobacterium	60-90

The analysis of the lipid content of the mycobacterial cell wall has been widely used for identification purposes. The various techniques used are based on the

physical partitioning between two phases (stationary and mobile) of single lipids present in the mycobacterial cell wall.

The extraction of the lipids from the bacterial colonies is the preliminary step in all the techniques described below.

Thin-layer chromatography (TLC)

The principle

TLC uses silica plates (stationary phase) on the surface of which the mycolic acids extracted from the mycobacterial strain are separated as a result of their different affinity for a solvent (mobile phase), advancing by capillarity. Once the plate has been stained, each species displays a particular dot pattern according to its mycolic acid content that can be identified by comparison with patterns of reference strains with known mycolic acid composition run in parallel (Minnikin 1975) (Figure 14-28).

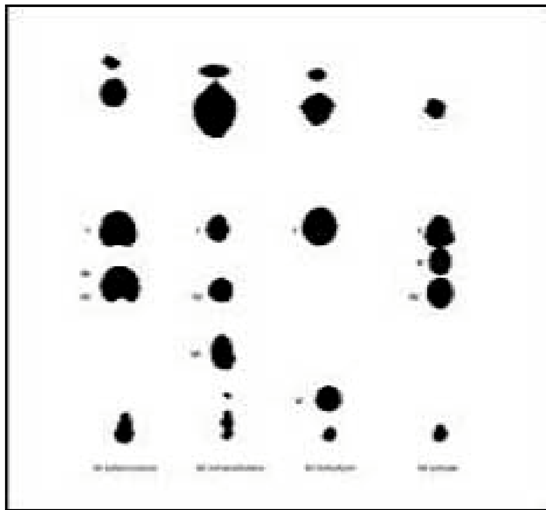


Figure 14-28: TLC of mycolic acids

The limitations

The high number of mycobacterial species, already over 130, has substantially scaled down the relevance of TLC for identification at the species level. With only seven types of mycolic acids and with most mycobacteria including no more than two or three of them, the number of TLC patterns shared by more than one, and often by many, species is high.

Gas-Liquid Chromatography (GLC)

The principle

In GLC, a gas (mobile phase) is used to carry the sample through a liquid (stationary phase) contained in a column. Once the lipids extracted from a mycobacterial strain have been injected, the high operating temperature of the column (about 300°C) produces the cleavage of the mycolic acids in saturated methyl esters 22-, 24- and 26-carbon atoms long. Along with such mycolic acid fragments, saturated and unsaturated fatty acids (including tuberculostearic acid) and alcohols are eluted.

The recognition of different elution products is usually obtained by mass spectrometry. Cleavage products, which are unvaried within single mycolic acid types, produce, along with fatty acids and alcohols, patterns consistent to single species and suitable for their differentiation (Guerrant 1981, Lambert 1986, Larsson 1985) (Figure 14-29).

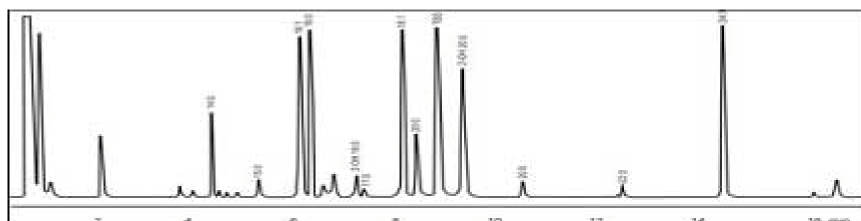


Figure 14-29: GLC pattern of *M. avium*

The limitations

The major problems concerning the GLC are the limited inter-laboratory reproducibility and the insufficient discriminative power related to the high number of *Mycobacterium* species.

High-Performance Liquid Chromatography (HPLC)

The principle

The HPLC uses high pressure to carry a liquid (mobile phase) containing the extracted sample, through the particulate (stationary phase) present in the column. The various types of mycolic acids, previously saponified, extracted and derivatized to bromophenacyl esters, are separated in the column and eluted at different times. On the basis of individual ultraviolet absorbance, the detector plots single fractions as peaks arranged in a profile. The profile of each species is sufficiently different from those of other species (Figure 14-30) to provide identification when visually compared with profiles of known mycobacteria (Butler 1988, Butler 1991, Butler 2001, Tortoli 1996). A fluorescence-based detection system may also be used that is more sensitive than the ultraviolet-based system.

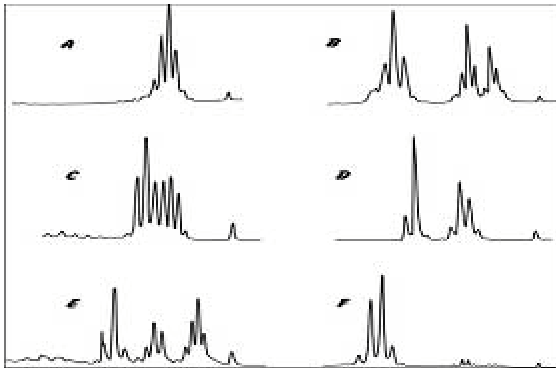


Figure 14-30: HPLC Representative patterns: A, *M. tuberculosis complex,* B, *M. intracellulare;* C, *M. gordonae;* D, *M. chelonae;* E, *M. simiae;* F, *M. nonchromogenicum*

The limitations

For many years, HPLC has been considered the only phenotypic method suitable for differentiating almost all mycobacterial species. The number of species hardly differentiable or not distinguishable at all, which was insignificant until a few years ago, has recently increased, due to the continuous description of new species, in particular, of rapidly growing mycobacteria.

A wide library of HPLC profiles, including more than 100 mycobacterial species, is available on the internet at http://www.mycobactoscana.it/page4.htm.

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Chapter 15: Tuberculosis in Adults

Afranio Kritski and Fernando Augusto Fiuza de Melo

15.1. Introduction

Tuberculosis (TB) is a disease with deep social and economical roots. Low-income people with large families, living in dense urban communities with deficient housing conditions, have a high probability of becoming infected, developing active disease, and dying from TB. Also, the risk of becoming infected and ill with TB is higher among people that live in congregated institutions, such as prisons, youth correctional facilities, nursing homes for elderly people, social shelters, day nurseries and schools; the same is valid for elderly people, diabetics and people living with Human Immunodeficiency Virus/Acquired Immunodeficiency Syndrome (HIV/AIDS) (Dye 1999, American Thoracic Society 2000, Castelo-Filho 2004, World Health Organization 2006).

The lung is the main entrance gate of the tuberculous bacillus, which causes a focal infection in the site where it is deposited after inhalation. If the infection cannot be contained at the local level, bacilli dissemination is produced initially by hematogenic route, probably inside phagocytic cells, towards different organs and, eventually, to the contiguous pleura. It reaches hilar lymph nodes via the lymphatic route, and from there, a second systemic dissemination can occur, through the thoracic duct and superior vena cava, with the development of local foci in the lungs. Extrapulmonary foci can also be produced by hematogenic and lymphatic dissemination. The clinical manifestations of TB depend on the local organic defenses on the sites of bacilli multiplication (Rich 1944, Bates 1980, Stead 1984). It has been emphasized that the use of bacille Calmette-Guérin (BCG) vaccination may play a role in this phase, avoiding dissemination and the occurrence of extrapulmonary forms of TB.

15.2. The initial lesion

Once inhaled, most tubercle bacilli are trapped in the mucosa of the upper respiratory tract, trachea and bronchi, especially when inhaled in clumps, and are eliminated by the mucocilliary defense mechanisms. Tiny particles or droplet nuclei smaller than 5 µm behave as a gas and overcome this barrier and reach the inferior respiratory tract, especially inside the alveoli, where they are readily phagocytosed by alveolar macrophages.

The survival of the infectious agent in the lung will depend on its pathogenicity/virulence and on the ability of the host cells to eliminate it. The alveolar macrophages are the first line of defense against *Mycobacterium tuberculosis*. This initial response, if completely effective, will cause the elimination of the pathogen through the phagocytic action inherent to such macrophages (see Chapter 5). If the alveolar macrophage is not capable of arresting bacterial growth, a localized pro-inflammatory response is formed through the activity of Toll-like receptor agonists, abundant on the surface of bacteria. Tumor necrosis factor alpha (TNF-α) and inflammatory chemokines produced by the infected macrophages recruit white blood cells, which phagocytose bacilli and eventually return to the bloodstream causing the primary hematogenic dissemination (Figure 15-1).

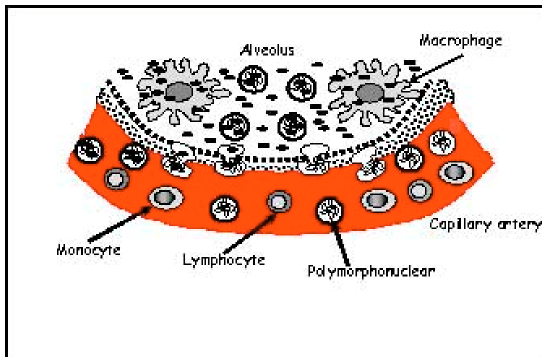

Figure 15-1: Non-specific inflammation with blood white cell migration and primary hematogenic dissemination

The recruited cells produce their own complement of chemokines and cytokines that amplify cellular recruitment and remodel the infection site into a cellular mass, the tubercle or granuloma. The granuloma initially formed consists of a core of infected macrophages surrounded by foamy macrophages, with an external layer of lymphocytes encircled by collagen and other extracellular matrix components (Russell 2007).

Tubercle bacilli can disseminate by the lymphatic route to regional lymph nodes, constituting the tuberculous primary complex of Ranke, composed by the original granuloma at the inoculation site (Gohn's nodule), the lymphangitis and the hilar lymph node enlargement (Figure 15-2). Although in some cases these lesions may become evident on chest X-ray, most cases of primary tuberculous infection are

clinically and radiologically unapparent, with a positive TST being the only indication of the occurrence of the infection.

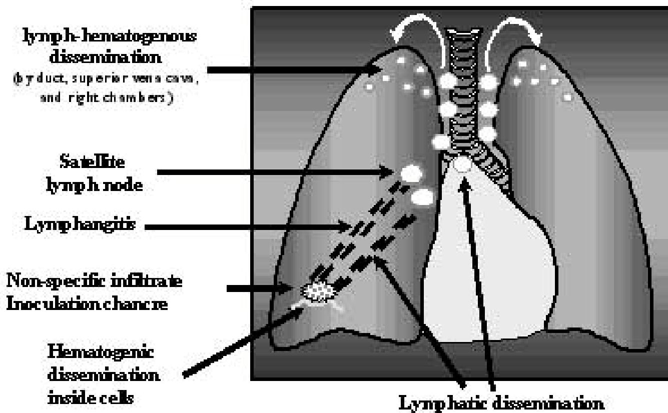

Figure 15-2: Primary infection, inoculation lesion, primary complex, and initial TB dissemination

From the hilar lymph nodes, tubercle bacilli disseminate to tracheal and vertebral lymph nodes. Through the thoracic duct, they reach the blood stream, spreading to the upper areas of the lungs or to different organs, such as kidney, brain, and bones. At these sites, they find a favorable atmosphere for implantation that combines a satisfactory oxygen tension and a low local perfusion: this is an ideal association that hinders the access of defense cells.

15.3. The inflammatory response

With the development of specific cellular immune response and production of interferon-gamma (IFN-γ), a mature stable granuloma is formed, which is responsible for the immune containment of the pathogen. Mature granulomas present neovascularization, epithelioid and giant multinucleated cells. An extensive fibrotic capsule develops and infected macrophages trapped inside granulomas eventually die. Tubercle bacilli tend to locate in the center of the granuloma, but bacteria and antigens are also associated with macrophages in the peripheral infiltrate (Russell 2007). The necrotic material present in the center of TB lesions contains high

amounts of fat representing the lipids liberated from bacillary catabolism. This material, which has a soft, dry and cottage cheese texture, is known as caseous necrosis. On microscopy, large amounts of epithelioid and giant multinucleated cells can be observed in the granulomas, located mainly around the caseous material. The nature of the host immune response will determine whether the infection will progress or be contained.

The discussion regarding the participation of T lymphocytes in the development of infectious diseases has lead to the paradigm, initially developed in the murine model, which characterizes CD4+ T lymphocytes in two subpopulations named T helper 1 (Th1) and T helper 2 (Th2). In the human infection, however, such a clear division into two different cytokine response patterns is not observed. Unlike the murine model, a wide spectrum of cytokines are produced in response to infection by *M. tuberculosis* in the human host, and their function in containing the infection is not yet completely understood (Chacon-Salinas 2005).

Individuals who are able to mount an immune response adequate enough to contain *M. tuberculosis* bacilli at this stage will develop a clinical form of infection, characterized as latent infection, in which bacilli will stay for an undetermined period of time. The risk of progressing to clinical TB is highest during the first 3-5 years after the infection, especially among immunosuppressed individuals.

In view of this, the disease can occur:

- **during the initial phase of infection** - due to excessive bacillary load, increased bacterial pathogenicity/virulence and/or factors that decrease host immune response. In this case, a host-parasite balance is not achieved, which results in the development of **primary TB** a few months after the infection.
- **posterior to the initial phase of infection** – due to a rupture of the host-parasite balance in individuals with latent TB, resulting in **post-primary TB**.

15.4. Tuberculosis infection

In most individuals, TB infection is clinically irrelevant and seldom recognized. It commonly occurs during childhood (see Chapter 16), and may occasionally cause malaise, low-grade fever, erythema nodosum, and phlyctenular conjunctivitis. Erythema nodosum is a toxic allergic nodular lesion 2 to 3 cm large, located in or under the skin. These lesions are spontaneously painful and very painful under

pressure, and are usually located bilaterally on the anterior surface of feet and legs. In most individuals, however, primary TB infection causes no apparent symptoms and the infection stays latent for life or until reactivation (Bates 1980, Melo 1993, Lima 1993). Chest X-rays can present several manifest ations. The classical presentation is known as the primary Ranke's complex, including a calcified peripheral lung nodule (Gohn's primary focus), lymph tracts toward the hilus (lymphangitis), and enlarged local lymph nodes (Figures 15-3 and 15-4).

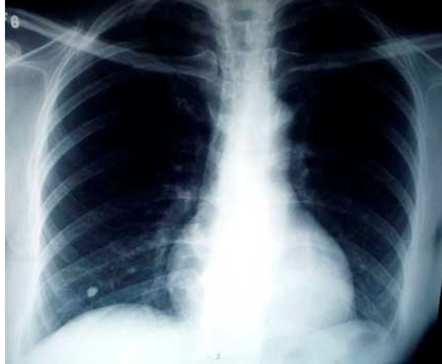

Figure 15-3: Chest X-ray showing a calcified peripheral nodule in the lower right lung (Gohn's primary focus) (Reproduced from Melo 2005a)

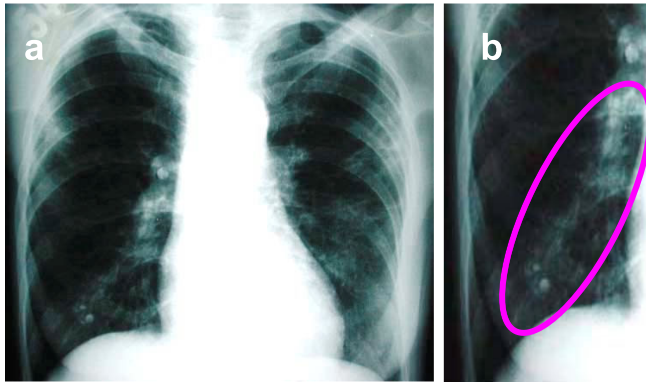

Figure 15-4: a: Chest X-ray showing a calcified peripheral nodule in the lower right lung, lymphangitis (encircled in b) and hilar involvement (Ranke's complex) (Reproduced from Melo 2005a).

In the initial phase of *M. tuberculosis* infection, some tubercle bacilli can reach the upper lobes of the lungs, creating small metastatic foci referred to as Simon foci, visible on chest X-ray (Figure 15-5).

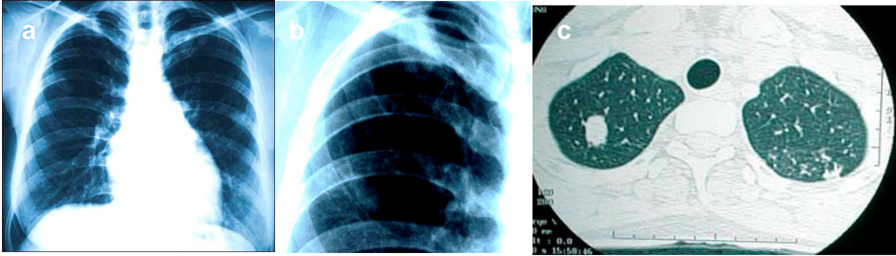

Figure 15-5: Simon nodule in upper lobe of the right lung in an asymptomatic adult. **a** and **b**: chest X-ray, **c**: computerized tomography.

For additional images of TB infection see Presentation 1 at http://www.tuberculosistextbook.com/pdf/Presentation_1.pdf.

15.5. Tuberculosis disease

The development of clinical TB will occur in 5 %-10 % of infected persons at some point in their lives, for reasons that are not completely clear. Some factors involved in increased risk of developing TB have been established, of which the most important are those interfering directly with host immunity.

Diseases and conditions that weaken immunity, such as malnutrition, alcoholism, advanced age, HIV/AIDS, diabetes, gastrectomy, chronic renal insufficiency, silicosis, paracoccidioidomycosis, leukemias, solid tumors, immunosuppressive drug treatments, and hereditary features, are factors that facilitate the development of TB disease. Additional factors include the infective bacterial load, pathogenicity/virulence of bacilli, and host genetic susceptibility (ATS- 2000).

15.5.1. The primary disease

Adult primary TB is paucibacillary, practically non-contagious, difficult to diagnose, and of variable severity, as described in children (see Chapter 16). In seriously immunodepressed patients, but also in individuals with IFN-γ or IL-12 re-

ceptor deficiency, it can develop into a disseminated form, which is sometimes fatal. High morbidity in the primary form was also observed in patients whose ancestors were not previously exposed to the tubercle bacillus, as reported in the Yanomami Indians in the Amazon Region (Sant´Anna 1988, Souza 1997).

For images of primary disease see Presentation 2 at http://www.tuberculosistextbook.com/pdf/Presentation_2.pdf.

15.5.2. The post-primary disease

The existence of post-primary TB, also known as secondary TB, means that the infection can progress after the development of an adequate specific immune response. This TB episode can develop in two ways: by inhalation of new bacilli or by reactivation of the primary focus. Recently, in African countries, using molecular typing methods, it has been shown that the transmission is community driven, and not solely through households, and that reinfection with novel *M. tuberculosis* strains may occur in 40 % of relapsing cases (Verver 2005). The recurrence/relapse caused by new strains highlights the possibility that the progression to disease can be enhanced by multiple infections, especially among high-risk persons, such as HIV infected individuals.

Pulmonary TB is the most common form of post-primary disease. Lymphatic dissemination can occur, but in this case the hilar lymph nodes are usually not affected. The response to bacillary multiplication provokes caseous necrosis that eventually blends and progresses to liquefaction. Tubercle bacilli, whose multiplication had been until then inhibited by granuloma formation, find favorable conditions for population growth after liquefaction of the caseum and subsequent cavitation, and may produce more than 10^8 bacilli per cavity with a diameter of less than 2 cm. The development of tuberculous cavities in the lung characterizes the post-primary TB and, from this lesion, infectious material can spread through bronchi, resulting in the continuous production and elimination of sputum. The natural evolution of post-primary lesions in immunocompetent persons can lead to dissemination and death in about 50 % of cases, and to chronicity in about 25 % to 30 %. Natural cure can also occur in 20 % to 25 % of cases, when the host immune response is able to re-establish control of the disease (Bates 1980, Melo 1993).

In most non-immunosuppressed persons infected by the tubercle bacillus, disease will occur in the first three to five years after the initial exposure. In HIV positive persons infected with the tubercle bacillus, however, 7 % to 10 % will develop active TB annually (ATS 2000). The remaining cases occur at any time during a

lifetime, especially when there are other diseases or weakening conditions, for example malnutrition, diabetes, prolonged treatment with corticosteroids, immunosuppressive therapy, chronic renal disease, gastrectomy, and others. The post-primary disease presents a great spectrum of manifestations, which are related to the affected organ. The lungs are most commonly affected, usually in the upper lobes or apical segments of inferior lobes. The disease can also affect other organs, including lymph nodes, pleura, kidneys, the central nervous system, and bones.

In pulmonary TB, the patients often present with an insidious clinical onset, sometimes with minimal or non-specific complaints in the initial phase. With the development of the disease, two types of signs and symptoms can be recognized. The most frequent are: lack of appetite, low-grade evening fevers, and night sweats. Additional symptoms are asthenia, irritability and migraine. With respect to respiratory signs and symptoms, the patient may complain of cough of insidious evolution, at any hour of the day, which as initially dry and later on productive with purulent or mucous expectoration. Hemoptysis and bloody sputum occur in less than a quarter of patients, with the worst cases originating from lesions invading blood vessels. Chest pain can be localized and dependent on breathing movements (Hopewell 2006). At the beginning of the disease, lung auscultation is of little help. Few crackles can be noticed on auscultation after deep inspiration and also ronchi and tubular sounds. In most cases, the patient may be symptomatic for one to three months before diagnosis. Such delays in diagnosis may be due to low diagnostic suspicion by the medical personnel, lack of access to health services, because the patient may not acknowledge being sick or may not seek medical help due to economic or cultural reasons. An early diagnosis is critical for controlling transmission of the disease in the community, especially in congregated institutions, such as hospitals, prisons, and shelters.

It is crucial to perform the diagnosis in the initial phase of this type of presentation in patients with recent symptoms (less than four weeks) (Figure15-6). If diagnosis is delayed, the disease may evolve rapidly, destroying the pulmonary parenchyma (Figures 15-7 and 15-8).

The parenchymal infiltrates from post-primary TB in adults resemble a pyramid, with the base towards the lung periphery and the apex looking at the hilar area. In the past, it was recognized as a sign of the tubercle bacilli seeking a route for airborne dissemination (Figure 15-7).

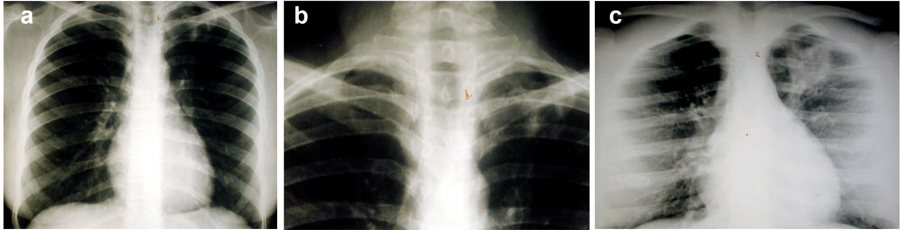

Figure 15-6: Parenchymal infiltrate in the upper left lung, in posteroanterior (**a** and **b**) and lordotic position (**c**).

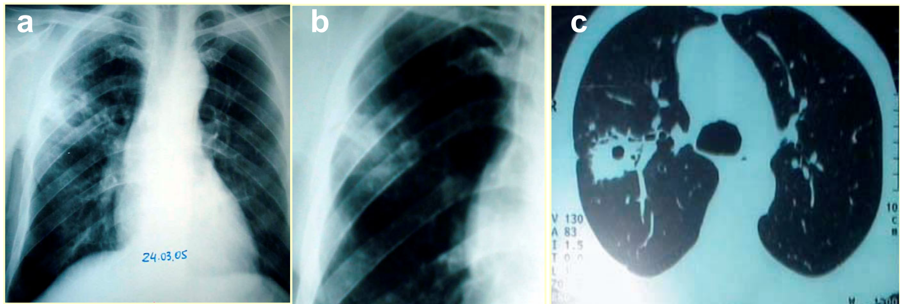

Figure 15-7: Lung infiltrate and cavitation in the upper lobe of the right lung. **a** and **b**: chest X-ray, **c**: computerized tomography (Reproduced from Melo 2005b).

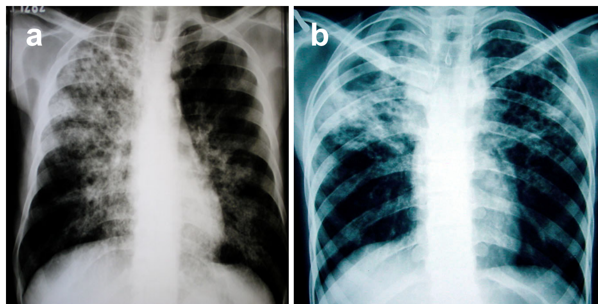

Figure 15-8: Chest X-ray showing parenchymal infiltrate and cavitation in the right upper lobe (**a**) and in both upper lobes (**b**) (Reproduced from Melo 2005b).

For additional images of post-primary disease see Presentation 3 at http://www.tuberculosistextbook.com/pdf/Presentation_3.pdf.

Sometimes, patients with acquired multidrug resistant TB (MDR TB), after several treatment schemes with available anti-tuberculosis medication, need to be submitted to thoracostomy, as shown in Figure 15-9.

After achieving cure, respiratory symptoms such as a productive cough persist in some patients for several years. When the patient refers to recurrent hemoptysis with elimination of more than 15-50 mL of sputum per day, bronchiectasis and/or a fungus ball may be present (Figure 15-10).

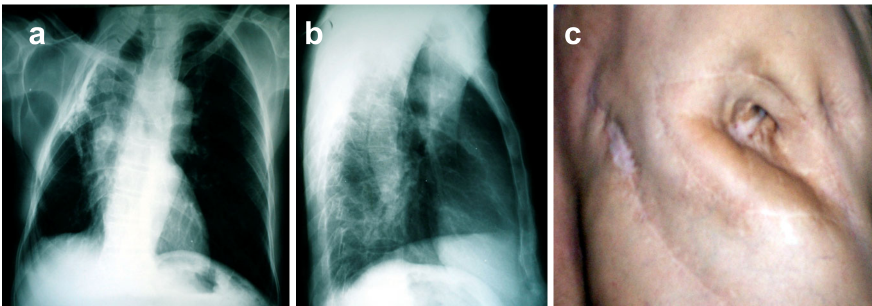

Figure 15-9: Patient with MDR TB showing sequelae resulting from chronic disease and thoracostomy.

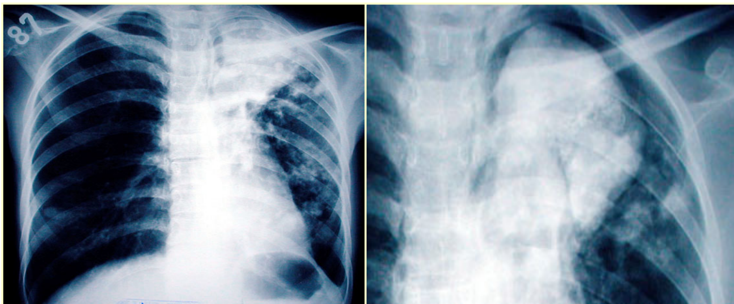

Figure 15-10: Chest X-ray showing fibrotic infiltrate and cavity with a fungus ball in the upper left lobe.

For additional images of sequela of TB see Presentation 4 at http://www.tuberculosistextbook.com/pdf/Presentation_4.pdf.

15.5.3. Extrapulmonary tuberculosis

After penetration into the organism through the respiratory route, *M. tuberculosis* can settle and multiply in any organ during the primary infection, before development of the specific immune response. After this, tubercle bacilli can multiply at any time when there is a decrease in the host's immune capacity to contain the bacilli in their implantation sites. The specific signs and symptoms will depend on the affected organ or system, and are characterized by inflammatory or obstructive phenomena. Systemic symptoms are much less frequent than in pulmonary TB, except in the disseminated form of the disease.

The majority of the extrapulmonary forms of TB affect organs with suboptimal conditions for bacillary growth. For this reason, the extrapulmonary disease generally has an insidious presentation, a slow evolution and paucibacillary lesions and/or fluids. Access to the lesions through secretions and body fluids is not always possible, and for this reason, invasive techniques may be necessary in many cases, to obtain material for diagnostic investigation. Tissues and/or body fluids should be submitted to laboratory examination, in particular bacteriological culture for mycobacteria and histopathological analysis. In the immunocompetent patient, the TST response is usually positive (induration ≥ 10 mm).

Imaging studies provide valuable information for the diagnosis of extrapulmonary TB, although specific radiological patterns are not observed. In immunocompetent patients, the extrapulmonary forms only occasionally coexist with active pulmonary TB. Nevertheless, the chest X-ray is mandatory for the evaluation of evidence of primary infection lesions, which provide a good verification to support the diagnosis (Rottenberg 1996).

Miliary tuberculosis

Miliary TB results from the massive hematogenic dissemination of the Koch bacillus during the primary infection. Its onset may be either insidious or abrupt, depending on the bacillary load and/or the host immune situation, with unvaccinated infants, elderly and immunodeficient patients being the most susceptible (Lester 1980, Thornton 1995). The variable and often nonspecific symptoms include fever, anorexia, weight loss, and asthenia. Other specific symptoms depend on the organs affected, and involvement of the central nervous system occurs in 30 % of cases. The physical examination is unspecific, and the patient can present

with variable degrees of wasting, fever, tachycardia and toxemia. The observation of bacilli on smear microscopy examination is rare, and culturing mycobacteria provides a higher probability of bacteriological confirmation of the diagnosis of TB.

In the advanced stages of HIV/AIDS (CD4+ cell count lower than 200 cells/mm^3 or peripheral blood lymphocyte count lower than 1000/mm^3), the bacilli circulate in the bloodstream, and tubercle bacilli are often isolated from blood when appropriate culture media are used (see Chapter 17). Chest X-ray shows a characteristic diffuse, bilateral and symmetrical micronodular infiltrate (Figure 15-8). Other characteristic TB lesions can be found simultaneously, such as cavities, focal parenchymatous condensations, and pleural effusion. Bilateral involvement is highly suggestive of miliary dissemination. Mediastinal and hilar lymphadenopathy appear more frequently in patients with recent lung infection (primary TB) or HIV co-infection (Figure 15-11). The diagnosis of TB can also be obtained when caseous granuloma is found in biopsy material (Lester 1980, Thornton 1995).

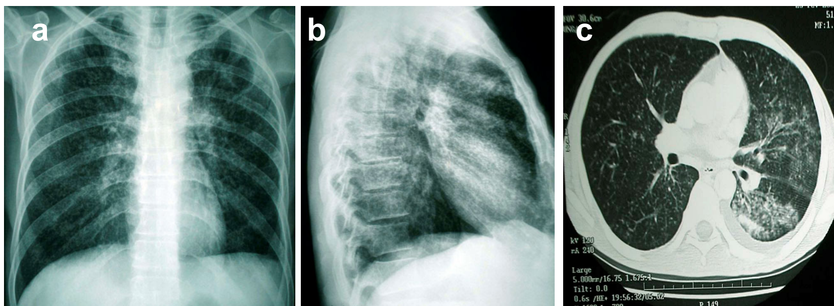

Figure 15-11: Miliary pattern of primary TB in adults. **a** and **b**: chest X-ray, **c**: computerized tomography.

Pleural tuberculosis

This is the most common form of extrapulmonary TB, and can either result from the rupture of a primary sub-pleural lung focus (evident or not on conventional chest X-ray) or be secondary to lymphohematogenic dissemination. The presence of a pleural TB effusion has also been related to hypersensitivity (Light 1990). Most cases occur several months after the primary infection, and frequently the patient relates having contact with an active pulmonary TB case in the two years preceding the current episode. The simultaneous presence of active pulmonary TB

may be related to recent infection followed by disease. The onset of the disease may be insidious or abrupt, with fever, systemic complaints, dyspnea, dry coughs, and pleuritic thoracic pain. The physical examination shows signs characteristic of pleural effusion. With regard to diagnosis, the result of the TST may be negative at the diagnosis of the disease and become positive during anti-tuberculosis treatment. The pleural effusion is generally unilateral and moderate, and can easily be detected by conventional chest X-ray examination (Figure 15-12). In one third of cases, an underlying lung infiltrate can be observed.

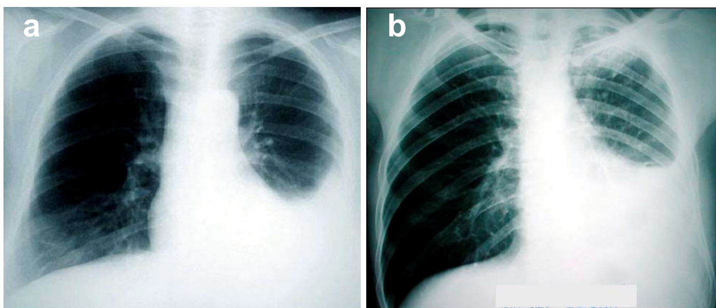

Figure 15-12: Pleural involvement with no parenchymal lesion (**a**) and with upper lobe lung infiltrate (**b**).

Thoracocentesis and puncture pleural biopsy should be indicated. The pleural liquid has a typically citrine yellow aspect and sometimes may be sero-hemorrhagic. It is generally an exudate with a predominance of lymphomononuclear cells, often negative for acid-fast bacilli (AFB) on microscopic examination. The etiological diagnosis is confirmed by the isolation of *M. tuberculosis* by culture of the fluid. The histopathological finding of granulomatous lesions in the pleural biopsy also confirms diagnosis, especially in countries with a high TB prevalence (Light 1990, Uehara 1993).

An important auxiliary method in the diagnosis of pleural TB is the determination of adenosine deaminase (ADA), an enzyme liberated by activated lymphocytes. This examination has a sensitivity and specificity above 90 %. Thus, ADA activity above the cut-off level in the pleural liquid is highly suggestive of pleural TB. If the patient is below 45 years of age and the pleural liquid shows predominance of

lymphomonocytic cells, the specificity and positive predictive value of ADA may approach 100 % (see Chapter 12).

The differential diagnosis for pleural effusions includes para-pneumonic pleural effusions, mycoses, malignant diseases, and, especially in young women, collagen vascular diseases. Most of the time, the effusion is resolved, even if not treated, leaving minimal or no radiological sequelae. Nevertheless, there is a high risk of reactivation of pulmonary TB in the following years if pleural TB is not adequately treated with anti-tuberculosis drugs (Light 1990, Uehara 1993).

Lymph node tuberculosis

This is the second most common form of extrapulmonary TB in HIV seronegative patients and the most frequent in patients living with HIV/AIDS. The preferential localization is the anterior cervical lymph node chain with little predominance of the right side chain. Initially, lymph nodes grow slowly, and are painless and mobile. Later on, as their volume increases, they tend to coalesce and some develop fistulas (Figure 15-13). Patients mainly complain of fever and the increasing volume of lymph nodes, but other symptoms may be absent. In general, the TST is strongly positive, except in immunosuppressed patients.

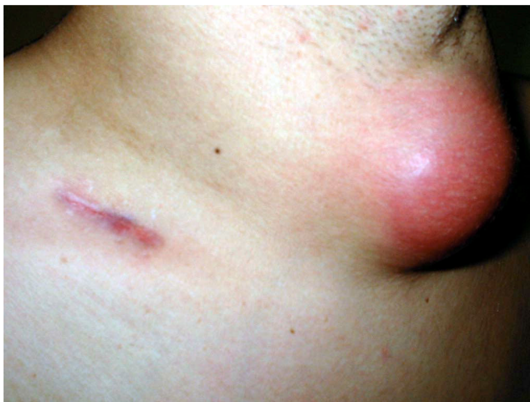

Figure 15-13: Lymph node TB in cervical area.

The images are similar to those described in primary TB in children: enlargement of hilar and mediastinal lymph nodes (Figure 15-14). The etiological diagnosis can

be made by aspiration puncture biopsy, which is AFB positive in only 10 % to 25 % of cases, but *M. tuberculosis* may be isolated by culture in 50 % to 85 % of cases. Cytopathology may be suggestive of the disease if there is a high proportion of Langhans cells. The histopathological analysis of the lymph node biopsy is usually conclusive, showing granuloma with caseous necrosis in 91 % to 96 % of cases (Lester 1980, Light 1990, Kang'ombe CT 2004, Greco 2004).

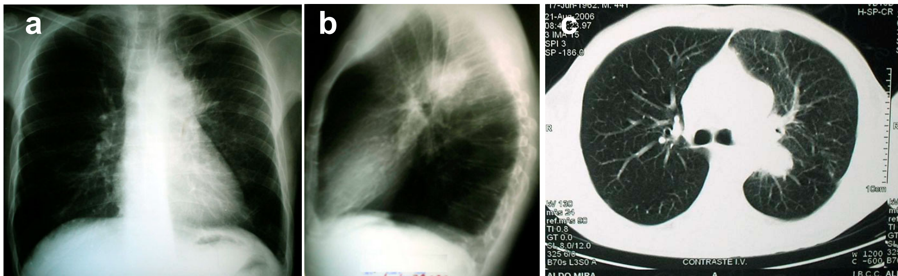

Figure 15-14: Enlargement of left hilar lymph node in an HIV-infected patient. **a** and **b**: chest X-ray, **c**: computerized tomography.

Renal tuberculosis

Renal TB is rare in children and predominantly affects individuals in the fourth decade of life. Renal disease occurs after a long latency period and is frequently secondary to hematogenous dissemination. The localization is almost always bilateral, but can be asymmetric. The lesions often start in the renal cortex and progress slowly toward the central region. Dissemination can occur to the bladder and even to the genital system.

Symptoms and signs may vary in duration and severity. The patient generally complains of dysuria, polacyuria, and lumbar pain, whereas systemic symptoms occur less frequently. Frequently, the disease presents as a urinary infection that does not respond to routine broad spectrum antimicrobial treatment. Purulent urine is frequently found, with urine culture negative for common germs (aseptic pyuria). Hematuria occurs in 10 % to 15 % of the cases.

Excretory urography can either be normal or present a wide variety of alterations that include parenchymatous cavities, dilatation of the pyelocalicial system, renal calcifications of irregular contours, decreased capacity of the urinary bladder, and multiple ureter stenoses (Figure 15-15). Due to the high association between renal

TB and urinary bladder TB, cystoscopy is indicated. In the cystoscopy, edema and diffuse hyperemia are observed, which are more intense around the orifice (golf hole sign), often accompanied by irregular ulcerations and/or infiltrates and vegetations. In these cases biopsy is indicated (Wise 2003).

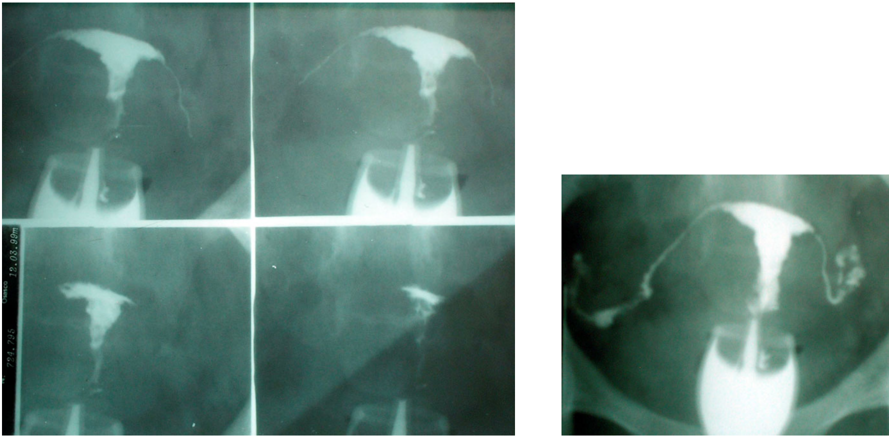

Figure 15-15: Infertility patient hysterosalpingogram, revealing proximal dilatations of the fallopian tubes ("rigid pipe stem" appearance) and distal enlargments/constrictions ("beaded" appearance). Association with antecedents of anterior contagion with TB and TST = 22 mm, allowed the establishment of the diagnosis of TB salpingitis

The diagnosis is confirmed when the urine culture is positive for *M. tuberculosis*. Culture of three to six specimens of first morning urine are together as reliable as the culture of a single 24-hour urine sample. TST is generally positive, except in patients with HIV/AIDS (Smith 1994, Simon 1977, Wise 2003).

Tuberculosis of the central nervous system

The compromise of the central nervous system occurs in two basic forms: meningoencephalitis and intracranial tuberculoma. Since the introduction of modern chemotherapy and especially massive BCG vaccination, a lower proportion of the meningoencephalitis has been observed, but the frequency of this form of TB is higher among young adults with HIV/AIDS (see chapter 17, Simon 1977, Smith 1994).

The clinical manifestations are due to the inflammatory process induced by the mycobacterial infection, and the symptoms depend on the site and intensity of

inflammation. Granulomas can be located in the cerebral cortex or in the meninges. Meningoencephalitis generally has an insidious onset and a slowly progressive course, with symptoms including apathy, lethargy, fever, and mental disturbances such as irritability, understanding difficulties, personality alterations, disorientation, and progressive mental confusion. Vertigo, migraine and vomiting can also be observed.

Findings on physical examination are related to the stage of the disease and the affected area, such as cranial nerve involvement (the most affected are the 2nd , 3rd , 4th, and 8th nerve pairs), focal neurological deficits, and signs of meningeal and cerebellar irritation.

The cerebrospinal fluid is generally clear, with a predominance of lymphocytes, an increase in proteins and a decrease in glucose levels. Microscopic examination for AFB is generally negative and cultures are positive in only 15 % to 30 % of cases.

In the differential diagnosis the following conditions should be considered: other infectious meningitis, vascular pathologies, the collagen vascular disease sarcoidosis, metastatic carcinoma, acute hemorrhagic leucoencephalopathy, and lymphoma.

In the case of intracranial tuberculoma, the clinical manifestations depend on the location of the lesion, which generally grows slowly. When there is no compromise of the sub-arachnoid space, the cerebrospinal fluid is normal and the computerized tomography exhibits a mass, which is generally difficult to differentiate from neoplasia (Azambuja 1993, Kasik 1994, Norris 1995).

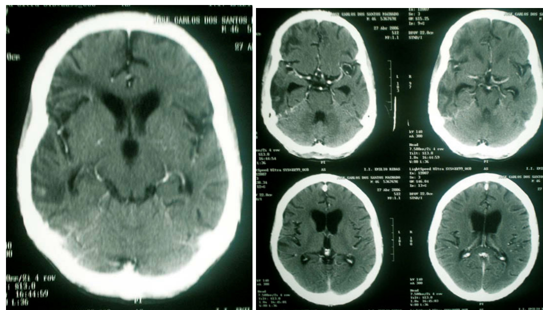

Figure 15-16: Computerized tomography of the skull in young adult patient with cerebral TB, with hydrocephaly, hypodense central areas, and atrophic lesions.

Osteoarticular tuberculosis

Involvement of the osteoarticular system is most commonly found in children and the elderly, and is generally secondary to hematogenous seeding, but can also occur as a consequence of lymphatic dissemination or direct spread from a contiguous lesion. Bone involvement consists of osteomyelitis, and arthritis can occur either by extension of the osseous lesion to the joint or by direct hematogenic inoculation.

The most frequent sites of bone involvement are the vertebrae (Pott's disease) and the proximal extremities of the long bones. Spinal TB frequently affects more than one vertebra. With evolution, it presents a wedged flattening and gibbus formation that can be associated with a paravertebral cold abscess (Figure 15-17). Paresthesia and paraplegia are reported when the cervical and upper thoracic area are affected. Image on X-ray is characterized by erosion of the anterior vertebral body margins with no preservation of the intervertebral space. The definitive diagnosis should be obtained by biopsy for culture and histopathological analysis (Ridley 1998, Schlesinger 2005).

The peripheral joints most frequently affected by TB are the hip and the knee. Pain, with or without movement limitation, fever and systemic symptoms are frequent. Monoarticular involvement is much more frequent than multiarticular disease.

The diagnosis of osteoarticular TB is usually delayed because this etiology is often overlooked in the differential diagnosis of joint disease. In most cases, the TST is positive, and approximately 50 % of cases also have abnormal chest X-rays, suggesting previous pulmonary disease. Cold abscesses occurring in the advanced phase of osteoarticular TB can develop into cutaneous fistulae, which are frequent in this form of the disease. The diagnosis is established by puncture, biopsy, histopathological examination, and culture (Zylbersztejn 1993, Davidson 1994, Ridley 1998, Schlesinger 2005).

Other extrapulmonary localizations

Tuberculous involvement of other tissues, such the eye, skin (*lupus vulgaris*), genital, and digestive tract, may also be the result of hematogenous dissemination, but there are other possible routes of infection.

Intestinal TB can be acquired by the oral route, and in countries with a high prevalence of bovine TB. Before the generalization of milk pasteurization, this was a rather common form of zoonotic TB (produced by *Mycobacterium bovis*), particularly in infants.

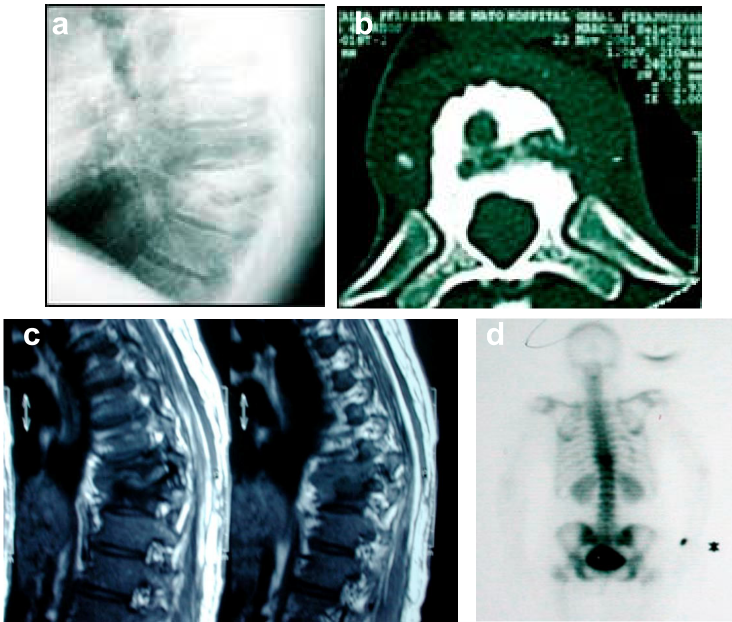

Figure 15-17: X-ray (a), computerized tomography scan (b), nuclear magnetic resonance (c), and scintilography (d) showing images of spondylitis and meningocele in a patient with Pott´s disease (from the archives of Instituto Clemente Ferreira, Brazil)

Eye and skin TB may be the consequence of accidental inoculation, particularly among medical and veterinary professionals, and genital TB may be produced by spread from renal TB.

15.5.4. Special conditions

During the past few decades, TB has been observed in association with immunosuppression, malignant neoplasms (i.e. lung cancer, head and neck cancer, Hodgkin's lymphoma), malnutrition (more than 15 % loss of usual weight), old age, diabetes mellitus and silicosis. The occurrence of TB in these patients is likely to be the result of both increased susceptibility and longer patient survival, due to the increased frequency of organ transplantation and the accompanying immune suppression, more effective cytotoxic treatments for neoplastic disease, and immuno-

suppressive treatment of autoimmune diseases, such as systemic lupus erythematosus or the anti-TNF antibodies used against chronic inflammatory arthritis.

In many low-income countries, patients with these immunosuppressed conditions receive care in large urban health centers (ambulatory or hospitalized) where health professionals are insufficiently trained in the diagnosis and treatment of TB. Meanwhile, the staff in TB clinics has scarce information about the management of immunosuppressed patients, other than those infected with HIV. Particularly in the elderly (> 60 years old), there has been an increase in morbidity and mortality from pulmonary TB in patients with diseases such as chronic renal failure, chronic liver diseases, malignant neoplasm, diabetes mellitus and organ transplant (Carvalho 2002). The clinical, radiological and laboratory presentation of these patients tend to be atypical, making diagnosis very difficult, delaying the start of anti-tuberculosis treatment, and increasing TB transmission within the hospital or ambulatory clinic environment.

In countries with a high TB prevalence, TB occurs 30 to 40 times more frequently among patients receiving organ transplantations such as heart, kidney and bone marrow. The immunosuppression may be caused by the original disease (i.e. chronic renal insufficiency), by the immunosuppressive radiotherapy/chemotherapy necessary to avoid transplant rejection, or by a combination of both. In these patients extrapulmonary TB is more frequent than pulmonary disease, and the disease manifests itself in the first or second year after transplantation. The mortality rate is especially high when the diagnosis and treatment are delayed.

Tuberculosis in patients with diabetes mellitus

Before the advent of anti-tuberculosis chemotherapy and the generalized use of insulin therapy, the incidence and mortality rates of TB among patients with diabetes mellitus were high. In patients whose diabetes is difficult to control, the immune defects are presumably more severe, and pulmonary TB tends to be more aggressive producing cavities and extensive lesions in the lower third of the lungs (Ikezoe 1992, Al-Wabel 1997, Singla 2006).

Tuberculosis in patients with chronic renal insufficiency

In patients with chronic renal insufficiency, TB often has a slow onset with low-grade fever, dry cough, dyspnea, pleuritis and/or pericarditis. TB occurs frequently in patients undergoing long periods of dialysis (on average, after 22 months), and extrapulmonary presentations (i.e. ganglionar) are common. As the mortality rate is high when the diagnosis is delayed, TB must always be considered a possibility,

and appropriate invasive and non-invasive procedures should be employed to ensure an early diagnosis (Moore 2002, John 2002, Erkoc 2004).

Tuberculosis in elderly patients

In middle-income countries, the increased survival rates have resulted in larger elderly populations, which can be expected to lead to an increase in TB reactivation. However, TB in the elderly may be due to a newly acquired infection. The lack of attention given to TB in the elderly in these countries is due to:

- disinformation of healthcare personnel, because TB is considered a problem of young adults
- inexperience of health personnel in diagnostic and therapeutic approaches to tuberculosis in elderly patients
- unavailability of laboratory facilities to diagnose TB in nursing homes and/or support houses
- confusing TB symptoms with other pulmonary, heart or malignant diseases
- impaired communication ability of some elderly patients

Chest X-ray findings can be similar to those observed in young adults with pulmonary TB, although with a lower frequency of cavitation and higher frequency of pleural involvement. Other diseases, especially lung tumors among smokers, can also be located in the lung apices, mimicking TB; so a prompt diagnosis is critical. Patients with neoplasia in the lung apex often present with referred pain, while this symptom is rare in patients with TB (Perez-Guzman 1999, Zevallos 2003).

It should be stressed that undetected pulmonary TB in the elderly commonly results in the transmission of the disease to close contacts in households, hospitals or nursing homes. The mortality rate is high among elderly patients who develop TB in nursing homes and support houses, probably because inadequate diagnostic procedures and facilities lead to a late diagnosis, which is followed by inadequate therapeutic measures. Standardization and legislation on the appropriate procedures for diagnosis and management of TB in these specific settings are required. A chest radiograph should be performed in every elderly patient admitted in a health center, complemented by bacteriological tests (AFB smear microscopy and, when available, culture for mycobacteria) in patients with pneumonia not responsive to broad spectrum antibiotics, fever of obscure origin, or a productive cough of more than three weeks duration, with or without associated weight loss.

15.6. Diagnostic approaches

15.6.1. Systemic symptoms and signs of tuberculosis

Although systemic signs and symptoms are classically ascribed to TB in medical textbooks, and are indeed very important for diagnostic suspicion, it should be kept in mind that they are nonspecific and can be present in other diseases of insidious evolution, particularly other bacterial and mycotic bronchopulmonary infections, lung cancer, and chronic diseases with lung involvement.

Fever and sweating

It is believed that bacillary multiplication increases in the afternoon, with the daily circadian rhythm cortisol peak, which is followed by the evening fever characteristic of the disease. *M. tuberculosis* multiplies at a slow pace in comparison with other bacteria and therefore the inflammatory process is moderate and is accompanied by a low-grade fever. The body responds to the evening fever with night sweats to maintain the body temperature. However, when there is massive hematogenous or endobronchial dissemination, peaks of high fever can occur at any time of the day and are accompanied by chills.

Weight loss

Consumption was the name given to TB many years ago because it appeared to consume those affected, and anorexia and weight loss are still frequent in TB patients (about 70 % of the cases). Weight loss is proportional to the duration and extent of the disease and is frequently accompanied by adynamia.

15.6.2. Respiratory symptoms and signs of pulmonary tuberculosis

Cough is present in virtually all patients with pulmonary TB. Cough results from the stimulus caused by the alveolar inflammatory process or from the granulomatous impingement into the respiratory airways. At the onset of the disease, the cough is dry; but with progression, it becomes productive with mucous or mucopurulent expectoration, generally in small amounts, and sometimes with blood. Cough is less frequent in the pleural form of the disease. It is worth mentioning that cough tends to be ignored or minimized by smokers, who may have a chronic cough, so questions about changes in the usual pattern can be of great value in increasing suspicion of pulmonary TB. In the WHO Guidelines, it is recommended that in low and middle income countries, community TB screening be performed by AFB

smear microscopy in all respiratory symptomatic persons, defined as those with a productive cough of at least three weeks duration. The diagnostic yield in this population ranges from 4 % to 10 %.

Hemoptysis

When hemoptysis occurs, the blood volume is variable, from bloody streaks mixed in the sputum (hemoptoic sputum) to massive hemoptysis (more than 400 mL/day), which is rare. A higher volume of hemoptysis is generally caused by erosion of Rasmussen's aneurysms, which are free terminations of arteries within lung cavities. Bleeding can also occur in small lesions during the formation of the cavities, when hemoptysis can be the first manifestation of the disease, which was known by the old phthysiologists as alert hemoptysis or bark.

Dyspnea

Although the inflammatory process of TB causes global parenchyma destruction of both alveoli and blood vessels, there is no gross alteration in the ventilation/perfusion ratio, except in cases of atelectasis, large cavities or lesions with a large acute inflammatory infiltration. Therefore, dyspnea is not a common symptom, but can be caused by pleural effusions, pneumothorax or restriction caused by fibrosis in advanced disease. Dyspnea may be more frequent in the miliary form, due to diffuse interstitial disease and consequent hypoxemia. An obstructive pattern of airway disease can result from the bronchial hyperresponsitivity that often accompanies TB and its sequelae.

Thoracic pain

Thoracic pain occurs when there is pleural involvement, but as the TB pathological process begins in the alveoli, very close to the pleural surface, this is an early and relatively frequent symptom. Generally of low intensity, it disappears within two or three weeks after effective treatment has begun.

Hoarseness

This occurs when the larynx is affected, which is frequent with pulmonary TB. It rarely occurs in other forms of the disease. When cough and other symptoms are overlooked by the patient, hoarseness may be the sole reason for seeking medical assistance.

15.6.3. Physical examination

Physical signs in TB are related to the extent of the lesions, the duration of the disease and the form of presentation. The longer the duration of the disease, the more evident are the classic signs of consumption, such as pallor and weight loss.

The extent and the form of the disease in the lung parenchyma determine the presence of specific pulmonary signs. The most common auscultation findings are: coarse crackles in the area corresponding to the lesion (generally apical and posterior); wheezing and ronchi in the area of compromised bronchi; clinical signs of lung condensation in the forms with caseous pneumonia; decreased vesicular murmur and broncophony or tubular blow when pleural effusion is present; as well as the classic amphoric breath sounds near cavities.

Hepatosplenomegaly can occur in the disseminated forms.

Some findings are caused by delayed-type hypersensitivity to tubercle bacilli components, although the lesions themselves do not contain *M. tuberculosis*. These TB-associated conditions are: erythema nodosum (inflammation of the subcutaneous adipose tissue), phlyctenular conjunctivitis, erythema induratum of Bazin (nodular vasculitis) and polyserositis (Figure 15-18). These lesions are mostly associated with primary TB infection, although they may also be observed in re-activation TB disease and sometimes are recurrent.

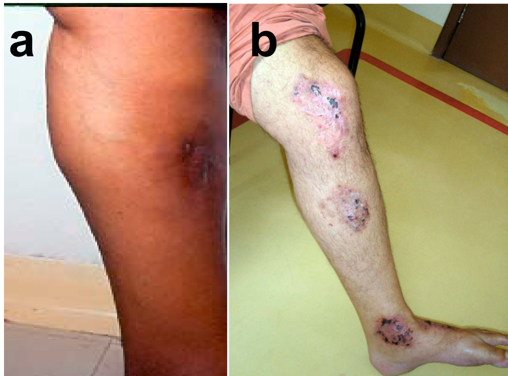

Fig 15-18: Erythema nodosum (**a**) and erythema induratum of Bazin (**b**).

15.6.4 Sputum smear microscopy and culture

The principal method of pulmonary TB diagnosis is microscopic examination of Ziehl-Neelsen stained sputum samples for AFB. In low-income countries, under routine conditions, sputum smear is positive for AFB in 30 % to 60 % of respiratory TB cases. When this is thoroughly performed on two sputum samples collected on consecutive mornings in patients with pulmonary cavities on chest X-ray and respiratory symptoms, the sensitivity of the sputum smear microscopic examination can be higher than 70 % (WHO 2005, Stop TB/WHO 2007). If the sensitivity of AFB smear microscopy does not approach these levels, the quality of the mycobacteriology laboratory or of the TB diagnosis itself should be questioned (see Chapter 12).

Sputum smear examination should be requested when (Castelo-Filho 2004, Hopewell 2006):

- the patient seeks assistance at the health services complaining of respiratory symptoms with cough and expectoration for more than three weeks
- chest X-ray alterations are present that are consistent with pulmonary TB

Whenever it is possible from an operational point of view, and especially when there is a high clinical suspicion, sputum should also be cultured for mycobacteria, as it increases the diagnostic yield by 15 % to 20 %. Cultures are most commonly performed on solid media (Löwenstein-Jensen or Ogawa Kudoh), giving results on an average of 30 days. Cultures in liquid media give faster results and may be more sensitive (see Chapter 12). Drug susceptibility testing is indicated when infection with drug resistant strains is suspected (see Chapter 19), but as suspicion for drug resistance is not always easily evaluated, susceptibility testing should be systematically performed on isolates from patients with associated risk factors:

- previous history of anti-tuberculosis treatment
- failure of chemotherapy given with direct supervision
- contact with patients with multi-drug resistant TB [*M. tuberculosis* strain resistant to at least rifampicin (RIF) and isoniazid (INH)].

15.6.5. Induced sputum

When the patient does not produce expectorant, it is advisable to induce sputum by nebulization with hypertonic (3 % to 5 %) saline solution. Recent studies showed that induced sputum has a diagnostic yield equal to or higher than that of material

collected by fiberoptic bronchoscopy. When miliary TB is suspected but the smears are negative for AFB, fiberoptic bronchoscopy with bronchial biopsy is recommended for a definitive diagnosis (Al Zahrani 2001).

15.6.6. Radiological examination

The chest X-ray examination may help to make the diagnosis in respiratory symptomatic patients that are repeatedly negative on direct microscopy sputum examination. It may also help in those individuals that cannot produce sputum for the bacteriological examination. In patients with positive smear microscopy, the chest X-ray exam may be indicated to exclude an associated lung disease, and also allows the evaluation of the disease evolution, especially in patients not responding to TB treatment (Rottenberg 1996).

Initial chest X-ray studies should include posteroanterior and lateral views. Lordotic and oblique views may be helpful for further evaluation of the extent of lung involvement, especially in patients with apical lesions or extensive hilar adenopathy (Figure 15-6). If pleural effusion is present, lateral decubitus views may aid determination of the nature of effusion (i.e. free moving, loculated) (Correa 1997, Schluger 1994, Vallejo 1994).

The results of the chest X-rays may be described as:

- **Normal**: absence of pathological images in the lung fields
- **Sequelae**: presence of images suggestive of old scarred lesions
- **Suspect**: presence of images suggestive of active TB
    - single or multiple condensations in the upper third of one or of both lungs and in the apical segment of the lower lobe
    - cavities in the upper third or in the apical segment of the lower lobe
    - unilateral or bilateral pleural effusion
    - miliary pattern
- **Other diseases**: presence of images suggestive of non-tuberculous pneumopathy.

15.6.7. Computerized tomography and ultrasonography

Computerized tomography of the chest is mainly used for diagnosis of pulmonary TB in patients who do not expectorate or that have a negative AFB sputum smear, and are suspected of having tuberculous lymphadenitis or miliary TB. It can also be useful for the differentiation of pulmonary sequelae due to old, inactive TB from active disease. Also, when the presence of an associated lung cancer is suspected, high-resolution computerized tomography with the analysis of the secondary lung lobule becomes an important diagnostic aid (Sinan 2002, Busi-Rizzi 2003).

Both abdominal computerized tomography and ultrasonography are useful for the investigation of fever in HIV/AIDS patients suspected of having disseminated TB. Visceral focal lesions and heterogeneous, low density adenomegalies are common in patients with TB and HIV/AIDS (see Chapter 17). In diseased lymph nodes, pathological images are preferentially located in the peripheral areas. Computerized tomography of the skull in patients with cerebral TB reveals hydrocephaly in 60 % of cases, lesions with hypodense central areas and "ring image" in 45 %, and atrophic lesions or lack of alteration in 30 % of cases.

15.6.8. Tuberculin skin testing

A positive tuberculin skin testing (TST) only indicates infection and by itself is not diagnostic of TB disease (Huebner 1993, Menzies 1999, WHO 1999, Castelo 2004). The tuberculin, PPD RT23, in a 0.1 mL dose, equivalent to 2 TU (tuberculin units), or PPD-S, equivalent to 5 TU, is applied intradermally in the medium third of the anterior surface of the left forearm. When kept at a temperature between 4°C and 8ºC, tuberculin remains active for six months, but it should not be frozen or exposed to direct sunlight.

The TST test is read 72 to 96 hours after its application, measuring the largest transverse diameter of the palpable hardened area with a millimeter ruler.

Interpretation of tuberculin skin test results

In developed nations, TST induration is interpreted on the basis of a ruler showing 5, 10, and 15 mm divisions. For persons exposed to highly contagious TB patients, or HIV infected persons, with a history of previous TB, or with fibrotic images consistent with TB on chest radiography, a reaction equal or greater than 5 mm diameter is classified as positive. For other groups, the cut-off of 10 mm has been proposed.

Table 15-1: Tuberculin skin test cut-off of reactive area for a positive tuberculin reaction

Cut off area (mm)		
≥ 5 mm	**≥ 10 mm**	**≥ 15 mm**
Contacts of infectious case with or without symptoms	Residents or professionals at hospitals, shelters, prisons, long-term facilities, mental institutions, nursing homes	Persons who do not meet any of these criteria
Fibrotic image on chest radiography consistent with TB	Seronegative intravenous drug use	
HIV infected	At higher risk of TB development: high-dose corticosteroid therapy, bone marrow and solid organ transplant, chronic renal failure, lymphoma, Hodgkin disease, diabetes mellitus, head and neck carcinoma, weight loss of ≥ 10 % below ideal body weight	

In low and middle income countries, the tested persons may be grouped according to the resulting induration registered in millimeters:

- **non reactor** (0 to 4 mm induration): not infected with *M. tuberculosis* or on immunosuppression
- **weak reactor** (5 to 9 mm): vaccinated with BCG or infected with *M. tuberculosis* or other mycobacteria
- **strong reactor** (10 mm or more): infected with *M. tuberculosis*, with or without clinical disease

Positive TST decreases as the interval between vaccination and tuberculin skin testing increases (because vaccination-induced reactivity wanes over time and is unlikely to persist for > 10 years). It has been proposed by some authors from developing nations, that TST indicates infection with *M. tuberculosis* when the induration area is greater than 10 mm in adults not vaccinated with BCG or those vaccinated more than two years prior to the examination; or the induration is greater than 15 mm in adults vaccinated with BCG less than two years prior to the examination (Castelo 2004). In adults who have been revaccinated with BCG, interpretation is difficult. In summary, for high burden countries, the TST should be recommended:

- in contacts of smear-positive TB cases
- in persons with radiographic or clinical findings consistent with TB, especially extrapulmonary forms
- in HIV infected persons
- as part of the medical examination of all health personnel.

In some circumstances, the sensitivity of the TST can be reduced, for instance:

- Immunosuppressive diseases, such as sarcoidosis, HIV/AIDS, head and neck cancer, lymphoproliferative diseases and other neoplasias
- Transient or age-related immunodeficiencies, such as after live virus vaccination, pregnancy, corticosteroid and immunosuppressive therapy, children less than two months old and persons above 65 years old.

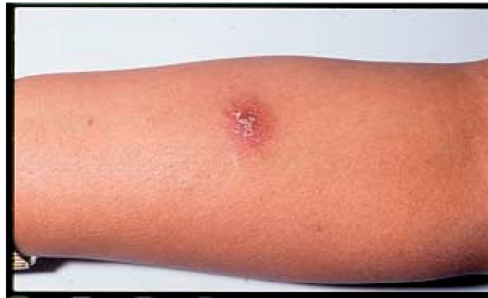

Figure 15-19: Phlyctenular tuberculin reaction with 2UT of PPD RT-23

15.6.9. The use of alternative methods for diagnosis of latent TB.

IFN-γ release assays are alternatives to TST now available for detection of latent TB. They have been used in developed nations, but data about the evaluation of its usefulness in high burden countries are scarce (Oxlade-2007, see Chapter 13).

15.6.10. Use of nucleic acid amplification for TB diagnosis

In industrialized countries, the advent of rapid nucleic acid amplification (NAA) tests for tuberculosis is seen as a major breakthrough in the management of PTB (Brodie 2005). Several commercial NAA tests have undergone validation and are licensed for routine testing of sputum. They have specificities of > 95 % for smear-positive specimens, but sensitivities are variable, especially in smear-negative disease, where a rapid diagnostic test is most needed. *In house* NAA tests have been proposed for developing countries due to their low cost. In a recent meta-analysis of *in house* NAA tests for the detection of *M. tuberculosis* in sputum specimens, the use of *IS6110* as the target appeared to be associated significantly with higher diagnostic accuracy (Flores 2005).

The majority of studies on the use of NAA for TB diagnosis have been performed in industrialized countries, where there is low burden of TB/HIV and history of pulmonary TB, and where false positive NAA results may occur due to *M. tuberculosis* DNA detection in the absence of active TB (Sloutsky 2004). Few series have estimated the potential clinical utility of these tests in relation to different levels of clinical suspicion and pretest probability (Cantazaro 2000).

For developing countries, where almost 100 % of smear-positive results represent TB disease, it has been emphasized that NAA tests increase sensitivity among smear-negative pulmonary TB cases. In these settings, the published evaluations of NAA techniques for smear-negative TB diagnosis have mainly been based on laboratory criteria for the diagnosis of disease (Kivihya-Ndugga 2005), with or without the use of clinical records to evaluate discrepant results (Laifer 2004). In addition, it has been emphasized that many laboratories in those countries did not use adequate quality controls to evaluate the performance of PCR (Suffys 2000).

Automated NAA or *in house* PCR might be more widely introduced in developing nations only after a proper evaluation of cost-effectiveness, together with analysis of clinical and radiographic characteristics, to refine estimates of the likelihood of TB disease in those settings, as proposed by others (Van Cleef 2005).

15.7. Treatment of latent tuberculosis infection

Treatment of latent TB infection with INH (10 mg/kg/day or, at most, 300 mg/day) for six to nine months is indicated in every adult person with a high risk of developing active disease (International Union Against Tuberculosis and Lung Disease 1994, Horsburgh 2004, Castelo-Filho 2004, Hopewell 2006).

The treatment of latent TB with INH reduces the risk of developing disease from endogenous reactivation, but it does not protect against exogenous exposure. Therefore, when there is the possibility of recent new exposure to the tubercle bacillus, the patient must be evaluated for the need to extend the treatment of latent TB (when receiving INH) or the instauration of a new treatment (in case the previous one has already been discontinued).

The candidates for treatment of latent TB infection are:

- household contacts of AFB smear-positive pulmonary TB patients, who have not recently been vaccinated with BCG and who have TST induration > 10 mm; or contacts that were BCG vaccinated within the previous two years with TST induration > 15 mm
- individuals with TST conversion (a positive test with > 10 mm induration after a previously negative test, applied 12 months earlier)
- HIV-infected individuals with a reactive TST of > 5 mm
- HIV-infected individuals that report close contact with a smear-positive TB patient, regardless of the TST response
- individuals with a chest X-ray image consistent of residual TB, without a history of previous anti-TB treatment

In every case, before starting preventive chemotherapy with INH, the physician should confirm the absence of active TB disease:

- the chest X-ray should be normal
- the individual should be asymptomatic

Before beginning preventive chemotherapy, it is important to exclude active TB, either pulmonary or extrapulmonary, particularly in patients with moderate/severe immunodeficiency. Moreover, appropriate follow-up of the patient is necessary to ensure a regular drug supply and at least 70 % adherence to the preventive treatment regimen.

Risk-benefit analyses demonstrated cost effective benefits for INH preventive therapy of low risk reactors and substantial cost effective benefits in higher risk reactors (Rose 1992, Jordan 2001), in addition to substantial long-term public health

benefits (Salpeter 1993). However, the overall effectiveness of INH preventive therapy in low or middle income countries has not been well established.

15.8. Contact tracing and control

Even for developed nations, competing demands restrict the resources that can be allocated to contact tracing. Therefore, public health officials must decide which contact investigations should be assigned a higher priority (Guidelines for contact investigation 2005). The WHO proposes that the control of contacts and treatment of latent TB should be considered a priority only in areas of high prevalence of TB and HIV co-infection, where TB control programs are using DOTS, and have cure rates higher than 85 % or defaulting of treatment lower than 5 %. A decision to investigate an index patient depends on the presence of factors used to predict the likelihood of transmission. When exposure is related to households, congregate living settings, or cough-inducing medical procedures, contacts are designated as high priority. Household contacts are defined as anyone who lived with a patient with pulmonary TB during their symptomatic phase, or shared the same bedroom at least once a week. The contacts of AFB smear-positive patients are at higher risk of becoming ill with TB and therefore a priority for active case finding (Arnadottir 1996, International Union Against Tuberculosis and Lung Disease 1991, Reichler 2002, Guidelines for the Investigation of Contacts 2005). In regions with high TB prevalence, assuming that the threshold for acquiring TB infection is 10 % means that 50 hours of contact with index cases could be used as a limit.

In the last years, it has been demonstrated that TST results helped identify persons in a BCG-vaccinated population who had had recent exposure to persons with pulmonary TB, were probably infected with *M. tuberculosis*, and could benefit from treatment of their latent TB (Garcia Sancho 2006).

The following contacts could be investigated with TST, chest X-ray and smear microscopy/culture, according to each group included in contact investigation procedures

- Household contacts of AFB smear-positive pulmonary TB and larynx TB patients
- Contacts under 15 years old (particularly < 5 years) and over 60 years old in settings where DOTS has not yet been well implemented
- Contacts with conditions that predispose them to develop the illness (HIV/AIDS, severe malnutrition, diabetes mellitus, patients under pro-

longed corticosteroid therapy or other immunosuppressive drugs, and patients with primary immunodeficiency)

- Contacts at congregating living settings, such as healthcare workers or medical students that report contact with AFB smear-positive pulmonary TB cases in their routine activities in the absence of biosafety measures

15.9. The limits between infection and disease

In developed countries is fairly easy to distinguish TB infection from TB disease. TB infection is characterized by the presence of a positive TST in the absence of symptoms and/or progressive lesions consistent with TB disease. This classification is useful for control strategies in areas of low prevalence of infection and low incidence of new cases. Chemoprophylaxis is indicated for recently infected persons in high risk groups in order to protect them from primary TB.

The application of such control strategies is very difficult in low and middle resource countries with high rates of infection and high incidences of new infectious TB cases. Wide-scale TST and chemoprophylaxis for all tuberculin-positive individuals would be neither innocuous nor cost-effective. Large scale TST testing demands a sophisticated system of production, quality control, distribution, handling and application of reagents. This strategy is not feasible in low resource countries where health attention systems have scarce economic, operational and human resources. In such countries, contact investigation should be introduced more widely after an evaluation of its cost-effectiveness and refined estimates of the likelihood of TB infection and TB disease in different settings.

In summary, in low and middle income countries, whenever possible, the control of the contacts should be done during the follow-up period of the index case, and at least all contacts younger than 15 years old (or particularly under 5 years old) with a TST > 10 mm (if not vaccinated with BCG or vaccinated more than two years before), asymptomatic and with normal chest X-ray, should be given prophylactic INH for at least six months.

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Chapter 16: Tuberculosis in Children

Nora Morcillo

16.1. Introduction

The incidence and prevalence of pediatric tuberculosis (TB) worldwide varies significantly according to the burden of the disease in different countries. It has been estimated that 3.1 million children under 15 years of age are infected with TB worldwide. According to the World Health Organization (WHO), children with TB represent 10 % to 20 % of all TB cases. The majority of these cases occur in low-income countries where the prevalence of Human Immunodeficiency Virus/Acquired Immunodeficiency Syndrome (HIV/AIDS) is high. TB occurs frequently among disadvantaged populations, such as malnourished individuals, and those living in crowded areas. According to WHO reports, India, China, Pakistan, the Philippines, Thailand, Indonesia, Bangladesh, and the Democratic Republic of the Congo account for nearly 75 % of all cases of pediatric TB (World Health Organization 2006, Dye 1990). Furthermore, it has also been reported that TB is responsible in Sub-Saharan countries for between 7 % and 16 % of all episodes of acute pneumonia in HIV-infected children, and for approximately one fifth of all deaths in children presenting with acute pneumonia (Chintu 2002, Jeena 2002).

On the other hand, in developed countries such as the United States (US), while an increase in the incidence of TB of approximately 13 % was reported in all ages from 1985-1994, the rate among children younger than 15 years old increased by 33 %. This was mainly attributed to the HIV epidemic, which increased the risk of developing active TB among persons with latent TB infection and HIV co-infection (American Thoracic Society/Centers for Disease Control and Prevention 2001, Taylor 2005). As in adults, TB equally affects children of both genders (males and females), but an increased risk of mortality exists at the extremes of age. Therefore, young children and especially newborns are at a high life risk when they are exposed to a contagious source (Dye 1999). Since most pediatric cases occur due to a rapid progression of a recent infection with a short incubation period, this implies a high rate of recent transmission in the community. Therefore, the infected and ill children in the community are an indirect, useful parameter for assessing the impact of Tuberculosis Control Program activities.

Pulmonary TB in children has a low bacillary load and cavities are also rarely present. Children also lack the forceful cough mechanism seen in adults. Adolescents and older children are important exceptions since their disease closely resembles

that of adults. In these cases, the disease is frequently associated with unfavorable conditions, such as bad nutrition (Correa 1997).

Most risk factors for the acquisition of TB are usually exogenous to the patient. Thus, the likelihood of being infected depends on the environment and characteristics of the index case. However, the development of active disease also depends on the inherent immunologic status of the host (Alcaiis 2006, Alet 2003).

16.2. Etiology, transmission and pathogenesis

In about 95 % of cases, TB is an airborne disease, transmitted by particles, or droplet nuclei that are expelled when persons who have pulmonary or laryngeal TB sneeze, cough, speak or sing (Feja 1999). When the recipients are persons without previous natural contact with *M. tuberculosis,* the infectious process is denominated primary infection. When this infection evolves to the disease, it is called primary TB (Vallejo 1994).

Droplet nuclei containing between one to 10 bacilli and a diameter close to 10 µm are expelled with the cough, suspended in the air and transported by air currents. Normal air currents can keep them airborne for prolonged periods of time and spread them throughout rooms or building. Some of these droplet nuclei, usually larger than 10 µm, are inhaled and anchored in the upper respiratory tract (Wells 1995). The mucus and the ciliary system of the respiratory tract avoid further progression of mycobacteria.

The effective infective droplet nucleus is very small; measuring 5 µm or less, it is able to avoid the mucus and ciliary system action and produce the anchorage in bronchioles and respiratory alveoli. The small size of the droplets allows them to remain suspended in the air for prolonged periods of time. Although theoretically a single organism may cause disease, it is generally accepted that about five to 200 inhaled bacilli are necessary for a successful infection. After inhalation, the bacilli are usually installed in the midlung zone, into the distal and subpleural respiratory bronchioles or alveoli.

Subsequently, alveolar macrophages phagocytose the inhaled bacilli. However, these first macrophages are unable to kill mycobacteria and the bacilli continue their replication inside these cells. Logarithmic multiplication of the mycobacteria takes place within the macrophage at the primary infection site. Thereafter, transportation of the infected macrophages to the regional lymph nodes occurs leading to the lymphohematogenous dissemination of the mycobacteria to other lymph nodes and organs such as kidneys, epiphyses of long bones, vertebral bodies, jux-

taependymal meninges adjacent to the subarachnoid space, and, occasionally, to the apical posterior areas of the lungs. In addition, chemotactic factors released by the macrophages attract circulating monocytes to the infection site, leading to their differentiation into mature macrophages with increased capacity to ingest and kill free bacteria (Correa 1997, Starke 1996, Vallejo 1994).

Two or three weeks after the initial *M. tuberculosis* infection, a cell-mediated immune response is fully established. While CD4+ T helper cells activate the macrophages to kill the intracellular bacteria and finally cause epithelioid granuloma formation, CD8+ suppressor T cells lyse the infected macrophages, resulting in the formation of caseous granulomas with central necrosis. Due to the fact that mycobacteria are not able to grow under the adverse conditions of the extracellular environment, most infections are controlled by the host immune system. The only evidence of a real and effective infection is a positive TST (Correa 1997, Seibert 1932, Seibert 1934). However, the initial pulmonary infection site, which is denominated "primary complex or Ghon focus" and its adjacent lymph nodes, sometimes reach sufficient size to develop necrosis and calcification demonstrable by radiographs (Feja 2005, Schluger 1994).

As in adults, childhood TB is mostly due to *M. tuberculosis.* The proportion of both pediatric and adults TB cases caused by *M. bovis* is very low. It is generally associated with close contact with cattle, and is variable from one country to another and even from region to region inside the same country (see Chapter 8).

Bacille Calmette-Guérin (BCG) vaccination applied to newborns reproduces a natural infection under controlled conditions in an attempt to avoid a first productive contact - leading to a severe disease - between children and virulent *M. tuberculosis* strains spread in the community. During further contacts with *M. tuberculosis* from a natural infectious source, a child's immune system, already prepared by BCG vaccination, will be more capable of controlling this new infection. BCG vaccination can sometimes cause a disease clinically indistinguishable from TB, but this usually occurs in patients with severe impairment of their immune system (Jacobs 1993, Vallejo 1994, see Chapters 8 and 10).

16.2.1. Infection acquisition

Most pediatric TB cases can be traced to a household relative contact. In general, it is believed that the younger the child with a positive tuberculin skin test (TST), the higher the probability of an infectious source within the home. This situation occurs when repetitive or constant contact with the infectious source - generally fam-

ily members - takes place. Therefore, when a child is diagnosed, a search should be performed for an adult case with a high bacillary load in the respiratory tract (Alet 1986). On the other hand, older children may become infected from an external source, such as schoolmates, team leaders or young adults outside the home.

The presence of extensive pulmonary lesions, such as cavities, is the most important individual human factor in determining the infectious power, since these lesions are associated not only with an important concentration of oxygen that allows active bacillary multiplication, but also with a rapid pathway to the external environment. The amount of bacilli released into the atmosphere under these conditions is enough to produce the transmission from person to person (Correa 1997, Schluger 1994).

The degree of pulmonary involvement is another important factor, since the extension of the lesions is related to the bacillary load, the intensity and frequency of coughing, and the number of cavities that may propagate these bacilli. Rarely, nonpulmonary localization of the disease with high infectious power, such as the laryngeal form, becomes an infectious source. In this case, simple actions such as talking can cause the elimination of an important amount of mycobacteria (Correa 1997).

Socioeconomic factors as well as the overcrowded living places in urban areas increase the risk of infection allowing larger contacts with infected persons. Race may not be considered an independent risk factor (Brailey 1996).

The infectious capacity of the source case is also associated with the virulence of the bacilli.

Environmental factors also contribute to the likelihood of acquiring the infection. The concentration of bacilli depends on ventilation of the surroundings and exposure to ultraviolet light. Thus, overcrowding, congregation in schools, poor housing and inadequate ventilation predispose individuals to infection and development of TB (American Academy of Pediatrics 2003).

16.2.2. Infection development

Defects in the level of immunocompetence, especially in cell-mediated immunity, such as HIV infection, are major determinants for development of TB. In general, TB case rates for persons who are co-infected with HIV and *M. tuberculosis* exceed the lifetime risk of persons without HIV co-infection. It has been estimated that for

patients with HIV infection, the risk of developing TB is 7 % to 10 % per year (American Thoracic Society 2000, see Chapter 17).

Children under steroid therapy, cancer chemotherapy, and hematological malignancies have an increased risk of developing TB. Something similar happens with malnutrition, which interferes with cell-mediated immune response and therefore accounts for much of the increased frequency of TB in impoverished patients.

Other infections, such as measles, varicella, and pertussis, may activate quiescent bacilli with subsequent TB development. Individuals with certain human leukocyte antigen types and hereditary factors, including the presence of a *bcg* gene, seem to have a predisposition for TB acquisition (American Thoracic Society 2000, Caminero 2003, see Chapter 6).

In pediatric TB, it is possible to clearly distinguish among three basic stages: exposure, infection, and disease. From a public health point of view, these stages have absolutely different transmission implications and epidemiologic consequences.

Exposure is related to the fact that the child has been in contact with adolescents or adults with suspected or confirmed contagious pulmonary TB. Household is the most frequent setting for exposure although several places that allow a close contact with potentially contagious adults such as school, day care centers and other environments become occasional exposure places. During the 18th century, the "familial hypothesis" raised by the occurrence of familial clustering, dominated medical thinking. However, it was not until the '30s that rigorous epidemiological studies provided solid evidence for the contribution of genetic factors in addition to exposure in the development of TB (Alcaiis 2006).

In populations that do not include BCG vaccination as part of the infant vaccination scheme, the TST is negative during the exposure period, the chest radiograph is normal, and there are neither signs nor symptoms of disease (World Health Organization 1982). Since a positive TST may take up to three or four months to develop from the time of infection, it is not possible to be precise about whether the child is truly infected during this period.

The hallmark of TB infection is a reactive TST in the absence of signs or symptoms of the disease, and in the presence of a chest radiograph that could be either normal or showing only a granuloma-compatible image (Correa 1997, Starke 1993).

Infection is then clinically different from disease. Disease is the presence of signs and symptoms or radiographic abnormalities after the infection. In adults, the distinction between infection and disease becomes less difficult because the latter may

be the result of dormant bacilli acquired during a past infection. In children, the distinction may not be so clear because the disease more often progresses from an initial or primary infection. From a practical point of view, adults with TB almost always manifest significant radiographic abnormalities and/or clinical symptoms, whereas up to 50 % of pediatric patients may remain asymptomatic with subtle abnormalities on the chest radiograph. Sometimes, erythema nodosum may be the only clinical finding in a child recently infected with *M. tuberculosis* (Jacobs 1993, Centers for Disease Control and Prevention 1999).

16.3. Primary pulmonary tuberculosis

Unfortunately, children younger than five years old may develop disseminated TB in the form of miliary disease or tuberculous meningoencephalitis before the TST result becomes positive. Thus, a very high index of suspicion must be adopted when pediatric patients have a contact history. Children with asymptomatic infection usually have a positive TST result but do not have any clinical or radiographic manifestations. These children may be identified on a routine medical examination, as children who have recently emigrated from a high prevalence country, or adopted children, or they may be identified subsequent to TB diagnosis in household or other contacts (Centers for Disease Control and Prevention 1999, Saltik 1991).

Pulmonary TB in children can range from an asymptomatic primary infection to a progressive primary TB. Primary TB is very often characterized by the absence of signs on clinical evaluation. Asymptomatic presentations are more common among school-age children (80-90 %) than in infants less than one year old (40-50 %) (Correa 1997, Vallejo 1996).

Disease should be suspected if the child has been exposed to a contagious source and if the TST is positive. In contrast to pulmonary TB in adults, the TST following standard procedures is an important element for TB diagnosis in children. Sometimes these patients are identified by a positive TST that may be associated with allergic manifestations such as erythema nodosum and phlyctenular conjunctivitis. Erythema nodosum is a toxic allergic erythema with nodular lesions in the skin or under it, 2 to 3 cm large. These lesions are spontaneously painful and very painful under pressure, and are usually located bilaterally in feet and legs. The erythema nodosum is usually accompanied by pharyngitis, fever and joint inflammation and is more frequent in girls over six years. Phlyctenular conjunctivitis is an allergic keratoconjunctivitis characterized by the presence of small vesicles that usually evolve to ulcers and resolve without scars. The more frequent symptoms

associated to the phlyctenular conjunctivitis are photophobia and an excessive lacrimation (Peroncini 1977).

Progression of the primary infectious complex may lead to enlargement of hilar and mediastinal lymph nodes with resultant bronchial collapse. Progressive primary TB, which is considered to be a serious form of the disease, may develop when the primary focus cavitates and bacteria spread through contiguous bronchi. Lympho-hematogenous dissemination, especially in young patients, may lead to miliary TB when caseous material reaches the bloodstream from a primary focus or a caseating metastatic focus in the wall of a pulmonary vein (Weigert focus). Tubercular meningoencephalitis may also result from hematogenous dissemination (Newton 1994, Smith 1992).

When the disease is controlled by the host immune system, those bacilli spread by the bloodstream may remain dormant in all areas of the lung or other organs for several months or years. Afterwards, in adult life, a progression to the disease may occur from an endogenous reactivation. Primary TB includes various presentations of the disease as described in the following sections.

16.3.1. Endobronchial tuberculosis

This form of pulmonary TB occurs when the infected lymph nodes erode into a bronchus. Enlargement of lymph nodes may result in signs suggestive of bronchial obstruction or hemidiaphragmatic paralysis. Dysphagia due to esophageal compression may be observed. Vocal cord paralysis may also occur as a result of local nerve compression.

A partial or complete bronchial obstruction can also occur. Usually it is the result of deposition of caseous material within the lumen. Obstructive hyperaeration of a lobar segment or a complete lobe is less common in pediatric patients while cavities, bronchiectasis and bullous emphysema are occasionally seen. Even in the presence of extensive pulmonary disease, many older children are asymptomatic at the time of diagnosis. In general, however, children are more likely to present with wheezing, cough, fever, and anorexia as part of the symptoms (Lincoln 1958, Starke 1996, Vallejo 1995).

Persistent cough may be indicative of bronchial obstruction, while difficulty in swallowing may result from esophageal compression. Hoarseness or difficult breathing may suggest vocal cord paralysis.

16.3.2. Progressive primary pulmonary tuberculosis

Progression of the pulmonary parenchymal component leads to enlargement of the caseous area and may lead to pneumonia, atelectasis, and air trapping. This is more likely to occur in young children than in adolescents. The child usually appears ill with symptoms of fever, cough, malaise, and weight loss.

This form presents classic signs of pneumonia, including tachypnea, dullness to percussion, nasal flaring, grunting, egophony, decreased breath sounds, and crackles.

16.3.3. Pleural involvement

Pleural effusion due to TB usually occurs in older children and is rarely associated with miliary disease. Typical history reveals an acute onset of fever, chest pain that increases in intensity on deep inspiration, and shortness of breath. The pain accompanies the onset of the pleural effusion, but after that the pleural involvement is painless. Fever usually persists for 14-21 days.

The signs of pleural effusion include tachypnea, respiratory distress, decreased breath sounds, dullness to percussion, and occasionally, features of mediastinal shift.

16.3.4. Reactivated pulmonary disease

Chronic pulmonary or adult-type TB is rare in children. This condition generally occurs in children who are at least seven years old when they develop TB, but is more common in older children and adolescents. Usually, it has a subacute presentation with weight loss, fever, cough, and, rarely, hemoptysis.

When the primary infection has not been treated properly, the lesion can reactivate from dormant bacilli in either lymph nodes or parenchymal nodules. In contrast to primary disease, the characteristic feature of reactivation is the parenchymal involvement, which usually evolves to cavities or diffuse infiltrates, without significant radiograph changes in pulmonary adenopathies (Peroncini 1979).

Physical examination may show no abnormalities or may reveal posttussive crackles.

16.3.5. Primary tuberculosis complications

TB complications are dependent on the delay in diagnosis and start of treatment.

Miliary disease and tubercular meningoencephalitis are the earliest and most deadly complications of primary TB. Pulmonary complications of TB include the development of pleural effusions and pneumothorax. Complete obstruction of a bronchus can result if caseous material extrudes into the lumen. Bronchiectasis, stenosis of the airways, bronchoesophageal fistula, and endobronchial disease caused by penetration through an airway wall are other complications that may occur in primary TB. When dissemination of the disease occurs, perforation of the small bowel, obstruction, enterocutaneous fistula, and the development of severe malabsorption may complicate TB of the small intestine.

Pericardial effusion can be an acute complication or can resemble chronic constrictive pericarditis. Renal complications, including hydronephrosis and autonephrectomy usually do not occur in children. Paraplegia may arise as a complication of TB located in the spine (i.e. tubercular spondylitis) (American Academy of Pediatrics 2003, Correa 1997, Jacobs 1993, Lincoln 1958).

16.4. Non-respiratory disease

Non-respiratory disease implies the dissemination of the bacilli through the circulatory and lymphatic systems. Localizations other than pulmonary are more frequent in children than in adults. Extrapulmonary TB includes peripheral lymphadenopathy, miliary TB, tubercular meningitis, skeletal TB, and other organ involvement (Caminero 2003, American Academy of Pediatrics 2003, American Thoracic Society 2000).

16.4.1. Peripheral lymphadenopathy

In fact, the high tropism that *M. tuberculosis* shows to lymph nodes in children under five years old is remarkable. In the majority of these cases, the localization is intrathoracic affecting mainly the mediastinal lymph nodes. Close to 25-35 % of these forms have extrathoracic localizations, such as on the neck lymph nodes called scrofula. However, it is important to remark that when scrofula affects children under five years old, it is caused by non-tuberculous mycobacteria (NTM) in 75 % to 80 % of cases. In different geographic areas, the prevalence of NTM varies greatly, being more prevalent in hot climate regions. It has been estimated that 65 % to 80 % of children under 12 years old may be infected with *Mycobacterium*

avium complex; 10 % to 20 % with *Mycobacterium scrofulaceum;* and 10 % with *M. tuberculosis*. In contrast, more than 90 % of culture-proven mycobacterial lymphadenitis in adults and children older than 12 years are caused by *M. tuberculosis* (Johnson 1998, Saltik 1991).

Although in developed countries the scrofula presentation is mostly caused by *M. avium* and *M. scrofulaceum*, the real situation in low-income countries still remains to be elucidated. To distinguish between NTM and *M. tuberculosis* infected lymph nodes is frequently difficult; therefore, surgical dissection and culture of the biopsy material is usually necessary for both diagnostic and therapeutic reasons (Smith 1992, Starke 1995).

Patients with scrofula may complain of enlarged nodes. Fever, weight loss, fatigue, and malaise are usually absent or minimal. Lymph node involvement typically occurs between six to nine months following the initial infection. Lymphadenopathy usually involves the anterior or posterior cervical and supraclavicular nodes. Less commonly involved are the submandibular, submental, axillary, and inguinal lymph nodes. The infected lymph nodes are typically firm, non-tender, and painless, with non-erythematous overlying skin. The nodes are initially non-fluctuant. Lymph node suppuration and spontaneous drainage may occur after caseation and necrosis development (Freixinet 1995, Starke 1995).

16.4.2. Miliary tuberculosis

As was mentioned before, miliary TB can be a complication of primary TB in young children. A rapid onset of fever and associated symptoms may be observed. When the lungs are involved, respiratory signs may evolve to include tachypnea, cyanosis, and respiratory distress, so miliary TB should be considered in a child with a history of cough and respiratory distress.

Miliary TB can also develop from an extrapulmonary form, leading to a disease in two or more organs, usually the brain and liver. Infants are particularly prone to the bacilli spreading throughout their body and development of the miliary form of the disease. Both pulmonary and extrapulmonary miliary forms are particularly severe diseases (Correa 1997, Rodrigues 1993).

16.4.3. Tuberculous meningitis

This is one of the most dangerous complications of TB. Between 30 % and 50 % of children with miliary TB have meningitis at the time of diagnosis. It occurs in up to

5 % to 10 % of cases of TB in children younger than two years old. Thereafter, the frequency drops to less than 1 %.

Because of the frequent insidious onset of the disease, a very high index of suspicion is required to make a timely diagnosis. A subacute presentation can also occur within three to six months after the initial infection. The clinical presentation comprises a variety of signs and symptoms with an insidious or acute start. The signs and symptoms include low-grade persistent fever, malaise, anorexia, weight loss, fatigue, hepatomegaly, splenomegaly and generalized lymphadenopathy, alteration in consciousness and sensorium, stupor and the emergence of focal neurological signs.

As the disease progresses, a deterioration of mental status is accompanied by headache and neck stiffness, photophobia, seizures, coma, and death may occur if a proper diagnosis and early intervention are not promptly started.

Typical cerebrospinal fluid findings include a moderate lymphocytic pleocytosis, low glucose level and an elevated protein concentration. Hyponatremia caused by inappropriate excretion of antidiuretic hormone is frequently seen. Abnormal chest radiographs are seen in 50 % of children with meningitis, but TST can be negative in 40 % of children at the time of diagnosis.

Three stages of tubercular meningitis have been identified:

- in the first stage, no focal or generalized neurological signs are present. Possibly, only nonspecific behavioral abnormalities are found.
- the second stage is characterized by the presence of nuchal rigidity, altered deep tendon reflexes, lethargy, and/or cranial nerve palsies. TB meningitis most often affects the sixth cranial nerve, resulting in lateral rectus palsy. This is due to the pressure of the thick basilar inflammatory exudates on the cranial nerves or to hydrocephalus. The third, fourth, and seventh cranial nerves may also be affected. Fundoscopic changes may include papilledema and the presence of choroid tubercles, which should be carefully sought.
- the final stage comprises major neurological defects, including coma, seizures, and abnormal movements (e.g. choreoathetosis, paresis, paralysis of one or more extremities).

In the terminal phase, decerebrated or decorticated posturing, opisthotonus, and death may occur.

Patients with tuberculomas or tubercular brain abscesses may present with focal neurological signs. Spinal cord disease may result in the acute development of spinal block or a transverse myelitis-like syndrome. A slowly ascending paralysis may develop over several months to years (Correa 1997, Vallejo 1994).

16.4.4. Skeletal tuberculosis

Osteoarticular TB complications appear in 1 % to 6 % of untreated primary infections. Clinical and radiographic presentations vary widely and depend upon the stage of the disease at the time of diagnosis. Skeletal TB may remain unrecognized for months to years because of its lack of specific signs and symptoms and indolent nature.

Bone or joint TB may present acutely or subacutely. Sites commonly involved are the large weight-bearing bones or joints including the vertebrae (50 %), hips (15 %), and knees (15 %). Less common skeletal sites are the femur, tibia, and fibula. Destruction of the bones with deformity is a late sign of TB. Manifestations may include angulation of the spine or “gibbus deformity” and/or the severe kyphosis with destruction of the vertebral bodies or “Pott’s disease”.

Cervical spine involvement may result in atlantoaxial subluxation, which may lead to paraplegia or quadriplegia. TB of the skeletal system may also lead to involvement of the inguinal, epitrochlear, or axillary lymph nodes. (Correa 1997, Vallejo 1995).

16.5. Congenital tuberculosis

Congenital TB is considered a rare event in the whole spectrum of TB presentations. This infection is caused by lymphohematogenous spread during pregnancy from an infected placenta or aspiration of contaminated amniotic fluid.

Symptoms typically develop during the second or third week of life and include poor feeding, poor weight gain, cough, lethargy, and irritability. Other symptoms include fever, ear discharge, and skin lesions, failure to thrive, icterus, hepatosplenomegaly, tachypnea, and lymphadenopathy. Congenital TB diagnosis is based on clinical features and the infant should have at least one of the following proven TB lesions (Correa 1997, Cantwell 1994):

- skin lesions during the first week of life, including papular lesions or petechiae, necrotic or purpuric lesions
- choroidal tubercles in the retina
- documentation of TB infection of the placenta or the maternal genital tract
- presence of a primary hepatic complex (liver and regional lymph-node involvement)
- exclusion of the possibility of postnatal transmission

16.6. Diagnosis

16.6.1. Clinical disease evaluation

Pediatric patients with pneumonia, pleural effusion, or a cavitary or mass lesion in the lung that does not improve with standard antibacterial therapy should be evaluated for TB. This evaluation is also indicated for children with fever of unknown origin, failure to thrive, significant weight loss (more than 10 % of normal weight), or unexplained lymphadenopathy. An adequate clinical history should look for household or adult infectious cases, immigration from high prevalence countries, living in shelters or other risk factors (American Academy of Pediatrics 2003, American Thoracic Society 2000, American Thoracic Society /Centers for Disease Control and Prevention 2001, Correa 1997, Feja 2005, Jacobs 1993, Taylor 2005, Vallejo 1994).

16.6.2. Diagnostic laboratory tests in pediatric tuberculosis

The cornerstone of the diagnosis of pulmonary TB in adults is based on the demonstration of *M. tuberculosis* by means of microbiological and/or molecular methods. Pediatric TB is usually considered a paucibacillary disease, which makes bacteriological diagnosis of TB extremely challenging because of the difficulty in isolating *M. tuberculosis* from clinical specimens. This difficulty decreases as the age of the child increases. Therefore, all tools available in laboratories must be used to diagnose pediatric cases, especially in the very young.

Despite innovations in rapid diagnosis, many of the classic diagnostic tools continue to be useful in the evaluation of TB patients (Caminero 2003).

Specimen collection

The first step in detecting and isolating mycobacteria is to obtain appropriate specimens for bacteriological examination. These specimens are: sputum, gastric lavage, bronchoalveolar lavage, lung tissue, lymph node tissue, pleural fluid, bone marrow, blood, liver, cerebrospinal fluid, urine, and stool, depending on the location of the disease.

Children under 12 years old are rarely able to produce sputum and voluntarily expectorate, and therefore gastric lavage is often used to obtain a specimen in very young children (< 6 years old). The rationale for this presumes that the child has coughed up and swallowed their bronchial secretions. The use of the correct technique for obtaining the gastric lavage is important because of the scarcity of bacilli in children compared to adults. The technique requires a nasogastric tube inserted in an inpatient setting, because the sensitivity of outpatient gastric lavages has not been evaluated. Early morning samples, optimally from three consecutive days, should be obtained before the child has had a chance to eat or move, as these activities dilute the bronchial secretions accumulated during the night. Initially, the stomach contents should be aspirated, and then a small amount of sterile water injected through the nasogastric tube. This aspirate also should be added to the specimen. Since gastric acidity is poorly tolerated by the tubercle bacilli, neutralization of the specimen with 10 % sodium carbonate or 40 % anhydrous sodium phosphate should be performed immediately. Even under the best technical conditions, tubercle bacilli can be only recovered in 70 % of infants and in 30 % to 40 % of ill children.

Sputum specimens may be used in older children who are able to expectorate. Clear instructions for collecting the sputum sample must be given in order to avoid obtaining nasopharyngeal secretions and saliva, which are not acceptable for analysis. More recently, the use of induced sputum, obtained after nebulization with a hypertonic NaCl solution to provoke a productive cough, has been proposed, as it produces high yield results similar to those obtained in adults (SAP 2002). Another technique to obtain bronchial secretions is by stimulating cough using an aerosol solution of propylene glycol in 10 % sodium chloride, or by bronchoalveolar lavage. The bronchoalveolar lavage (instilling a total of 180 mL of saline solution and obtaining the sample by aspiration of the bronchial contents) is an invasive technique and requires the use of anesthesia, so its use in children must be well justified. Besides, bronchoscopy yield has been lower than properly obtained gastric lavages and its role remains controversial in evaluating pulmonary TB.

Bronchoscopy may be useful in determining endobronchial involvement and also in distinguishing *M. tuberculosis* from other opportunistic mycobacterial infections in immunocompromised patients (Abadco 1992, Alet 1986, American Academy of Pediatrics 2003, American Thoracic Society 2000, Newton 1994, Saltik 1991, Smith 1992).

Renal disease is a rare event in children, but when it is suspected, overnight urine specimens must be collected in the early morning and immediately sent for analysis, as the tubercle bacilli poorly tolerate the acid pH of urine.

Other body fluids (e.g. cerebrospinal, pleural, or peritoneal) must be centrifuged and the sediment used to prepare smears to evaluate the presence of acid-fast bacilli (AFB). Smears of cerebrospinal fluid are positive in fewer than 10 % of patients with TB meningitis. Enhancement of the yield may be possible by staining any typical clot (bride veil) formed in cerebrospinal fluid specimens. Increased yield may also be obtained from cisternal or ventricular fluid (Newton 1994, Starke 2000).

Bacteriological techniques

Finding AFB on a stained sputum smear provides a strong preliminary confirmation of TB diagnosis, especially in low-income countries where it has a high positive predictive value for TB (> 98 %). Staining can also give a quantitative assessment of the number of bacilli being excreted. Nevertheless, in children in whom bacilli in the respiratory secretions are sparse, results may be negative. In these cases, a single organism on a slide is highly suggestive and warrants further investigation.

Culture of *M. tuberculosis* is the definitive method to diagnose the disease and must be performed whenever possible in pediatric cases, because it is more sensitive than microscopic examination, and also allows species confirmation and drug susceptibility testing. Conventional cultures on Löwenstein-Jensen solid medium are commonly used in low-income countries, while automated culture methods are widely employed in high-income countries for the rapid detection and recovery of mycobacteria (Caminero 2003) (see Chapters 12 and 14).

Specimens from body sites naturally contaminated, such as sputum and urine, require a decontamination process prior to culture in order to allow the growth of mycobacteria in the culture media, without overgrowth of the commensal flora.

The culture isolation rate from body fluids in children with extrapulmonary TB is usually lower than 50 % (Correa 1997), and it is estimated that only 10 % to 20 % of all pediatric forms can be diagnosed by culture.

Molecular methods

Nucleic acid amplification methods, such as the polymerase chain reaction (PCR), have shown sensitivity and specificity greater than 90 % for detecting smear-positive pulmonary TB in adults. Although the use of this technique in children has not yet been extensively evaluated, several studies have reported sensitivity ranging from 25 % to 83 % in children with pulmonary TB (American Thoracic Society 2000). According to several reports, the sensitivity and specificity of the nucleic acid amplification methods in smear-positive cases may exceed 95 %, but the sensitivity in smear-negative cases, which includes most of the pediatric cases, varies from 40 % to 70 % (Eisenach 1990, Morcillo 2001, Saltini 1998).

To distinguish TB infection from disease has been particularly difficult with the currently available in-house and commercial nucleic acid amplification tests. Specificity is even more controversial, and false positive results have been observed in up to 20 % of controls (Smith 1996).

16.6.3. Tuberculin skin testing

The American Academy of Pediatrics has issued the following guidelines for pediatric testing (American Academy of Pediatrics 2003):

- TST is indicated in children who have been in contact with persons with active TB
- TST is indicated in immigrants from regions in which TB is endemic (e.g. Asia, the Middle East, Africa, Latin America) or children with travel histories to these regions
- TST is indicated in children with radiographic or clinical findings suggestive of TB (Arnadottir 1996, Guvenc 1993)
- Annual TST is indicated in children who are infected with HIV or those living in a household with persons infected with HIV; also in incarcerated adolescents
- Testing at two- to three-year intervals is indicated if the child has been exposed to high-risk individuals, including those who are homeless, adults who are infected with HIV, drug users, residents of nursing homes, and incarcerated adolescents or adults
- Testing in children 4-6 years old and 11-16 years old living in high-prevalence areas is indicated irrespective of the presence of risk factors

Performing an initial TST before the initiation of immunosuppressive therapy is recommended in any patient. TST application should follow the principles defined in the following paragraphs.

Mantoux technique

In the accepted protocol (see Chapter 13) for TST by the Mantoux technique, a standardized antigen preparation containing two tuberculin units of purified protein derivative (PPD) should be injected intradermally into the volar aspect of the forearm using a 27-gauge needle. The test should read by skilled personnel 48-72 hours after administration. The size of induration and not erythema must be measured by placing the ruler transversally to the long axis of the forearm (ruler-based reading).

The Mantoux test is the only skin test acceptable in children evaluation. Multiple puncture techniques should no longer be used because of its intrinsic limitations and inaccuracy (Arnadottir 1996, International Union Against Tuberculosis and Lung Disease 1991, World Health Organization 1963).

nterpretation of tuberculin skin test results

The US Centers for Disease Control and Prevention, and the American Academy of Pediatrics have made recommendations on the size of TST induration that is considered to be a positive result and indicative of disease in different groups of children (Table 16-1). The TST reactivity is interpreted on the basis of a ruler showing 5, 10, and 15 mm. divisions.

For children that have been exposed to highly contagious TB patients, a reaction equal or greater than 5 mm diameter is classified as positive. For other high-risk groups, such as children with increased environmental exposure, or those younger than four years old, a reaction equal or greater than 10 mm is a positive result. For children over 4 years of age who are not at risk of TB, a reaction equal or greater than 15 mm is a positive result (Centers for Disease Control and Prevention 2000, Centers for Disease Control and Prevention 1999).

TST false-positive reactions are often attributed to asymptomatic infection by environmental non-tuberculous mycobacteria (NTM) due to cross-reactivity. False-negative results may be caused by recent vaccination with live-attenuated virus, anergy, immunosuppression, immune deficiency, or malnutrition (Flament 1994). Other factors that may cause a false-negative result include improper administration (e.g. subcutaneous injection, injection of too little antigen), improper storage of the PPD material, and contamination. PPD has been recognized to have an initial false-negative rate of 29 % (Batra 2000).

BCG vaccination is used in all developing countries, and is not a contraindication for the TST, but differentiating tuberculin reactions caused by BCG vaccination from those attributable to *M. tuberculosis* infection is sometimes difficult. A history of contact with a person with contagious TB or emigration from a high prevalence country increases the likelihood that a TST induration of 10 mm or more is due to a true infection with *M. tuberculosis*. The reactivity caused by BCG vaccination, which is usually less than 10 mm, generally wanes with time, but multiple BCG vaccinations can perhaps cause a positive TST.

Table 16-1. Tuberculin skin test: cutoff size of reactive area for positive tuberculin reaction

Cut off area (mm)		
≥ 5 mm	**≥ 10 mm**	**≥ 15 mm**
Contact to infectious cases with or without symptoms.	Children from high prevalence countries.	Children ≥ 5 years without risk factors
Abnormal chest radiograph consistent with TB.	Residents of shelters and institutions.	
HIV co-infected and other immunosuppression.	Close contact with high-risk adults or adolescents.	
Previous TB.	At a higher risk of TB dissemination. Immunocompromised patients (lymphoma, Hodgkin disease, diabetes mellitus, malnutrition)	
Clinical evidence of active TB		
	Children < 5 years of age	

The tuberculin reactivity may be boosted after several Mantoux administrations (Guvenc 1993), and may even lead to false positive reactions, and routine annual TST in children is not used in developed countries. Cost-benefit analyses have shown that universal school-based skin testing programs are not effective in finding ill children, and the targeted screening of high risk children is more efficient and less costly than screening all students.

Children showing significant reactions to TST (≥ 10 mm) should have a thorough physical examination, a chest radiograph, and an exhaustive review of contacts for possible exposure to adults with TB or environments with a high risk for TB exposure.

When TB is discovered in a child, it is crucial to search for an infectious source, which should be properly identified, diagnosed and treated. Bacteriological diagnosis and drug susceptibility testing of the mycobacterium causing the disease in the index case is extremely important. It is often impossible to obtain a sputum from young children, so analyzing the strain isolated from the index adult case may be the only way to determine the appropriate treatment for the child (Chadna 2003, Comstock 1974, International Union Against Tuberculosis and Lung Disease 1991, Jacobs 1993).

16.6.4. Imaging Studies

Chest X-ray is a classic diagnostic tool when evaluating patients for pulmonary TB.

Radiographic manifestations

Lymphadenopathy involving the hilar and paratracheal lymph nodes is the hallmark of primary TB in children. Nevertheless, the hilar region may be difficult to evaluate by a posteroanterior radiograph view, so the systematic inclusion of a lateral view radiograph is necessary. When one or several granulomas or calcifications are detected in the lung parenchyma or hilar/mediastinal lymph nodes (primary bipolar complex), these could just be evidence of a past infection with *M. tuberculosis* and do not necessarily indicate active disease. However, the absence of calcification in the lesions lends support to the possibility of active primary disease.

A fan-shaped lesion on the radiograph is a manifestation of bronchial obstruction, leading to segmental disease characterized by atelectasis and consolidation of the involved area. Other chest radiographic observations include linear, interstitial and nodular densities, cavities with consolidation, empyema, bronchiectasis or focal masses.

Meningitis mainly affects the base of the brain. Computed tomography imaging can reveal basal cistern inflammation, hydrocephalus and meningeal enhancement, as well as focal parenchymal abnormalities, such as tuberculomas and infarction.

The radiographic findings in skeletal TB often include irregular areas of destruction, sclerosis, osteopenia, minimal periosteal reaction and slow enlargement of the focus. In adults, tuberculous osteomyelitis usually originates in the epiphysis of long bones with spread into the adjacent joint space. Instead, in children, TB typically affects the metaphysis and can intrude into the growth plate.

Other imaging tools

Computerized tomography scans and magnetic resonance imaging are other valuable tools for the diagnosis of respiratory and non-respiratory TB. In patients with pulmonary TB, these imaging studies can help demonstrate hilar lymphadenopathy, endobronchial TB, pericardial invasion, and early cavitations or bronchiectasis. However, computed tomography scans and magnetic resonance imaging are superfluous when chest radiograph findings are diagnostic.

16.7. Pediatric tuberculosis treatment

Children of five years old or younger, with proven exposure to an active case of pulmonary TB, should immediately begin treatment regardless of the TST or chest X-ray findings. The decision to empirically treat exposed older children is more controversial. There are several aspects of treatment that are markedly different in children and require special consideration, such as the availability of pediatric formulations, dosing, side effects, and follow-up (Correa 1997, Blumberg 2004).

16.7.1. Treatment of asymptomatic tuberculosis infection

The purpose of treating asymptomatic infection is to prevent the development of active disease in the future. This treatment has also been called preventive therapy, chemoprophylaxis or latent TB treatment. The decision to treat children in the different stages of TB - exposure, infection and disease - is based on the risk-benefit ratio and the side effects of the treatment. Since infected children are at a high risk of developing active disease, all infected children should receive preventive chemotherapy (Miller 1993, Starke 1995).

The risk of acquisition of TB is particularly high in very young children (< 5 years old) and in adolescents. Thus, patients in these age groups with a positive TST and no other manifestations should receive prophylactic INH therapy, but active TB must be carefully excluded prior to the initiation of preventive therapy (American Thoracic Society 1994, Comstock 1967, World Health Organization 2001).

Guidelines for the application of preventive chemotherapy vary among countries and even communities in relation to the age of the infected children (Arnadottir 1996, International Union Against Tuberculosis and Lung Disease 1991). But, in spite of age considerations, there is a general consensus about using isoniazid (INH) when the contagious source is a patient with a fully drug-susceptible TB strain (Pape 1993, Rieder 1999). It is extremely important To exclude active disease in order to avoid mycobacteria selection under drug pressure due to a chemo-

therapy based on INH alone, with the consequent development of INH-resistance (Alet 1986, Miller 1993).

In the US, before the advent of latent TB chemotherapy, the reported mortality rates for children under three years old were 16 % in African Americans and 8 % in Caucasians. In contrast, for children who received a year of daily INH, several studies reported no deaths and no disease, or a reduction of 90 % in the appearance of TB during the first year of treatment, with a protective effect lasting at least 30 years. Most of these studies used INH at 5.0 to 10.0 mg/kg/day, not exceeding 300 mg/day, in a single daily dose for one year (American Thoracic Society 1994, Comstock 1967, World Health Organization 2001).

Current recommendations for preventive therapy are based on a comparative analysis of the risk of administration of INH versus the risk of acquiring the disease. Adults with a positive TST and no clinical or radiographic manifestations, who received INH therapy, have 54 % to 88 % protection against the development of the disease, while children have been reported to have between 90 % to 100 % protection (Hsu 1995, Correa 1997).

INH is the drug of choice for prophylaxis worldwide, and is an extremely effective agent in preventing progression from infection to active disease. The only caveat is that the prevalence of primary INH resistance must be low enough to ensure that the prophylaxis will be effective. This is frequently unknown in several countries or regions, and should be evaluated before establishing a standard preventive treatment regimen in these areas. If the strain isolated from the source case is INH-resistant but still susceptible to rifampicin (RIF), this is then the recommended prophylactic agent.

Studies on the treatment of adults with latent TB demonstrated that six months of INH administration was less effective but more cost-effective than a 12-month treatment. Although there are no comparable data for children, based on the results in adults, many healthcare providers adopted the six-month regimen for children with TB infection. At present, a nine-month duration of INH treatment for children with TB infection is the general recommendation. A period of treatment of 12 months is recommended for patients with HIV co-infection.

Although there are no published studies that demonstrate the effectiveness of twice-weekly preventive therapy for TB infection, this may be justified in certain situations where the risk of progression to disease is high and non-adherence to the daily treatment regimen is suspected.

For childhood contacts of INH-resistant cases, preventive therapy with RIF is generally recommended, but specific efficacy data are still available in the literature.

Treatment for exposure

Although exposed adults are usually not treated, young children should receive chemotherapy during the exposure stage and until infection has been properly excluded.

For recent contacts of patients with contagious TB (i.e. contact within the last three months), INH chemoprophylaxis is indicated even if the TST is negative. This is especially true for contacts that are infected with HIV or for household contacts younger than five years old.

Children treated for exposure should receive at least three months of an effective drug after contact with the source has been interrupted. If TST is negative after this period (i.e. TST < 5 mm), treatment can be stopped. If TST becomes positive, the infection has occurred and treatment must be extended for a total of nine months.

Infection in newborns

Newborns may become infected through the mother or another family member with multibacillary pulmonary TB. In general, they can be treated in the same way as other exposed children, with the addition of pyridoxine to prevent neurological complications of INH treatment. There is no reason to restrict breastfeeding and contact between the infected mother and child must be encouraged.

Management of a neonate whose mother or another household contact has TB depends upon the status of the disease in the mother. The following general recommendations have been elaborated by the American Academy of Pediatrics (American Academy of Pediatrics 2003, Curtis 1984, Starke 1997):

- The mother has a positive TST and no evidence of active disease. Since a positive TST may be evidence of an unrecognized case of contagious TB within the household, careful screening and evaluation of the other members of the household should be performed. Perform a Mantoux test when the infant is aged four to six weeks and again at age three to four months. Consider giving INH (10 mg/kg/day) to the infant if the family cannot be promptly evaluated for the presence of active TB.
- The mother has active disease but is not contagious at the time of delivery. Evaluation of the infant includes chest radiograph and Mantoux test at age four to six weeks; if negative, the test must be repeated at age three to four months and again at six months. INH should be administered even if the

TST result is negative and the chest X-ray does not suggest TB. Progressive disease may not develop until six months of age.

- If the mother is receiving treatment and non-infectious, separation of the mother and infant is not necessary and breastfeeding should not be discouraged. The amount of drug in breast milk is very small, and there has been no good documentation of adverse effects, although the infant should be given pyridoxine. Mothers who have received anti-tuberculosis drugs are much less infectious than those who have not received any treatment, due primarily to the reduction in the bacillary population in the lungs (Correa 1997, Starke 1997).
- The mother has active disease and is contagious at the time of delivery. In this situation, separation of the mother and infant is recommended until the mother is no longer contagious. Thereafter, management is as described above.

Treatment of congenital infection

Congenital TB is not a frequent presentation, but if the possibility is suspected, a prompt Mantoux test and chest radiograph must be performed, and treatment of the infant begun immediately. INH should be administered until the infant is six months old, at which time TST should be repeated. If the TST result is positive, the infant should be treated with INH for a total of nine months.

Safety considerations for treatment of latent tuberculosis

Treatment of childhood TB infection with INH has proven to be very safe. The incidence of asymptomatic elevation in serum liver enzymes in children is usually lower than 2 %, and clinical hepatitis is less than 1 %. Routine tests of blood chemistry and serum hepatic enzymes are unnecessary unless the child has hepatic disease or dysfunction, or is also taking other potentially hepatotoxic drugs. Medical examinations are recommended every four to six weeks to check for adverse reactions as well as to assure adherence to the treatment. Simultaneous administration of pyridoxine is routinely prescribed only for breastfed babies, pregnant women and persons with poor dietary intake of this vitamin (Pape 1993).

Treatment of infection with a multidrug-resistant strain

The best treatment for latent TB in both adults and children infected with a multidrug-resistant strain (MDR-TB) is uncertain because it requires the use of less effective drugs that are associated with adverse reactions that are both more frequent and more severe. Careful follow-up and observation of the children is rec-

ommended, as none of the second-line drugs have been evaluated for preventive therapy. Drugs have been used in these circumstances include pyrazinamide, fluoroquinolones, and ethambutol, depending on the strain susceptibility pattern. (Correa 1997, Starke 1997).

16.7.2. Treatment of pediatric tuberculosis disease

Treatment of pediatric TB follows the same general principles as the treatment of adults. The specific therapeutic regimen should be individually designed according to available drug susceptibility testing results, the tolerance of the patients for the drugs, and the continuous supply and availability of drugs for the whole duration of treatment (Canetti 1969, Heifets 2003, American Academy of Pediatrics 2003, American Thoracic Society 1994, American Thoracic Society /Centers for Disease Control and Prevention 2001, Blumberg 2004).

Following the standard guidelines for new patients, the child must be given at least three drugs during the first phase of the treatment. As in the HIV co-infected population, injections are avoided in children when possible, and therefore streptomycin, which also has ototoxicity, is not recommended in this age group. The second phase must include two drugs, which can be administered twice a week (Table 16-2).

Table 16-2: Recommended doses in therapeutic regimens for TB in children according to the localization and seriousness of the disease (American Academy of Pediatrics 1994; American Thoracic Society 1994).

Disease localization		**1st phase (month)**	**2nd phase (month)**	**Total (month)**
Pulmonary disease	Moderate*	2 HRZ	4 HR	6
	Serious*	2 HRZE (or S)	4 HR	6
Extrapulmonary		2 HRZ	7-10 HR	9-12
Meningitis		2 HRZE	7-10 HR	9-12
Associated with HIV		2 HRZE	7-10 HR	9-12

H: isoniazid; R: rifampicin; Z: pyrazinamide; E: ethambutol; S: streptomycin.

Treatment of respiratory disease

Since the '70s anti-tuberculosis treatment has become shorter, but with more drugs included in the treatment regimen. The most commonly prescribed regimen for pulmonary TB in children is a six-month course of INH and RIF supplemented during the first two months with pyrazinamide. This intensive first phase with three drugs is followed by four months of the continuation phase with INH and RIF alone (Vallejo 1994).

Poor adherence to TB treatment can lead to relapse and to the development of drug-resistance. This problem becomes exacerbated in the pediatric population by the unavailability of pediatric formulations for all first-line drugs, the lack of symptomatology and the poor radiographic improvement commonly seen in this age group. To ensure adherence to and completion of therapy, all children must be treated under directly observed therapy (DOT), which is based on the medication delivery by a healthcare worker, a responsible family member or a school employee. DOT as an activity inside DOTS (directly observed therapy of short course) strategy, has proven to be the most effective and safest way to administer anti-tuberculosis therapy (Correa 1997, Kochi 1991, Starke 1995).

There is no data about pediatric regimens using twice-weekly treatment under directly observed therapy (DOT), but in adults these regimens have proved to be as effective and safe as daily therapy. In the published clinical trials the overall success rate has been greater than 97 % for complete clinical and radiographic cure and 99 % for significant radiographic improvement during a two-year follow-up period. The incidence of relevant adverse events, mostly gastrointestinal upset or mild skin rash, was less than 2 %.

Short courses of corticosteroids may be effective for children with enlarged hilar lymph nodes that compress the tracheal bronchial tree causing respiratory distress, localized emphysema, or severe segmental pulmonary disease. The most commonly prescribed regimen is prednisone 1 to 2 mg/kg/day for 4 to 6 weeks with gradual tapering (Starke 2004).

Special situations: HIV, drug resistance

The optimal treatment for HIV co-infected children has not yet been established, although most experts consider that the initial regimens should be the same as those for non HIV-infected children, but extended to nine to 12 months.

When it is possible to obtain cultures from older children, drug susceptibility testing should be performed. However, when an isolate can't be obtained from the child or while waiting for cultures to grow, there are situations that raise suspicions

that the child may be infected with a drug resistant strain that could compromise the efficacy of the standard treatment regimen: an unidentified contagious source, several possible contagious sources in the child's environment, or when the likely source case has a drug-resistant strain.

If the risk for initial INH or other drug resistance is significant, a fourth drug, usually ethambutol, should be given until drug susceptibility information is available. If the strain is eventually shown to be susceptible to INH and RIF the fourth drug can be discontinued (Correa 1997, Vallejo 1995, Vallejo 1996).

For pulmonary disease caused by INH-resistant but RIF-susceptible strains, a nine- to 12-month regimen containing RIF, pyrazinamide and ethambutol or streptomycin is usually highly effective. Although streptomycin is a bactericidal drug, its use in children is restricted due to its parenteral administration and ototoxicity, so ethambutol is preferred.

Treatment of children infected with multidrug resistant TB (MDR-TB) – strains, resistant to at least INH and RIF, is as difficult as in adults. MDR-TB requires the administration of three to six drugs to minimize the probability of failure and relapse, and the selection of the drugs should be based on the results of drug susceptibility testing (Palomino 2000). Drug treatment should last between 12 to 24 months, depending upon the anatomic location of and severity of the disease, and when the patient becomes bacteriologically negative, both by direct smear examination and by cultures, if possible. Drugs associated with frequent side effects, such as cycloserine and ethionamide may be started at low doses and if tolerated, gradually increased to the recommended dose. The use of fluoroquinolones in children remains controversial because of their potential for damaging cartilage growth. However, the later generation fluoroquinolones, such as moxifloxacin, have good bactericidal activity against *M. tuberculosis*, so their use in second-line treatment regimens for pediatric MDR-TB is being recommended, However, the use of fluoroquinolones must be individualized for each case to minimize the risk of cartilage damage.

Treatment of non-respiratory disease

There are virtually no controlled clinical trials comparing different treatments for extrapulmonary TB in children, but, the three-drug, six-month schemes used for pulmonary TB appear to be effective for most forms of the disease. The exceptions are bone and joint involvement and TB meningitis. For bone and joint TB, recommended treatment is for nine to 12 months, while meningitis should be treated for no less than 12 months (American Thoracic Society 1994, American Thoracic Society /Centers for Disease Control and Prevention 2001, Blumberg 2004).

Corticosteroids are useful in the treatment of some children with TB under effective anti-tuberculosis drugs and probably are used more commonly for children than adults with TB (Starke 2004). Corticosteroids are useful when the host inflammatory reaction contributes significantly to tissue damage or impairment of organ function. In these cases, the most commonly prescribed regimen is prednisone 1 to 2 mg/kg/day for 4 to 6 weeks with gradual tapering. There is convincing evidence that corticosteroids decrease mortality rates and long-term neurological sequelae in patients with TB meningitis (Starke 2004).

16.7.3. Surgical treatment

Surgical intervention in children is a rare event. Hemoptysis, though rare in pediatric cases, is the most frequent situation requiring surgical intervention, but surgery may also be indicated to remove tubercular abscesses and close bronchopleural fistulae (Freixinet 1995, Starke 1996).

16.7.4. Monitoring pediatric cases under chemotherapy

Routine examinations and drug toxicity

In children without any co-morbidity, the rate of drug-related adverse effects is low enough to make frequent, routine, biochemical monitoring unnecessary. Hepatotoxicity from INH is age related, and rare in children. However, if the child has had hepatitis or a chronic hepatic illness, it is necessary to obtain baseline serum levels of liver enzymes before initiating TB therapy. When patients or their families report any symptoms that might be attributable to the drugs, a physical examination and serum liver enzyme determinations must be performed. Two- to three-fold elevations in serum liver enzymes are common and, in the absence of other abnormal findings, do not require discontinuation of the drugs. However, the levels should be checked again after several weeks to make sure they are stable.

Mild arthralgias are usually caused by pyrazinamide and are transient, even without discontinuing the drug. Ethambutol is well known for causing blurred or altered vision and color blindness, but ophthalmologic toxicity in children has not been reported with an ethambutol dose of 15 mg/kg/day. Nevertheless, children taking ethambutol should be carefully monitored for decreased visual acuity and color blindness, although in a child less than six years old, it is hard to know if they are having visual side effects. Ethionamide often causes gastrointestinal disorders and can also cause hepatitis. Cycloserine is usually well tolerated by children but can cause changes in mood and a variety of neurological complaints. Several doctors

think that serum cycloserine levels should be monitored whenever the drug is given (Correa 1997). Only RIF is available in pediatric formulation.

Radiographic control

Chest X-rays should be obtained at the time of diagnosis and repeated one to two months after beginning treatment, to ensure that no progression or complications have occurred. When the results are satisfactory, it is not necessary to repeat the chest radiograph until the planned end of the treatment. However, chest radiographs are often not useful to verify treatment success, because radiographic improvement of pulmonary TB and intrathoracic adenitis can occur at different speeds, and is generally very slow.

The majority of children with intrathoracic adenopathy presumably attributable to TB will have abnormal radiographic images during a period ranging from one to three years after successfully completing treatment. For this reason, it is not necessary to achieve a normal chest radiograph before discontinuing treatment. If clinical improvement has occurred after six months of treatment, the drugs can be stopped and the chest radiographs repeated at 6- to 12-month intervals until they become stable (Correa 1997).

16.8. Vaccination

Although BCG vaccination has been in use since 1921, and approximately three billion doses have been administered, its efficacy continues to be debated. Several trials performed to assess the efficacy of the vaccine have produced results that vary from country to country. While controversy exists about its efficacy against pulmonary TB, it is generally accepted that BCG vaccination does not prevent infection with *M. tuberculosis* nor the development of the disease after infection (Trnka 1998). However, two meta-analyses of the various trials concluded that the vaccine is efficacious against miliary and meningeal TB (Rodrigues 1993). Therefore, the major role of BCG is the prevention of severe and life-threatening forms of TB in children (Curtis 1984, Fine 1999, World Health Organization 1982, Young 1986).

The Expanded Program on Immunization from the WHO recommends the administration of BCG only at birth. The vaccine is being used in more than 100 countries. In developed countries, such as the United States, BCG vaccination is currently recommended only in certain situations:

- when the child is exposed to persons with contagious pulmonary MDR-TB, has negative HIV and TST results, and cannot be removed from the exposure;
- the child is exposed to persons with untreated or ineffectively treated contagious pulmonary TB, has negative HIV and TST results, and cannot be removed from the exposure or treated with antitubercular medication.

From birth to two months of age, administration of BCG does not require a prior TST. Thereafter, a TST is mandatory prior to vaccination.

Adverse reactions due to the vaccine include subcutaneous abscess formation and lymphadenopathy. Contraindications to the administration of the vaccine include immunosuppressed conditions, such as primary genetic immunodeficiency syndromes or secondary immunodeficiency, for example from steroid use, and HIV infection. However, in areas of the world where the risk of TB is high, WHO recommends using the BCG vaccine in children who have asymptomatic HIV infection (Dourado 2003, see Chapter 8).

Rare complications of the BCG vaccination, such as osteitis of the epiphyses of the long bones or disseminated BCG, are generally associated with an immunocompromised status and may necessitate administration of anti-tuberculosis therapy, excluding pyrazinamide.

16.9. Prognosis of pediatric tuberculosis

The prognosis for children with TB varies according to the clinical manifestation. In general and under DOTS strategy conditions, primary TB caused by a fully drug-susceptible strain has a more than 95 % probability of being cured, but poor prognosis is associated with disseminated TB, miliary disease and tubercular meningitis. The prognosis of tubercular meningitis varies according to the stage of the disease at the time treatment is started. Stage one has good prognosis, while patients with stage three are usually left with sequelae, such as blindness, paraplegia, deafness, mental retardation, movement disorders, and diabetes insipidus. Higher mortality rates occur in children younger than five years old (20 %) and in those with a prolonged illness of more than two months (80 %) (American Academy of Pediatrics 1994, American Academy of Pediatrics 2000, Correa 1997).

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Chapter 17: Tuberculosis and HIV/AIDS

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17.1. Epidemiological background

Tuberculosis (TB) – known in the past as the "White Plague" – is an ancient and often neglected disease. Recent genetic evidence suggests that even our remote hominid ancestors, who lived three million years ago, may have suffered from TB (Gutierrez 2005). Paradoxically, the disease re-emerged in the late '80s fueled by the Human Immunodeficiency Virus/Acquired Immunodeficiency Syndrome (HIV/AIDS) pandemic. In a few years TB became – and continues to be – a leading cause of illness and death among people with HIV/AIDS in resource-poor areas of the world (Moore 2007, Quy 2007). This unexpected encounter between the ancient and the new plague is an intriguing biological issue (Heney 2006).

Taking a turn for the worse, the AIDS pandemic further promoted the emergence of multidrug-resistant TB (MDR-TB). The first AIDS-associated MDR-TB outbreaks were reported in the United States (US) in the early '90s (Frieden 1996). These were the first alarm signals of the decline of the TB control programs that were prevalent at that time not only in the US, but also in several other parts of the world. Indeed, a third epidemic has resulted from the interaction of TB and AIDS epidemics, i.e. the MDR-TB epidemic, which not only affects immunodepressed hosts, but also extends globally (Neville 1994). This is partly due to the airborne nature of TB transmission, which is so difficult to prevent, as well as to the growing waves of human migration from high to low TB prevalence areas. Today, drug-resistant TB is still threatening the efforts towards effective control of the disease worldwide (see the WHO Global tuberculosis control 2006 on the internet at http://www.who.int/tb/publications/global_report/2006/en/index.html).

An estimated 38.6 million people worldwide were living with HIV at the end of 2005. At that time, 4.1 million persons became newly infected with HIV, and 2.8 million lost their lives because of AIDS. Africa continues to be the global epicenter of the AIDS pandemic. South Africa's AIDS epidemic — one of the worst in the world — shows no evidence of declining. In this country, an estimated 5.5 million people were living with HIV in 2004 and almost one in every three pregnant women attending public antenatal clinics were HIV positive, with increasing prevalence trends. The epidemic also looks rampant in South-East Asian, East European and other Sub-Saharan African countries (see UNAIDS 2006 global

report on the internet, http://www.unaids.org/en/HIV_data/2006GlobalReport/default.asp).

A comparison between TB and HIV/AIDS statistics worldwide shows an overlap between both epidemics, mainly in Sub-Saharan Africa and South-East Asia, where a devastating synergy is observed between the kinetic of both diseases (see Chapter 7). Among all opportunistic diseases associated with HIV/AIDS, the distinctive feature of TB lies mainly in its airborne dissemination to other patients, to health-care workers and to the entire community (Pape 2004, Putong 2002, Sharma 2005). Poverty, social inequities, difficult access to public health systems, and lack of sanitary education leads to a critical public health situation that is hampering the international efforts aimed at controlling both diseases. The response of public and private health organizations to this burdensome association currently focuses on the reinforcement of TB and HIV/AIDS control activities, including a considerable increase in their budgets and in the interaction/partnership between both programs.

From the point of view of TB control, the emergence of MDR-TB, and especially of extensively drug-resistant TB (XDR-TB), has mobilized a strong partnership between public and private sectors on the international level. Global efforts brought together by an initiative of the World Health Organization (WHO) are currently being focused on the procurement of first quality drugs, the supervision of their administration and the development of new drugs (see the Stop TB Strategy on the internet at http://www.who.int/tb/strategy/en/).

17.2. Interactions between *M. tuberculosis* and HIV infection

A complex biological interplay occurs between *M. tuberculosis* and HIV in the co-infected host that results in the worsening of both pathologies. HIV promotes progression of *M. tuberculosis* latent infection to disease and, in turn, *M. tuberculosis* enhances HIV replication, accelerating the natural evolution of HIV infection (Goletti 1996, Mariani 2001, Nakata 1997, Rosas-Taraco 2006). HIV infection impairs *Mycobacterium tuberculosis*-specific IFN-gamma production, and this impairment is not reversed by anti-retroviral treatment (Sutherland 2006).

TB develops in HIV-infected hosts at a yearly rate of 8 % by either of the two pathogenic mechanisms: endogenous reactivation or exogenous reinfection (Small 1993, van Rie 1999). Eventually, both mechanisms can coexist. Indeed, it was shown that a single patient can be infected and/or re-infected with more than one strain of *M. tuberculosis* even during a single TB episode (van Rie 1999, van Rie 2005).

Unlike most other opportunistic diseases, which usually appear in the late stages of AIDS upon severe immunological impairment, TB can occur anytime during HIV infection. The clinical presentation of TB, however, differs according to the severity of the immunodepression associated with the HIV infection. Localized pulmonary disease is the most common presentation in the early stages of HIV infection. On the other hand, disseminated forms of TB, in particular TB meningitis, are more frequent in severely immunodepressed AIDS patients and, obviously, mortality in these cases is significantly higher (Whalen 1997).

17.3. Clinical characteristics

As mentioned above, the clinical presentations of TB in an HIV/AIDS patient is clearly related to the patient's degree of immunodepression, which is measured as the blood level of CD4+ T lymphocytes (Jones 1993). A level of 200 CD4+ T cells per µL represents an approximate threshold for severe immunodepression. Above this level, a complete TB granuloma is produced in response to *M. tuberculosis* infection, including multinucleated giant cells, macrophages, CD4+ and CD8+ T lymphocytes and a central caseous necrosis. On chest X-ray, the typical pulmonary localizations can be observed, often with images of lung cavitation (Figure 17-1). As in the immunocompetent host, the clinical presentation of the disease involves fever, night sweats and weight loss accompanied by productive cough with mucopurulent or hemoptoic sputum or even hemoptysis. In these early stages of HIV immunodepression, pleural and lymph node TB are the most frequent extrapulmonary localizations of the disease, whereas disseminated TB and meningitis are rarely seen.

With the decline of CD4+ T cell counts to below 200/µL, the formation of the granuloma is progressively impaired, the hematogenous and lymphatic dissemination of the disease is more frequent and the clinical picture changes drastically. The skin reaction to intradermal injection of Protein Purified Derivative (PPD) –, which is based on the cellular immune response, – is usually negative. Even in these cases with severe immunodepression, pulmonary localization is most common. However, the frequency of extrapulmonary and disseminated presentation scales up to near 50 % of cases and extrapulmonary involvement disease often coexists with pulmonary disease. The so-called "atypical" presentations are frequently observed in the chest X-ray (Figures 17-2, 17-3, 17-4) (Daley 1995). These include basal opacities, absence of cavitation, micronodular (miliary) patterns, hilar and mediastinal adenopathy, pleural and/or pericardial effusion. Still, up to 10 % of cases may present

with a normal chest X-ray, even with positive sputum acid fast bacilli (AFB) smear microscopy (Aaron 2004).

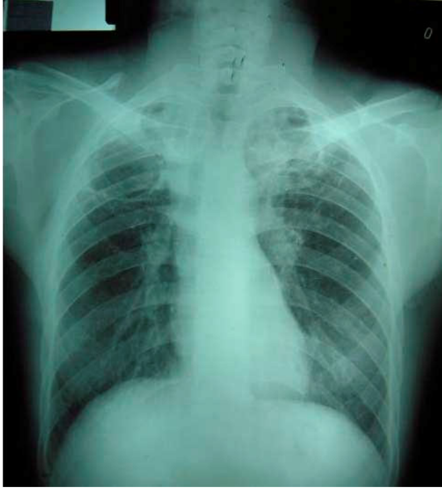

Figure 17-1: Chest X-ray of a male patient with HIV co-infection and 427 CD4+ cells/µL showing cavity images in both upper lobes.

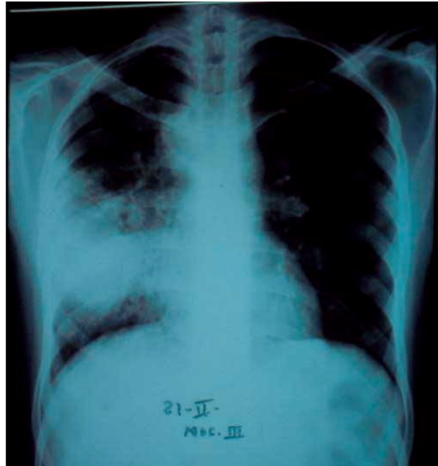

Figure 17-2: Chest X-ray of a male patient with 23 CD4+ cells/µL showing lower and medial lobe opacities with hilar and mediastinal lymph node compromise.

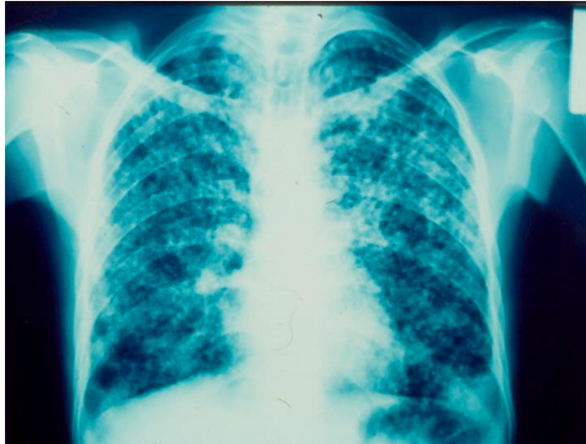

Figure 17-3: Chest X-ray of a 31 years old AIDS patient with 71 CD4+ cells/µL in blood and *M. tuberculosis* isolation from sputum: multiple pulmonary opacities in both lungs are typical of hematogenous dissemination of TB.

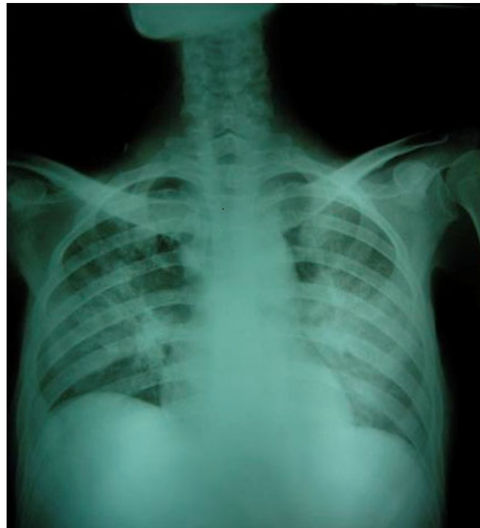

Figure 17-4: Chest X-ray showing bilateral opacities in a 27-year-old patient with AIDS and disseminated MDR-TB. On admission, he was severely ill with a CD4+ count of 23 cells/µL. The sputum smear microscopy was positive for acid fast bacilli, and *M. tuberculosis* resistant to isoniazid and rifampicin was identified in the culture.

The differential diagnosis of both typical and atypical presentations of pulmonary TB includes *Pneumocystis jirovecii* pneumonia and bacterial pneumonia. In particular, pulmonary nocardiosis closely resembles TB due to its subacute evolution and the presence of apical infiltrates with cavitation. The differential diagnosis in AIDS patients should also consider infrequent respiratory pathogens, such as *Rhodococcus equii*.

The cornerstone of TB diagnosis is the isolation of *M. tuberculosis* from tissues, fluids or secretions of the suspected patient. As pulmonary localization is the most frequent form of TB, even in severely immunodepressed AIDS patients, the respiratory secretions are the first target to examine when searching for tubercle bacilli. Sputum can be easily obtained by spontaneous cough, induced by hypertonic saline nebulization, or recovered through an early morning gastric washing after overnight fasting. Bronchoscopy is a technique that allows the visualization of the accessible respiratory tract, the obtention of bronchial washings, bronchoalveolar lavages and bronchial or transbronchial lung biopsies. Therefore, bronchoscopy offers the advantage of expanding the diagnostic spectrum to non-infectious diseases (sarcoidosis, lymphoma, endobronchial tumors).

In the advanced stages of AIDS, the most common extrapulmonary localizations of TB are serous effusions (pleurisy, pericarditis, ascites), lymphadenopathy, Pott's disease, osteomyelitis, arthritis and meningitis. Other organs may be involved, including the gastrointestinal tract, liver, kidneys, urinary tract, adrenal gland, larynx and genital (male and female) tract.

Serous effusions (pleural, pericardial and/or peritoneal) are quite frequent in HIV/AIDS patients and may be caused by various other etiological agents. TB pleurisy ranks among the most frequent cause of serous effusion, together with empyema, from which it has to be differentiated. In TB pleurisy, the aspirated fluid is exudative with a predominance of lymphocytes. Pleural biopsy and mycobacterial culture of the fluid are the most useful and specific diagnostic tools. Adenosine deaminase (ADA) levels above 50 U/L in non-purulent pleural fluid specimens have a high positive predictive value for the diagnosis of TB.

Cervical lymphadenitis is the second most frequent extrapulmonary localization of TB in AIDS patients, after pleurisy. Aspiration puncture of a swollen and fluctuant lymphadenopathy usually yields a purulent or caseous material with abundant AFB on microscopy examination (Figure 17-5).

Abdominal localizations of AIDS-associated TB (ileocecal area, peritoneum, mesenteric lymph node, liver) are the cause of unspecific presentations such as diarrhea, visceral enlargement, swollen abdomen and right lower quadrant pain. Diag-

nostic procedures such as peritoneal fluid aspiration, laparoscopy or fiber colonoscopy can be performed and provide samples for culture and biopsy.

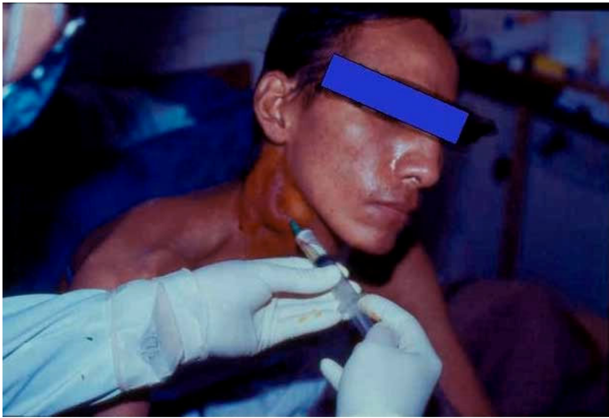

Figure 17-5: Aspiration procedure of a cervical lymphadenopathy in an AIDS patient with disseminated TB. The aspirate had a caseous aspect and was AFB smear microscopy + (10 AFB/field). Other demonstrated localizations in this case were pulmonary and a bilateral psoas abscess.

Spinal TB (Pott's disease) is a notoriously severe extrapulmonary TB presentation in AIDS patients because it can result in an accelerated destruction of vertebral bodies and intervertebral discs (Figure 17-6). The most common localizations are the thoracic and lumbosacral vertebrae, where there is risk of spinal cord compression and subsequent paraplegia. Progression through the psoas muscle can produce a cold inguinal abscess. The characteristic pain and the radiographic findings contribute to the diagnosis. The specimen for bacteriological confirmation is obtained by aspiration and/or biopsy of the affected vertebral body.

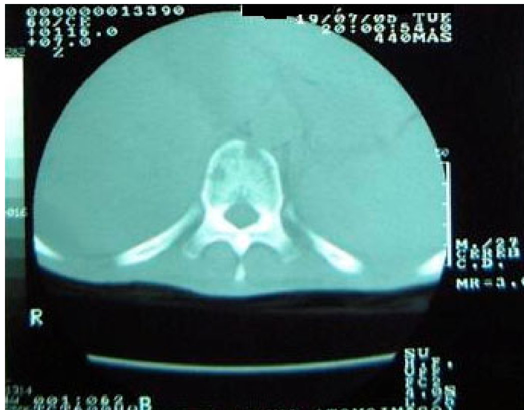

Figure 17-6: Computerized tomography scan showing an osteolytic lesion in the body of a thoracic vertebra in a patient with Pott´s disease and AIDS.

TB meningitis has a more insidious clinical presentation and higher mortality in AIDS patients than in immunocompetent patients. Headaches and mental confusion may be the first symptoms to induce the suspicion of a meningeal involvement. The classical meningeal syndrome with the Kernig and Brudzinsky signs and cranial nerve palsies, usually appears late in its evolution (Figure 17-7). The basal meninges are usually involved and cranial palsies of the 3rd and 6th nerves are common. Mono-, hemi-, or paraparesis can occur, as well as seizures. In addition to the lumbar puncture, brain computed tomography imaging is needed to rule out or confirm the diagnosis of tuberculous meningitis. The central nervous system involvement may include intracranial tuberculomas and brain abscesses that require brain biopsy and/or aspiration for bacteriological and/or histopathological confirmation. The cerebrospinal fluid is hypertensive with an elevated protein content, low glucose levels and mononuclear pleocytosis.

The differential diagnosis between meningitis caused by *M. tuberculosis* and *Cryptococcus neoformans* is extremely important in order to establish adequate treatment. Both etiological agents produce a subacute meningeal syndrome and very similar abnormalities in the cerebrospinal fluid. In most cases, however, a direct India ink coloration of the spinal fluid allows the immediate identification of the typically capsulated *Cryptococcus* cells. The culture for mycobacteria is frequently negative in tuberculous meningitis and the value of other diagnostic methods, such as adenosine deaminase dosage or PCR, is questionable. Therefore, many patients are empirically treated upon clinical suspicion of TB meningitis in view of its somber prognosis. Sequels, including cranial nerve palsy, deafness, hydro-

cephalus, altered mental status and paresis or paralysis are common in AIDS patients who survive to develop tuberculous meningitis.

Enlargement of the liver and spleen is often indicative of hematogenous dissemination of *M. tuberculosis*. Multiple nodular lesions (microabscesses) in both organs can be detected as hypoechoic images on the ultrasound ecography (Figure 17-8) and also on computed tomography scans. Another consequence of the hematogenous spread is the above-mentioned meningeal involvement, which has a poor survival prognosis (Berenguer 1992, Sanchez Portocarrero 1996, Cecchini 2007). Retroperitoneal, multiple adenopathies and psoas abscesses can be diagnosed by ultrasonography or computed tomography guided aspirate.

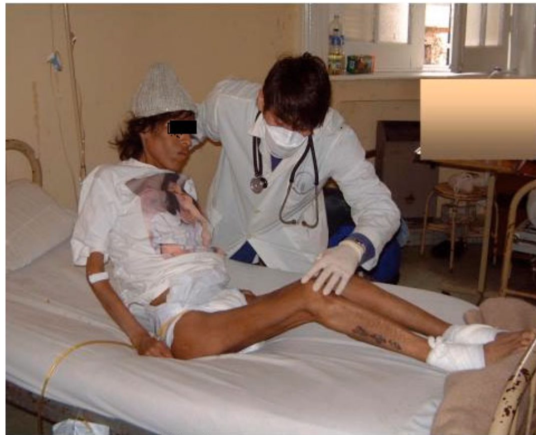

Figure 17-7: Kernig's sign positive appears late in the evolution of TB meningitis. In this particular case, the spinal fluid was positive for *M. tuberculosis* culture.

Polyserositis (pleural-pericardial-peritoneal involvement) is another manifestation of disseminated TB in AIDS patients, where *M. tuberculosis* can be recovered from any of the various serous effusions. The clinical presentation of this form of disseminated TB is unspecific: fever of unknown origin, anemia and wasting are usual manifestations in AIDS patients, common to several other co-morbidities and also to the HIV infection itself. In these cases, several bodily sources in addition to respiratory secretions are useful for *M. tuberculosis* isolation: blood, bone marrow, abscess punctures, urine and cerebrospinal fluid. In the pre-AIDS era, specimens such as blood or bone marrow aspirate specimens were unthinkable sources of *M. tuberculosis* isolation. In severely immunodepressed AIDS patients, however, they offer a considerable diagnostic yield ranging from 10 % to 20 % (Biron 1988,

Khandekar 2005). Figures 17-4, 17-6, 17-7, and 17-8 illustrate the case of a transvestite male sex worker with AIDS and disseminated MDR-TB with pulmonary, vertebral, liver, spleen, psoas muscle, and finally meningeal involvement.

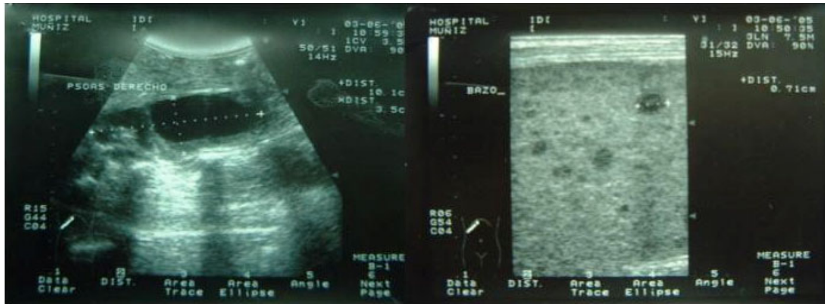

Figure 17-8: Abdominal ultrasonography showing a psoas muscle abscess and multiple hypoechoic lesions in spleen, suggestive of TB microabscesses in the same patient as in Figure 17-7.

The clinical manifestations of disseminated TB are very similar to those of disease caused by nontuberculous mycobacteria, mainly *M. avium*. For this reason, the presence of AFB in the smear microscopy examination is not enough for the diagnosis: the specimen must be submitted to cultivation, species identification and drug susceptibility testing. In addition to disease due to mycobacteria other than *M. tuberculosis*, the differential diagnosis includes disseminated cryptococcal disease, disseminated histoplasmosis and lymphoma.

17.4. Multidrug-resistant tuberculosis and HIV/AIDS

17.4.1. Definitions

A case of TB is more or less manageable according to the drugs to which the patient's isolate is resistant. In this respect, the disease can be classified as:

- **Monoresistant TB:** caused by *M. tuberculosis* resistant to a single drug
- **Polyresistant TB**: caused by *M. tuberculosis* resistant to at least two drugs, but not involving isoniazid (INH) and rifampicin (RIF) simultaneously
- **Multidrug-resistant TB (MDR-TB):** caused by *M. tuberculosis* resistant to at least two drugs, always involving INH and RIF

- **Extensively drug resistant TB (XDR-TB)**: defined as MDR-TB with additional resistance to any fluoroquinolone, and to at least one of the three following injectable drugs used in anti-TB treatment: capreomycin, kanamycin and amikacin (Raviglione 2007)

The most frequent drugs involved in mono-resistance are INH and streptomycin (SM) (see Chapter 19). Nowadays, SM is not regularly used in the standard therapeutic schemes, and resistance to INH has limited clinical or epidemiological relevance (Nardell 2005). Likewise, poly-resistance is relatively easy to overcome as long as susceptibility to RIF is preserved. In contrast, the standard antituberculosis chemotherapy often fails in patients with RIF-resistant TB, which are therefore at an increased risk of developing added INH resistance, that is, to become MDR. In many settings, resistance to RIF is a strong predictor of MDR-TB (Traore 2000) (see chapter 19). Furthermore, poor outcome and death are associated with resistance to RIF alone or in combination with resistance to other drugs (Espinal 2000). Monoresistance to RIF is rather unusual and occurs mainly in association with HIV/AIDS. The reasons for this association appear to be multiple, including malabsorption, drug interaction and previous administration of a related rifamycin (rifabutin) as a prophylactic treatment for *M. avium* disease (Ridzon 1998).

As for the epidemiological mode of *M. tuberculosis* resistance development, drug resistant TB is classified in two subgroups:

- **drug resistance in patients without previous treatment for TB** (formerly “primary” or “initial” drug resistance)

- **drug resistance in patients with previous TB treatment** (formerly “secondary” or “acquired” drug resistance)

Assumedly, a case of MDR-TB in a person without a previous history of TB treatment has been contracted from a source MDR-TB case (see Chapter 19). This kind of resistance is rather frequent in HIV/AIDS cases, in which MDR-TB may acquire epidemic dimensions (Frieden 1996). On the other hand, MDR-TB in a patient with previous TB treatment is usually the result of a prolonged history of inadequate treatment due to erroneous prescriptions, inadequate quality of medicines or irregular treatment compliance. Erratic behavior of certain populations with TB and HIV/AIDS co-infection is often associated with poor treatment compliance and acquisition of antituberculosis drug resistance. However, the distinction between “initial” and “acquired” drug resistance is not always clear in HIV/AIDS patients, who may become infected with a drug resistant strain in the same healthcare environment where they are being treated for pansusceptible TB. In fact, in certain settings, with a high incidence of both TB and HIV/AIDS, the relative contribution

of transmission to the burden of drug-resistant tuberculosis seems to be much higher than previously expected (Gandhi 2006, van Rie 2000).

17.4.2. The development of drug resistance

The mechanisms driving *M. tuberculosis* resistance to antituberculosis drugs are genetically controlled (see Chapter 18). A proportion of mutants resistant to a single drug are generated spontaneously in any bacilli population, even if not exposed to any antituberculosis drug. In *M. tuberculosis,* the average spontaneous mutation rate for resistance to RIF, INH, SM, and ethambutol (EMB) is 2.25×10^{-10}, 2.56×10^{-8}, 2.95×10^{-8} and 1.0×10^{-7} mutations per bacterium per generation, respectively. The probability of occurrence of simultaneous resistance to both INH and RIF (MDR-TB) is obtained by multiplying both mutation rates: $(2.25 \times 10^{-10}) \times (2.56 \times 10^{-8}) = 5.76 \times 10^{-18}$ (Canetti 1965). Thus, it is highly improbable that a patient with a pulmonary cavity lesion containing approximately 10^{-9} bacilli can be spontaneously multidrug-resistant.

Drug resistance emerges a result of a selection process that occurs within the lesions of a TB patient undergoing inadequate therapy. Usually, drug resistance is acquired stepwise through successive inadequate treatments. This is consistent with the finding of higher rates of drug resistance in previously treated TB cases. The selection process of *M. tuberculosis* resistant mutants requires an important bacillary load within the patient's lesions. This is the reason why drug resistance occurs mainly in cases of pulmonary TB and, in turn, is rare in latent TB infection and extrapulmonary localizations that usually have low bacillary loads (Centers for Disease Control and Prevention 1994).

For a long time, drug resistant strains were thought to be less fit than pansusceptible strains and therefore less likely to be transmitted. In particular, large mutations in the *M. tuberculosis* catalase-peroxidase (*katG*) gene have been associated to both an INH-resistant phenotype and a reduced virulence. Actually, mutations leading to antibiotic resistance may or may not have an effect on the fitness of drug-resistant tuberculosis strains (Cohen 2003) (see Chapter 18). The results from different studies are controversial regarding the risk of infection among contacts exposed to resistant bacilli (Burgos 2003, Snider 1985, van Soolingen 1999). Certain MDR *M. tuberculosis* strains, at least those bearing the most commonly occurring *katG* mutation S315T, are to be considered as infectious as wild pansusceptible strains (Gagneux 2006, Pym 2002, van Doorn 2006). In any case, the occurrence of drug-resistance in patients without previous treatment and the very occurrence of MDR-TB outbreaks undeniably denote ongoing transmission of drug resistant strains.

17.4.3. Early suspicion of drug-resistance in the HIV or TB clinic

The first outline of a probable case of drug resistant TB can be drawn in the clinical interview. Such is the case of treatment failure, which almost certainly denotes a case of drug-resistant TB or MDR-TB. Treatment **failure** is defined as the finding of a positive *M. tuberculosis* sputum culture at the end of the fourth month of chemotherapy in a patient under standard therapy in a DOTS regimen (World Health Organization 2003). A persistently positive AFB sputum smear microscopy result in a patient under a strict DOTS regimen can also predict treatment failure and consequently MDR-TB. Treatment **default** (interruption of the treatment for longer than a two-month period) and **relapse** (defined by a positive culture after the end of treatment) may also be suggestive of drug resistant TB. A history of one (or more) previous treatment(s) with several failing or discontinued regimen(s) is indeed a much stronger predictor of drug-resistant TB.

The exposure to a known source of drug resistant TB is another situation in which the investigation of drug resistant TB is mandatory. The risk of exposure is enhanced if the patient has a history of previous hospitalizations, stays in shelters or imprisonment. Once the patient's informed consent has been obtained, HIV testing should be indicated to all TB patients at the initiation of treatment (Caminero 2005) and conversely, antituberculosis drug susceptibility testing should be routinely performed on all HIV/AIDS patients in whom TB is suspected.

17.4.4. AIDS-associated multidrug-resistant tuberculosis outbreaks

The initial reports on MDR-TB outbreaks among HIV/AIDS patients were communicated in the early '90s in Florida (Pitchenik 1990) and New York City (Edlin 1992, Pearson 1992, Frieden 1993). A common feature in these and later publications was the hospital exposure of highly susceptible HIV/AIDS patients to infectious chronic MDR-TB cases. When seeking assistance repeatedly in health centers for infections diseases, AIDS patients with progressive immunodepression shared waiting rooms, wards and other hospital facilities with infectious MDR-TB patients. At that time, MDR-TB strains were considered of low infectivity and adequate biosafety measures were not in force. This erroneous concept – combined with the previous dismantling of TB control programs and TB clinics – paved the way for the early AIDS-associated MDR-TB outbreaks.

The most spectacular MDR-TB outbreak was caused by the so-called W strain, belonging to the *M. tuberculosis* Beijing family. This strain is resistant to multiple

drugs, and was identified as the main source of clustered MDR-TB cases in New York City throughout the first half of that decade (Ikeda 1995). The W strain is an eloquent example of the pathogenic potential of the Beijing lineage of *M. tuberculosis*. Evidence has been gathered supporting the idea that some Beijing strains, which are highly prevalent in East Asia and former Soviet Union Republics, have an increased potential for spontaneous mutation – which increases the possibility of selection for drug-resistant clones – and apparently an increased virulence, too (European Concerted Action 2006).

Analogous nosocomial outbreaks were described in other countries. A conspicuous example occurred in Argentina and was due to an MDR *M. tuberculosis* strain of the Haarlem lineage: the M strain (Ritacco 1997). In a single reference treatment center for infectious diseases, located in Buenos Aires, more than 800 cases were assisted with the association MDR-TB-AIDS from 1992 to 2005. In the early stages of the outbreak, most patients died before culture and drug susceptibility testing confirmed the diagnosis. Later on, methods for speeding up the diagnosis were implemented, adequate second-line drug treatment could be instituted promptly, and survival was substantially elongated. Also, the implementation of internationally recognized hospital infection control measures helped to contain the outbreak (Waisman 2005). Yet, the outbreak strain managed to disseminate in a large urban area not only among AIDS patients but also among HIV-negative patients, both with and without a history of TB treatment (Palmero 2005).

M. bovis, another member of the *M. tuberculosis* complex, was also involved in similar MDR-TB outbreaks. The *M. bovis* strain named B – resistant to 11 antituberculosis drugs – affected mainly hospitalized AIDS patients with advanced immunodepression in two big health centers in central Spain between 1993 and 1995 (Guerrero 1997). Afterwards, the outbreak spread to other cities in the country and even to Canada (Samper 1997, Long 1999, Rivero 2001). Sporadic cases in HIV-negative patients were also described (Palenque 1998, Robles Ruiz 2002). It has been hypothesized that the original strain developed INH resistance in the natural host as a consequence of the use of this drug as a growth promoter in cattle, which was once common practice in Spain. The treatment of the first human case with the standard antituberculosis therapy, which in addition to INH and RIF included pyrazinamide (PZA) – to which *M. bovis* is naturally resistant – would have been in fact a monotherapy with RIF that led to multidrug resistance (Romero 2006).

A deadly outbreak occurred more recently in Tugela Ferry, a rural district in Kwala Zulu-Natal province, South Africa. MDR-TB was diagnosed in 221 out of 1,539 patients recruited within a 15-month period (2005-2006). Of these 221, 53 had

extensively drug resistant TB (XDR-TB), an especially serious condition. Fifty-five percent of the patients had never been treated for TB and 67 % had had a recent hospital admission. All 44 patients with XDR-TB, who were tested for HIV, were co-infected and 52 of 53 patients with XDR-TB died, with a median survival of 16 days from the time of diagnosis. Genotyping of isolates showed that 85 % of patients with XDR-TB had similar strains (Gandhi 2006).

This South African outbreak underlined the severity and urgency of the current situation of MDR-TB in a number of developing countries. Hospital transmission between AIDS patients in the absence of adequate biosafety measures reproduces the major features of previous MDR-TB outbreaks. The risk of transmission of these highly resistant strains to healthcare workers and to the general population jeopardizes the efforts to control TB. As described later in the treatment section of this chapter, the current treatment of MDR-TB includes "injectable" compounds (aminoglycosides or capreomycin) and quinolones. Precisely these dangerous XDR *M. tuberculosis* strains are resistant to at least to one drug of either class.

In the course of an international survey, XDR-TB cases were identified in six continents and their treatment outcome was found to be significantly worse than that of other MDR-TB cases (Sarita Shah 2007). TB organizations worldwide are nowadays focusing their efforts on diagnosing, treating, and controlling this new enemy (see the WHO Global Task Force Report on XDR-TB 2006 on the internet http://www.who.int/tb/xdr/globaltaskforcereport_oct06.pdf).

The prevention of institutional transmission of TB and MDR-TB is outlined in the guidelines released by the US Centers for Disease Control and Prevention (Centers for Disease Control and Prevention 1994, Centers for Disease Control and Prevention 2005) and in the 1999 WHO guide for resource-limited settings. The classification of control measures in administrative, environmental and personal respiratory protection described in Chapter 11 is widely accepted and efficacy-proven. Basically, the first steps are:

- the prompt identification of the infectious TB case
- the adequate isolation and treatment of the patient
- the protection and control of personnel at risk of infection and disease

Paradoxically, in many developing countries, where TB is an important public health problem, airborne infection control measures are often neglected in view of many other more immediate sanitary problems, such as cholera, malaria, war and disaster. This allows the perpetuation of chains of transmission involving inpatients, outpatients, healthcare workers and community members.

17.5. Treatment of tuberculosis in HIV/AIDS patients

17.5.1. Special considerations

The application of Directly-Observed Treatment, Short-course (DOTS), the universally accepted intervention for TB treatment, is crucial in AIDS cases. In fact, the DOTS strategy recreates the sound idea of a supervised TB treatment that was delineated in the '70s by the eminent bacteriologist Wallace Fox. However, the DOTS strategy includes not only the observation of the patient's medicine intake but also other important issues that constitute a strategy launched in 1996 by the WHO (World Health Organization 2006). Its five essential elements are: 1) sustained political commitment, 2) access to quality-assured TB sputum microscopy, 3) standardized short-course chemotherapy, supervised, 4) adequate and continuous supply of quality assured drugs, and 5) a recording and reporting system with outcome assessment of patients (see Chapter 7).

TB clinics are excellent sites to detect HIV infection and also to apply the same directly observed therapy strategy to the initial antiretroviral therapy. There is an urgent need to complement the TB and the HIV programs worldwide in order to reinforce detection and control activities of both diseases.

Many patients with HIV/AIDS disease and TB have severe immunodepression and high plasmatic viral loads. The instauration of antituberculosis treatment is critical in these patients (Quy 2007). Indeed, if not treated promptly, an AIDS patient with disseminated TB will die from it in the short term. In turn, HAART substantially improves the prognosis of patients with *M. tuberculosis* co-infection by helping to restore the immune system. Nevertheless, the efficacy of HAART in these cases is often jeopardized by drug toxicity, pharmacological interactions, impaired drug absorption and paradoxical reactions. HAART has to be frequently combined with treatments for TB and other opportunistic infections caused by agents such as *Candida, Pneumocystis*, mycobacteria other than *M. tuberculosis*, Cytomegalovirus, *Toxoplasma*, herpes, fungus, etc.

Strong evidence has been gathered on the high efficiency of RIF in reducing the mortality of TB/AIDS patients (Wallis 1996). Consequently, RIF is considered an essential drug in the treatment of HIV/AIDS-associated TB. Unfortunately, this drug is a potent inducer of hepatic cytochrome P-450 (isozyme CYP3A) and as such, it interacts with two classes of antiretroviral drugs: protease inhibitors and non-nucleoside reverse transcriptase inhibitors. Within the family of rifamycins, rifabutin is a less potent activator of CYP3A and therefore can be used safely as a

surrogate for RIF in combination with protease inhibitors such as nelfinavir. All HIV infected patients with TB should be treated with a rifamycin-based combination regimen i.e., rifabutin reducing the dose to half (150 mg/d).

TB caused by fully susceptible strains of *M. tuberculosis* can be treated with a six-month standard scheme (2 months of INH, RIF, PZA, and EMB plus 4 months of INH and RIF) as recommended by international organizations, with the exception of meningeal, miliary or spinal TB, which should receive a nine-month treatment regimen (2 months of INH, RIF, PZA, and EMB plus 7 months of INH and RIF) (ATS/Centers for Disease Control and Prevention/IDSA 2003, World Health Organization 2004). Some authors have reported higher rates of relapse with standard treatment in HIV/AIDS patients and therefore recommend prolonging the second phase of TB treatment to seven months (Pulido 1997).

SM is seldom used in the initial phase due to both the discomfort caused by its application and the risk inherent to the handling of syringes and needles. In the continuation phase, drugs can be administered daily or intermittently, but this latter option is reserved for patients with a CD4 + T cell count above 100/µL. The risk of resistance to rifamycins increases when they are administered intermittently, especially in regimens consisting of rifapentin plus INH once weekly or RIF plus INH twice weekly. When intermittent therapy is indicated, regimens administered thrice weekly that include INH (10 mg/kg/d) plus RIF (usual dosage) are preferable (World Health Organization 2004, American Thoracic Society/Centers of Disease Control/Infectious Disease Society of America 2003). If the CD4+ T cell count is not available, intermittent therapy should not been used in HIV/AIDS patients.

At least in settings with a high prevalence of MDR-TB, antituberculosis drug susceptibility testing should always be performed upon isolation of *M. tuberculosis* from an HIV/AIDS patient. The early detection of resistance to RIF and INH prompts switching to a drug scheme containing second-line drugs. This often extends a patient's survival, even in the case of disseminated TB. If standard TB program guidelines were to be followed strictly, severely immunodepressed patients with MDR-TB would most probably die under a standard antituberculosis drug scheme before treatment failure is suspected and/or confirmed.

Treatment regimens for MDR-TB should preferably be tailored on the basis of the results of susceptibility testing. The initial phase of two to six months includes three or four drugs given orally together with an injectable drug such as an aminoglycosides (SM, kanamycin or amikacin) or capreomycin. In the second phase, the injectable drug is discontinued. The patient is discharged as cured after 18 to 24

months of uninterrupted therapy, only when five sequential cultures yield negative results.

There is a great deal of controversy on case management of MDR-TB in resource-limited high burden countries and simplified regimens have neither been evaluated nor standardized (Caminero 2006). TB control programs without adequate bacteriological support are compelled to apply empirical re-treatment schemes based on previous local susceptibility testing surveys. Prospective studies of this kind of approach evidenced poor treatment outcomes when compared with regimens tailored according to drug susceptibility test results (Mitnick 2003).

In the medical management of MDR-TB cases, some degree of empiricism associated with expertise is necessary for the design of the re-treatment regimens. Actually, most of the currently available rapid drug susceptibility methods only produce results for first-line drugs (SM, INH, RIF, EMB and PZA). Testing for second-line drugs is usually not available – or results only become available after a considerable delay because the tests are performed on traditional solid media. In addition, the results are less reliable than those of the first line drugs due to insufficient standardization and external quality control. In most cases, there is no control at all. Often, the specialist physician is constrained to select a drug scheme merely on the basis of the pattern of resistance to the first-line drugs.

17.5.2. Adverse reactions in HIV/AIDS patients

The HIV/AIDS patient with low CD4+ T cell counts is usually a multi-etiological case. Several opportunistic infectious and noninfectious agents coexist in addition to the HIV itself, which puts into action its own pathogenic mechanisms. Organs in the gastrointestinal tract, mainly the esophagus, are affected by pathogens, including *Candida sp,* cytomegalovirus, herpes virus, *Cryptosporidium*, etc. These infections contribute to the wasting of the patient and hamper the ingestion, tolerance and absorption of oral medicines. In such conditions, the gastric intolerance to antituberculosis drugs such as RIF or PAS, which itself is fairly frequent, is exacerbated. It should be highlighted that parenteral formulations of first-line drugs exist but are not currently available in any TB control program worldwide.

The potential hepatotoxicity of drugs such as INH, RIF, PZA, and ethionamide increases when administered to patients with concomitant hepatitis C or B.

The impairment of the renal function in an HIV/AIDS patient under treatment with aminoglycosides or capreomycin may be due either to drug toxicity or to an AIDS-associated kidney disease.

The involvement of the central and the peripheral nervous system is frequent in AIDS and may be caused by the HIV itself and/or by various opportunistic infections, including toxoplasmosis and cryptococcosis. Thus, the neurotoxicity of drugs, for example INH, cycloserine/terizidone or fluorquinolones, often aggravates a previous condition and the exact contribution of the drug adverse effect to the clinical picture is difficult to discern.

Moreover, the multiple treatments simultaneously required for different pathologies contribute to drug-drug interactions. RIF, a key drug for TB treatment, interacts with the protease inhibitor class of antiretroviral drugs and its surrogate, rifabutin, is not always available for TB treatment in developing countries.

The same general principles of antituberculosis drug toxicity applied to the general population are valid for HIV-positive patients with some peculiarities. For instance, RIF-induced gastrointestinal intolerance and toxic hepatitis are more frequent in HIV/AIDS patients. Also, skin reactions in HIV/AIDS patients have been attributed to the association of INH plus RIF (Pitche 2005).

In the heavily treated AIDS patient, it is difficult to accurately identify which drug is causing an adverse drug reaction. In view of this, a first-line antituberculosis drug should never be discontinued in the absence of solid evidence of such a drug being the cause of an adverse reaction (American Thoracic Society/Centers for Disease Control and Prevention/Infectious Disease Society of America 2003).

Thiacetazone is an antituberculosis drug widely used in developing countries, mainly in Africa. This drug frequently produces serious adverse events in the skin, including Stevens-Johnson and Lyell syndrome, and its use is very dangerous in AIDS patients (Lawn 1999).

The first- and second-line drugs used in TB treatment, dose and toxicity in HIV/AIDS patients are summarized in Table 17-2 (see also Chapter 18).

Table 17-2: Drugs used in the TB treatment and re-treatment (see also Chapter 18)

Drug	Daily dose	Toxicity in HIV/AIDS
Rifampicin	10 mg/kg	Gastric intolerance, hepatitis, rash, hemolytic anemia, acute nephritis, purpura, epidermolysis, potent inducer of CYP3A (drug interactions)
Rifabutin	5 mg/kg	Similar to RIF, less potent inducer of CYP3A
Isoniazid	5 mg/kg	Hepatitis, polyneuropathy
Pyrazinamide	25 mg/kg	Hepatitis, hyperuricemia
Ethambutol	20 mg/kg	Optical neuropathy
Aminoglycosides*	15 mg/kg	Renal and 8th cranial nerve
Capreomycin	15 mg/kg	Renal and 8th cranial nerve
Para-aminosalicylic acid	100 mg/kg	Gastric intolerance
Cycloserine/ Terizidon	10-15 mg/kg	Central nervous system (seizures, psychosis)
Ethionamide/ Prothionamide	15 mg/kg	Hepatitis
Levofloxacin	500 mg	Tendonitis, neurotoxicity, arrhythmia
Moxifloxacin	400 mg	Tendonitis, neurotoxicity, arrhythmia
Linezolid	600 mg**	Last chance drug, optical neuritis, bone marrow depression
Thiacetazone	Not recommended	Epidermolysis (Lyell syndrome)

* Streptomycin, Kanamycin, Amikacin

**Half of the recommended dose for bacterial infections (1,200 mg/d) seems to be effective in TB

17.5.3. When to start antiretroviral therapy in patients with tuberculosis

When TB and HIV/AIDS are diagnosed simultaneously, the treatment for TB should be started immediately. In principle, antiretroviral therapy should be started as early as possible in patients with low CD4+ cell counts. However, the simultaneous implementation of both treatment regimens conveys an elevated risk of adverse effects. There is neither consensus on a CD4+ cell count threshold below which therapy should be postponed nor on an optimal time-interval for the delay in the start of antiretroviral therapy. The issue is particularly controversial in the case of severely immunodepressed patients (CD4+ cell count below 100/µL) with TB. In one study, adverse events occurred in 54 % of 183 patients, one third of who changed or interrupted HIV and/or TB medication. Most of the adverse events occurred in the first two months and consisted of peripheral neuropathy, rash, hepatitis, and gastrointestinal upset (Dean 2002). In 2006, the International AIDS Society recommended starting HAART after the first month of antituberculosis therapy in patients with less than 100 CD4+ cells/µL, and after the initial phase of TB treatment (end of the second month) when the CD4 + T cell level is above 100/µL (Hammer 2006).

With regard to the optimal antiretroviral regimen in patients without previous antiretroviral treatment (initial treatment), two approaches yielded comparable performances: one included boosted protease inhibitors (not recommended in association with RIF) plus two nucleoside or nucleotide reverse transcriptase inhibitors, and the other included a non-nucleoside reverse transcriptase inhibitor such as efavirenz associated with two nucleoside or nucleotide inhibitors of reverse transcriptase (zidovudine plus lamivudine or tenofovir plus emtricitabine).

Rifabutin should be used instead of RIF in combination with protease inhibitors to minimize drug interactions. As RIF also interacts with non-nucleoside reverse transcriptase inhibitors, its association with nevirapine is not recommended. Efavirenz, however, can still be associated with RIF, preferably in a higher dosage i.e. 800 mg instead of 600 mg/d (Corti 2005). Fusion inhibitors such as enfuvirtide belong to a new class of antiretroviral drugs that has no interactions with RIF (Manfredi 2006).

17.6. Immune reconstitution inflammatory syndrome

This syndrome, also known as IRIS, was recognized early in the modern antituberculosis therapy era and consists of a paradoxical worsening of clinical disease shortly after the initiation of drug treatment. Irrespective of the HIV status, the

immune system is impaired in the advanced stages of TB as shown by low levels of circulating CD4+ T lymphocytes. Once the treatment starts to produce an effect, an "immune restoration" occurs that reflects the reconstituted immunity to *M. tuberculosis.* The syndrome includes an enlargement of the affected lymph nodes and of the lung lesions accompanied by an exacerbation of the general symptoms. This condition resolves spontaneously during the course of antituberculosis therapy.

Since the beginning of the HAART era, the immune reconstitution inflammatory syndrome has been observed with increasing frequency in AIDS. Although HIV/AIDS-related IRIS can be associated with other opportunistic infections – namely mycobacterioses and mycoses – TB accounts for one third of the cases, at least in settings with a high prevalence of HIV-TB co-infection (Colebunders 2006). This syndrome is observed most frequently when the treatment of both infections is started in close temporal proximity. The reactions usually occur in the first four to eight weeks after initiation of the antiretroviral therapy and do not differ from those associated with the classical TB immune restoration syndrome. They may include systemic manifestations, such as fever and malaise and/or local reactions in lymph nodes, lungs, pleura and the central nervous system, depending on the localization of the TB lesions (Narita 1998). New infections and other reactions to therapy must be taken into account in the differential diagnosis of this syndrome. As a consensus has not been reached on its clinical definition, the syndrome is probably being over-diagnosed (Lipman 2006).

In AIDS patients, the immune reconstitution inflammatory reactions are best managed with anti-inflammatory agents, including corticosteroids such as prednisone 20-40 mg/d, if necessary. Both antituberculosis and antiretroviral therapy should be continued during the entire reconstitution syndrome.

17.7. Treatment of latent tuberculosis infection in HIV/AIDS patients

The classical method for detection of TB infection is the skin test reaction with PPD RT23 2 UT or PPDS 5 UT. In HIV-infected persons, a nodule of 5 mm or more is considered positive. Particularly in this population, the reliability of the method of detection of latent infection is highly dependent on the level of immunosuppression. Quantiferon is a whole blood assay for the detection of interferon gamma produced by peripheral lymphocytes in response to specific *M. tuberculosis* antigens. This test often yields negative or indeterminate results in severely immunosupressed AIDS patients (Brock 2006). On the other hand, preliminary results

suggest that the performance of ELISPOT – a test that enumerates *Mycobacterium tuberculosis* antigen-specific IFN-γ-secreting T cells test – is not affected by HIV-associated immunosuppression (Dheda 2005). Further studies on improved versions of these tests are needed to fully assess the value of this kind of approach for the detection of latent TB in severely immunodepressed AIDS patients (see Chapter 13).

When latent TB infection is detected in an HIV-positive person, he/she should receive chemoprophylaxis. The treatment consists of a course of at least six months – preferable nine months – of INH. Alternatively, a four-month course of RIF may be indicated. Both drugs are administered in their usual dosages (Centers for Disease Control and Prevention 2000).

The protective effect of a number of TB chemoprophylaxis regimens in HIV-positive, PPD-positive persons has been sufficiently proven (Whalen 1997, Lim 2006). An interesting option is to administer TB chemoprophylaxis to AIDS patients with CD4+ counts below 100 cells/μL. The risk exists, however, of overlooking a sub-clinical TB, thus selecting INH resistant, or worse, RIF resistant mutants – depending on the drug used in chemoprophylaxis.

At the turn of the millennium, a simple and ingenious solution was evaluated for the treatment of latent TB infection in HIV/AIDS patients, consisting of a two-month course of RIF plus PZA. The use of two drugs was expected to prevent the development of resistance, while the short-course treatment would grant a better adherence. Indeed, this chemoprophylaxis regimen was successfully used in HIV infected persons (Gordin 2000). Unfortunately this regimen proved unsafe for the general population due to the high incidence of severe liver toxicity associated with its use (Centers for Disease Control and Prevention 2001).

17.8. Mycobacteriosis in AIDS patients

17.8.1. Non-tuberculous mycobacteria and AIDS

Mycobacteriosis is a term generally reserved for the disease caused by any mycobacteria other than *M. leprae* and those belonging to the *M. tuberculosis* complex. Non-tuberculous mycobacteria (NTM) – also called atypical or environmental mycobacteria – are ubiquitous organisms commonly found in soil and water. They are infrequent agents of human disease in patients other than HIV/AIDS. When present, they affect mainly predisposed hosts and produce disease in organs with underlying conditions. For instance, organisms in the *Mycobacterium avium* com-

plex, *M. kansasii* and other mycobacteria may cause a pulmonary disease resembling TB in patients with lung disorders, including bronchiectasis, chronic obstructive pulmonary disease, or residual granulomatous lesions produced by TB and mycoses.

Mycobacterioses became particularly relevant in relation to the global emergence of HIV/AIDS. *M. avium* is the most frequent etiological agent of NTM disease associated with AIDS, as shown by an early study where it accounted for 96 % of 2,269 NTM-AIDS cases (Horsburgh 1989). Indeed, early in the AIDS pandemic, *M. avium* was recognized to cause disseminated disease and death in advanced stages of immunodepression with blood CD4+ counts below 50 cells/µL (Chaisson 1992). In the course of HIV infection, the progression of this NTM disease seems to undergo several stages from mucosal entry, passing through early transient dissemination and tissue colonization, before the persistent and deadly bacteremia. *M. avium*-specific T cell responses apparently develop and still persist during disseminated disease. Yet, they are dysfunctional or insufficient to prevent persistence (MacGregor 2005).

Specific and effective therapeutic and prophylactic therapeutic schemes have been developed for AIDS-associated *M. avium* disease. In addition, the introduction of HAART and the subsequent improvement in the survival of AIDS patients lowered the incidence of most opportunistic associated diseases, including NTM. In the US, NTM diseases have fallen from 16 % before 1996 to 4 % soon after HAART implementation (Palella 1998). Nevertheless, the risk of these opportunistic infections remains high in undiagnosed HIV-infected patients and in patients who either have no access to, or do not adhere to HAART.

M. xenopi and *M. kansasii* are the next most frequent NTM producing opportunistic infections associated with AIDS. Several other mycobacterial species can cause local and/or disseminated disease in these patients, including *M. fortuitum, M. genavese,* and *M. chelonae* (Shaffer 1992). As the infection with NTM is acquired from the environment and interhuman transmission has not yet been demonstrated, the isolation of these patients is not necessary.

17.8.2. Clinical presentations

Disseminated *M. avium* disease usually appears with fever, malaise, weight loss (over 10 % of body weight), nocturnal sweats, abdominal pain, and diarrhea. Peripheral lymphadenitis with frequent abscesses as well as liver and spleen enlargement are frequently observed. Either abdominal ultrasonography or computed to-

mography scans reveal visceral enlargement with multiple focal hypoechoic or hypodense images, and retroperitoneal lymph node enlargement. Psoas abscess and vertebral compromise can also be observed. The laboratory results show anemia and leucopenia, reflecting bone marrow invasion by *M. avium*. Hepatic alkaline phosphatase is consistently elevated.

The immune reconstitution inflammatory syndrome or IRIS is frequently associated with *M. avium* disease in AIDS patients who start HAART (Karakousis 2004). In a study of 51 patients with mycobacterial disease (mainly *M. avium*), the incidence of nontuberculous mycobacterial immune reconstitution syndrome was 3.5 % among patients initiating HAART with a baseline CD4+ cell count of < 100 cells/μL. The main clinical presentations were peripheral lymphadenitis, pulmonary disease and intra-abdominal disease (Phillips 2005).

17.8.3. Diagnosis

The diagnosis of *M. avium* disease should be born in mind in all AIDS patients presenting with fever of unknown origin. The isolation of the agent from stool does not necessarily indicate disseminated *M. avium* disease but merely gastrointestinal colonization (Jacobson 1991). Similarly, the finding of *M. avium* in sputum requires repeated positive sputum cultures together with radiological and clinical manifestations to confirm its pathological involvement in progressive pulmonary disease. On the other hand, a positive culture from a sterile source, such as blood or bone marrow, is enough to confirm the diagnosis of disseminated *M. avium* disease (MacGregor 2005).

17.8.4. Treatment

With few exceptions, *M. avium* is resistant to the usual antituberculosis drugs. As is the case in TB, the treatment of *M. avium* disease is a combination therapy to avoid resistance due to selective pressure. The results of drug susceptibility testing often have a poor correlation with the clinical evolution and empirical treatment has to be used.

Empirical treatment schemes for *M. avium* disease are:

- clarithromycin (or azithromycin), EMB and rifabutin or
- clarithromycin (or azithromycin), EMB, fluoroquinolone and amikacin

These schemes are applied at least during the initial 6 to 12 weeks (Benson 2003, Gordin 1999, Katoch 2004). The treatment is generally prolonged for about one year, depending on the clinical evolution and CD4+ cell counts. As is the case in TB, the early initiation of HAART is of crucial importance in these severely immunodepressed patients. After finishing treatment of *M. avium* disease, secondary prophylaxis should be administered until the CD4+ cell count reaches 100 CD4+ cells/µL; this may consist of azithromycin 1,200 mg/once weekly or clarithromycin 1,000 mg/day. Paradoxically, secondary prophylaxis may ultimately not be necessary if the patient suffered IRIS during treatment. Indeed, together with a dramatic deterioration of the clinical status, this syndrome induces an inflammatory response that is often accompanied by a restoration of the immune response (Shelburne 2003).

Several pharmacological interactions should be considered: the macrolide clarithromycin interacts with RIF and rifabutin, increasing their serum concentration by 25 %. In turn, these rifamycins reduce serum concentrations of clarithromycin by 50 %. In addition, clarithromycin interacts with protease inhibitors, in particular with atazanavir, which increases its concentration by 95 %. Thus, the recommendation is to halve the macrolide dose.

Rifabutin can be discontinued after several weeks of treatment when clinical improvement is observed. The clarithromycin dose should not exceed 1,000 mg/d because high doses were found to be significantly associated with high rates of death (Cohn 1999).

Azithromycin has less drug-drug interactions and therefore can be used more safely in place of clarithromycin. It has been proven to have comparable efficacy in combination with ethambutol (Ward 1998). A promising new macrolide named thelitromycin has been proven to have activity against *M. avium* in vitro as well as in animal models (Bermudez 2004).

M. xenopi and *M. kansasii* are susceptible to INH, RIF, and EMB, with or without the addition of SM (Katoch 2004). A one year therapeutical scheme, similar to that used in TB can be applied with the exception of pyrazinamide, a drug to which these mycobacterial species are naturally resistant.

17.8.5. Primary prophylaxis

All AIDS cases with a CD4+ count below 50 cells/µL are at high risk of developing disseminated *M. avium* disease and must receive prophylaxis (Kaplan 2002). Before the introduction of effective prophylactic therapy, *M. avium* disease ap-

peared in more than 40 % of AIDS patients in developed countries with a low TB incidence. Large placebo-controlled clinical trials have shown that rifabutin, as well as the macrolides clarithromycin and azithromycin, significantly reduce the incidence of *M. avium* when used for primary prophylaxis in severely immunocompromised patients (Havlir 1996, Pierce 1996). There are substantial arguments against the use of rifabutin, a drug rich in pharmacological interactions with the additional disadvantage of selecting rifamycin monoresistant *M. tuberculosis* clones. Clarithromycin has also several drug-drug interactions. The safest drug for primary chemoprophylaxis of *M. avium* infection in AIDS patients is azithromycin, which has fewer interactions, and can be administered weekly at a dose of 1,200 mg, alternative to the conventional dose of 500 mg daily. Prophylaxis must be continued until the CD4+ count reaches levels above 100/µL sustained over time (Kirk 2002).

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Chapter 18: Drugs and Drug Interactions

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18.1. Introduction

The history of tuberculosis (TB) changed dramatically after the introduction of anti-mycobacterial agents. Drug treatment is fundamental for controlling TB, promoting the cure of the patients and breaking the chain of transmission when the antituberculosis drug regimen is completely and correctly followed.

Antituberculosis drug treatment started in 1944, when streptomycin (SM) and para-aminosalicylic acid (PAS) were discovered. In 1950, the first trial was performed comparing the efficacy of SM and PAS both as monotherapy or combined. The study demonstrated that combined therapy was more effective and resulted in the first multidrug antituberculosis treatment that consisted of a long course of both drugs. In 1952, a third drug, isoniazid (INH), was added to the previous combination, greatly improving the efficacy of treatment, but which still had to be administered for 18-24 months. In 1960, ethambutol (EMB) substituted PAS, and the treatment course was reduced to 18 months. In the '70s, with the introduction of rifampicin (RIF) into the combination, treatment was shortened to just nine months. Finally, in 1980, pyrazinamide (PZA) was introduced into the antituberculosis treatment, which could be reduced further to only six months.

Two biological features explain why combined drug therapy is more effective at curing TB than monotherapy. One is that treatment of active TB with a single drug results in the selection of drug resistant bacilli and failure to eliminate the disease. The other is that different populations of tubercle bacilli – each of them showing a distinct pattern of susceptibility for antituberculosis drugs – may co-exist in a TB patient (Shamputa 2006).

Soon after the introduction of the first anti-mycobacterial drugs, drug resistant bacilli started to emerge, but the launch of both combination therapy and new and more effective drugs seemed to be enough to control the disease. In fact, it was thought that TB could be eradicated by the end of 20th century. However, TB unexpectedly re-emerged in the '80s, and in the following years there was an important increase in the incidence of poly-, multiple-, and extensively drug resistant strains. Since 1970, no new drug has been discovered for antituberculosis treatment, which today seems insufficient to confront the disease. Fortunately, research efforts have been accomplished and today there is a wide range of new molecules with promising antituberculosis activity.

In our days, due to the worldwide re-emergence of TB and the increased incidence of multidrug resistant (MDR) and extensively drug resistant (XDR) strains of *Mycobacterium tuberculosis* (Centers for Disease Control and Prevention 2006, see also Chapter 19), new anti-mycobacterial agents (see section 18.6 below), new drug delivery systems (Gelperina 2005), and new treatment regimens are being investigated.

In this chapter, we describe the basic guidelines on TB treatment along with a description of the major antituberculosis drugs (the classical drugs) and their pharmacokinetic properties, toxicity, and interactions with other drugs. Mechanisms of drug resistance in the tuberculous bacillus are also described. In the final part of this chapter we review the main new antimycobacterial drugs that are being developed as candidates to be incorporated in the arsenal of anti-tuberculosis drugs.

18.2. Overview of existing treatment schemes

18.2.1. Rationale

Antituberculosis treatment has two main objectives (Onyebujoh 2005). First, there is a need to rapidly kill those bacilli living extracellularly in lung cavities, which are metabolically active and are dividing continuously; this is required in order to attain the negativization of sputum and therefore to prevent further transmission of the disease. Second, it is necessary to achieve complete sterilization and elimination of those bacilli replicating less actively in acidic and anoxic closed lesions, and to kill semi-dormant bacilli living intracellularly in other host tissues, otherwise these bacilli may persist and will be responsible for subsequent TB relapses. INH is the drug with the highest activity against rapidly dividing bacilli, whereas RIF and PZA have the greatest sterilizing activity against bacteria that are not dividing. These reasons, along with the prevention of drug resistance, support the use of a combination therapy for the treatment of TB.

Drugs for treating TB are usually classified as first- and second-line drugs. Traditionally, there are five first-line drugs: INH, RIF, PZA, EMB, and SM. Second-line drugs include the aminoglycosides kanamycin and amikacin, the polypeptide capreomycin, PAS, cycloserine, the thioamides ethionamide and prothionamide and several fluoroquinolones such as moxifloxacin, levofloxacin and gatifloxacin. Some reports, however, include SM among the second-line drugs, since its use has declined in recent years, due to the high rates of resistance, and also, because other more effective drugs have been incorporated into the anti-tuberculosis treatment. Similarly, new drugs such as the rifamycin derivatives rifapentine and rifabutin can

be considered among the first-line drugs, and in the near future, it is quite likely that some fluoroquinolones could be incorporated into the standard anti-tuberculosis treatment, thus being considered as first-line drugs.

The current short-course treatment for the complete elimination of active and dormant bacilli involves two phases:

- **initial phase**: three or more drugs (usually isoniazid, rifampicin, pyrazinamide and ethambutol or streptomycin) are used for two months, and allow a rapid killing of actively dividing bacteria, resulting in the negativization of sputum
- **continuation phase**: fewer drugs (usually isoniazid and rifampicin) are used for 4 to 7 months, aimed at killing any remaining or dormant bacilli and preventing recurrence

18.2.2. Dosing

There are five first-line drugs: INH, RIF, EMB, PZA, and SM. For standard regimes, first-line drugs should be used at the doses summarized in Table 18-1 (data from Martindale 2004, and Centers for Disease Control and Prevention 2003a).

Table 18-1: Recommended doses for first-line antituberculosis drugs

Drug	Adults or Children[a]	Daily dose (max. dose)	Three times per week (max. dose)	Twice per week (max. dose)
INH[b]	Adults	5 mg/kg (300 mg)	10-15 mg/kg (900 mg)	15 mg/kg (900 mg)
	Children	10-15 mg/kg (300 mg)		20-30 mg/kg (900 mg)
RIF	Adults	10 mg/kg (600 mg)	10 mg/kg[c] (600 mg)	10 mg/kg[c] (600 mg)
	Children	10-20 mg/kg (600 mg)		10-20 mg/kg (600 mg)
PZA[d]	Adults	18.2-26.3 mg/kg (1-2 g)	27.3-39.5 mg/kg (1.5-3 g)	36.4-52.6 mg/kg (2-4 g)
	Children	15-30 mg/kg (2 g)		50 mg/kg (2 g)
EMB[d]	Adults	14.5-21.1 mg/kg (800-1,600 mg)	21.8-31.6 mg/kg (1.2-2.4 g)	36.4-52.6 mg/kg (1.2-2.4 g)
	Children	15-20 mg/kg (1 g)		50 mg/kg (2.5 g)
SM	Adults	15 mg/kg[e] (1 g)		
	Children	20-40 mg/kg (1 g)		20 mg/kg (1 g)

[a] : Patients under 15 years of age.

[b] : INH can be given also once per week, on a 15 mg/kg basis, up to a maximal dose of 900 mg

[c] : For RIF, some manuals also recommend higher doses (10-15 mg/kg) intermittently (two-three days per week) having a maximum of 900 mg (Martindale 2004)

[d] : For PZA and EMB, doses have to be calculated precisely depending on the weight range (for details, see CDC 2003a)

[e] : SM: doses should be reduced to 10 mg/kg in people over 59 years old

When resistance to any of these first-line drugs is found or highly suspected, or when adverse effects to first-line drugs develop during therapy, the treatment should include other drugs known as second-line drugs (details of second-line drugs can be found in section 18.3). The doses and periodicity of second-line drugs and other drugs are given in Table 18-2 (Centers for Disease Control and Prevention 2003a).

Table 18-2: Recommended doses for second-line anti-tuberculosis drugs

Drug	Adults or children[a]	Dose (max. dose)	Days per week
Rifapentine[b]	Adults	10 mg/kg (600 mg)	One
Rifabutin[c]	Adults	5 mg/kg (300 mg)	Two, three or seven
Cycloserine	Adults and children	10-15 mg/kg (1 g)	Seven
Ethionamide	Adults and children	15-20 mg/kg (1 g)	Seven
Amikacin	Adults	15 mg/kg (1 g)	One, two, three or seven
Kanamycin	Children	15-30 mg/kg (1 g)	Two or seven
Capreomycin			
PAS	Adults	8-12 g	Seven
	Children	200-300 mg/kg (10 g)	Seven
Levofloxacin[d]	Adults	500-1,000 mg	Seven
Moxifloxacin[d]	Adults	400 mg	Seven
Gatifloxacin[d]			

[a] : Patients under 15 years of age.

[b] : This drug has not been approved for use in children.

[c] : Doses of rifabutin may need to be adjusted in HIV-positive patients receiving antiretroviral therapy.

[d] : This drug has not been approved for long-term use in children and adolescents.

18.2.3. Treatment regimens

There are many different anti-tuberculosis regimens described in the literature, mostly matching the premises, indications and doses indicated in the sections above (Centers for Disease Control and Prevention 2003a, World Health Organization 2003). Several drug regimens are recommended depending on many factors, such as disease localization and severity, result of sputum smear microscopy, human immunodeficiency virus (HIV) co-infection, prevalence of drug resistance in the setting, availability of drugs, cost of treatment and medical supervision, whether the patient has previously received any anti-tuberculosis drug, the country's budget, health coverage by public health services, and qualifications of health staff at the peripheral level. Then, the selection of a particular drug regimen must be done considering all these factors.

The World Health Organization (WHO) has established four TB diagnostic categories, assuming from a public health perspective that the highest priority of national

TB programs is to identify and cure those patients with sputum smear-positive pulmonary TB, i.e. infectious TB patients (World Health Organization 2003). **Category I** comprises those patients with a high priority for treatment who are new smear-positive patients, new smear-negative pulmonary TB patients with extensive parenchymal involvement, patients with concomitant HIV/acquired immunodeficiency syndrome (AIDS) disease or severe forms of extrapulmonary TB. Patients with a lower priority for treatment are classified as follows: **Category II** (relapse, treatment failure or default), **Category III** (new smear-negative pulmonary TB other than in Category I and less severe forms of extrapulmonary TB) and **Category IV** (chronic sputum-positive TB after re-treatment and proven or suspected MDR-TB). Preferred and optional treatment regimens for each category, as recommended by the WHO, are detailed in Table 18-3 at http://www.tuberculosistextbook.com/pdf/Table 18-3.pdf .

In addition to these guidelines for TB treatment, there are other alternatives. For example, the Center for Disease Control and Prevention (CDC) of the United States (US) also suggests continuation phases consisting of INH and rifapentine once per week for four months for patients in Category I (Centers for Disease Control and Prevention 2003a). This treatment can be used when sputum is negative for acid-fast bacilli (AFB) after the first two months of treatment but should be extended to nine months if the result of the culture at that time point is sill positive. These guidelines apply only to HIV-negative patients as regimens containing rifapentine should not be administered to HIV/AIDS patients.

In general, the duration of the continuation phase must be estimated once the first two months of treatment (initial phase) have been completed. If the patient had cavitations on initial chest radiography and cultures are still positive after two months of treatment, the continuation phase should be extended to 31 weeks (seven months).

When drug resistance develops, patients should be treated with a new combination containing at least three drugs that they had never received before (or that do not show cross-resistance with those to which resistance is suspected). In these conditions, the treatment is longer, more toxic, more expensive and less effective than regimens containing first-line drugs, and should be directly observed.

In children, drug regimens similar to those described above for adults can be given, although EMB is not recommended because of its ocular toxicity. Rifapentine has not been approved for pediatric use.

In case of pregnancy, similar drug regimens can be prescribed, although SM and other second-line aminoglycosides must not be given because they are ototoxic for

the fetus. Also, there has been concern about the use of PZA. Then, a drug regimen of nine months of INH and RIF supplemented with EMB during the first months has been proposed. All antituberculosis drugs are compatible with breast feeding, although babies should be given chemoprophylaxis for at least three months after the mother is considered non-infectious.

Since HIV/AIDS patients have a higher probability of acquiring TB (either pulmonary or extrapulmonary) or other mycobacterial opportunistic infections, particular drug regimens have been designed for treating active TB disease in them (Tuberculosis Coalition for Technical Assistance 2006). Also, the severity of adverse effects of anti-mycobacterial drugs (due to the interactions with anti-retroviral drugs) and mortality is higher among HIV-positive patients. Although, in general, HIV-positive patients respond well to a standard short-course treatment of TB, treatment failure due to malabsorption of antimycobacterial drugs has been reported. The WHO recommends not using SM or thiacetazone in HIV-positive patients in order to prevent the adverse effects of these drugs, often enhanced by anti-retroviral drugs; EMB can be used instead. Rifamycins (rifampicin, rifabutin, etc.) have clinically relevant interactions with some drugs used in the antiretroviral therapy, since they induce the metabolism of anti-retroviral agents such as zidovudine, non-nucleoside reverse transcriptase inhibitors, and HIV protease inhibitors, whose concentrations may fall to sub-therapeutic levels (see section 18.5 below). Then, rifamycin-free regimens have been suggested. They consist of INH, EMB, PZA, and SM, daily for two months, followed by INH, PZA, and SM two or three times weekly for seven months. However, it has also been described that the use of RIF throughout antituberculosis treatment improves outcome in HIV patients.

Chemoprophylaxis of TB is indicated for asymptomatic patients having a positive tuberculin skin test (TST) but not showing active disease (latent TB infection), especially when they are at risk of developing the disease (for example, HIV-positive patients) (Balcells 2006, Centers for Disease Control and Prevention 2003b, Stout 2004). This is aimed at preventing the occurrence of TB. Prophylaxis is most frequently achieved by the administration of INH only, at doses of 300 mg daily for six to nine months (although there is a risk of developing INH resistance). When resistance to INH is suspected, other regimens include RIF, PZA or EMB, can be administered, although there is a greater chance of having adverse effects. In TB prophylaxis, RIF can be given concurrently with INH, reducing the prophylaxis treatment to three months.

It is of prime importance to ensure the patient's adherence to the antituberculosis treatment in order to achieve complete elimination of the bacilli (and hence avoid disease relapse), and also to prevent the emergence of drug resistance. For this

reason, the antituberculosis treatment has to be supervised. Both the patient's adherence and supervision are often difficult to manage when the antituberculosis treatment has to be administered on a daily basis. Alternative treatments based on an intermittent administration of drugs (three times, twice and even once per week) facilitate the patient's adherence and the supervision of treatment. Intermittent treatment is possible because antituberculosis drugs have a marked post-antibiotic effect. After the tuberculous bacillus has been exposed to drugs, there is a lag period (up to several days) during which its growth is interrupted even after the drug concentration has fallen to sub-inhibitory levels. Thus, there is no need to maintain a continuous inhibitory drug concentration to kill the bacilli or prevent growth.

18.2.4. Drug preparations

Most drugs used in antituberculosis treatment – INH, RIF, rifapentine, rifabutin, PZA, EMB, and ethionamide (ETH) – are commercially available as tablets or capsules and can therefore be taken orally. INH is also available as an elixir, in granules for pediatric use, and in aqueous solution for intravenous or intramuscular injection. RIF is available in powder for preparing suspensions for oral administration, and also in aqueous solution for intravenous or intramuscular injection. The exceptions are the aminoglycosides – SM, kanamycin, and amikacin – and capreomycin, which are only available as aqueous solutions for intravenous or intramuscular injection. PAS is usually available as granules for mixing with food; tablets and solutions for intravenous administration can also be found. The fluoroquinolones are available as tablets or as aqueous solutions for intravenous injections.

The three main drugs used in the standard antituberculosis regimen – INH, RIF, and PZA – can also be found in fixed-dose combination preparations (Centers for Disease Control and Prevention 2003a, Panchagnula 2004, World Health Organization 2003). There are several combinations, containing for example, INH and RIF, INH and EMB, INH, RIF and PZA, and INH, RIF, PZA and EMB. When available, the use of combination preparations is recommended. Indeed, by reducing the number of tablets to be taken, they facilitate the patient's adherence to treatment and supervision of therapy. Most importantly, this form of preparation minimizes the possibility of monotherapy and therefore, reduces the risk of drug resistance development.

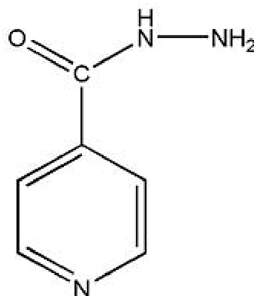
18.3. Drugs: structure, pharmacokinetics and toxicity

In this section, we describe the structure, general properties, pharmacokinetics, and toxicity of the main drugs used in the treatment of TB. More detailed information on drugs can be found in pharmacological books (Martindale 2004), reports on TB treatment (Centers for Disease Control and Prevention 2003a) or highly specialized reports (Douglas 1999, Forget 2006, Launay-Vacher 2005, Nuermberger 2004a, Saukkonen 2006, Zhang 2005).

18.3.1. Isoniazid

Structure and general properties

INH is a pro-drug that requires processing by the bacterial catalase-peroxidase to become active. Once activated, it inhibits the biosynthesis of mycolic acids, which are essential components of the mycobacterial cell wall. This drug is bactericidal against metabolically active bacilli and bacteriostatic against resting bacilli. INH is active against *M. tuberculosis, M. bovis* and *M. kansasii.* Susceptible *M. tuberculosis* strains show minimal inhibitory concentrations (MIC) between 0.02 and 0.2 mg/L.

Isoniazid (isonicotinic acid hydrazide; $C_8H_7N_3O$, MW: 137.1) is one of the most powerful drugs against TB. It is a white crystalline powder freely soluble in water. Solutions can be sterilized by autoclaving	

Pharmacokinetics

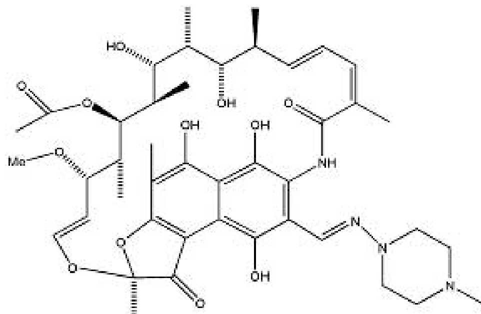
INH is readily absorbed from the gastrointestinal tract (although absorption is reduced by food) or following intramuscular injections. Peak concentrations of 3-8 mg/L appear in blood between 1-2 hours after ingestion of 300 mg of INH. It diffuses into all body tissues, including cerebrospinal fluid. The plasma half-life ranges from 1 to 6 hours. INH is metabolized in the liver and the small intestine: first, an N-acetyltransferase acetylates INH producing acetylisoniazid; this product is hydrolyzed to isonicotinic acid and monoacetylhydrazine, and the latter compound is further acetylated to diacetylhydrazine. None of these INH-derived metabolites have any antituberculosis activity. Within the population, there are two groups of patients, depending on whether INH is acetylated slowly or rapidly, a characteristic that is genetically determined. Plasma INH concentrations are lower in rapid acetylators than in slow acetylators, although this difference does not affect the efficacy of the treatment. INH and its metabolites are excreted in the urine.

Toxicity

INH is well tolerated at recommended doses, although slow acetylators can accumulate higher INH concentrations and then have a higher risk of developing adverse effects. Between 10 % and 20 % of patients may develop transient increases in liver enzymes at the beginning of treatment, and sometimes develop hepatic damage. In these cases, administration of INH should be stopped. Liver function should be monitored before and during treatment, especially in those patients with a history of hepatic or renal dysfunction, in whom doses of INH should be reduced to prevent further damage. Neurological or hematological adverse effects and hypersensitivity reactions occur less frequently. A daily dose of 10 mg of pyridoxine hydrochloride is recommended to reduce neurotoxicity and to treat adverse effects caused by INH.

18.3.2. Rifampicin

Structure and general properties

Rifampicin, often spelled rifampin, (5,6,9,17,19,21-Hexahydroxy-23-methoxy-2,4,12,16,18,20,22-heptamethyl-8-[*N*-(4-methyl-1-piperazinyl)formimidoyl]-2,7-(epoxypen-tadeca[1,11,13]trienimino)-naphtho[2,1-*b*]furan-1,11(2*H*)-dione21-acetate; $C_{43}H_{58}N_4O_{12}$; MW 822.9) is a red-brown crystalline powder poorly soluble in water; it is dissolved in methyl alcohol and can be stored at room temperature protected from light.	

RIF inhibits gene transcription, by interacting with the beta subunit of the ribonucleic acid (RNA) polymerase enzyme. It is bactericidal against dividing mycobacteria and also has some activity against non-dividing bacilli. *M. tuberculosis* strains are normally susceptible to 0.1-2 mg/L. The introduction of RIF, thus, allowed reduction of the duration of standard antituberculosis treatments from one year to nine months. This was later reduced to six months after incorporation of PZA. RIF is also active against a wide range of microorganisms, including staphylococci, *Neisseria* spp. *Haemophilus influenzae* and *Legionella* spp.

Pharmacokinetics

This drug is readily absorbed from the gastrointestinal tract (food may delay or decrease RIF absorption); within 2 to 4 hours after ingestion of a dose of 600 mg, peak plasma concentrations may reach 7-10 mg/L. It also can be given intravenously. In blood, RIF is bound to plasma proteins, and distributes into body tissues and fluids, including cerebrospinal fluid and breast milk, and crosses the placenta.

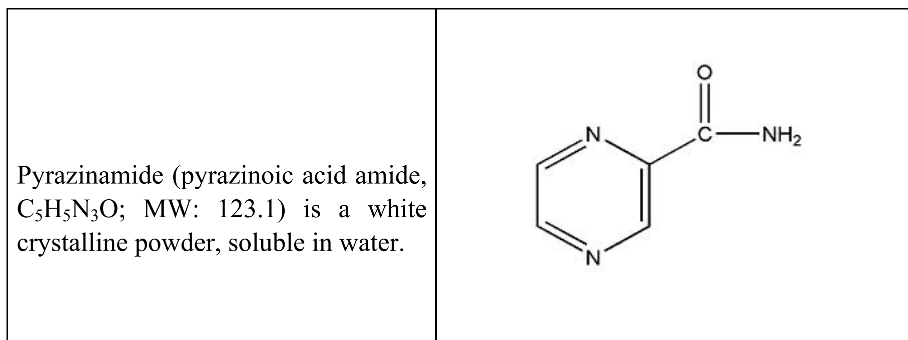
The half-life of RIF ranges from 2 to 5 hours. RIF is metabolized in the liver, and excreted in the bile, feces and urine.

Toxicity

RIF is well tolerated, although adverse effects may arise during intermittent therapy or when restarting an interrupted treatment. Adverse effects include diverse alterations in the gastrointestinal tract, skin, kidney and nervous system. It may also produce thrombocytopenia. RIF will cause a red-orange coloration of body fluids such as urine, tears, saliva, sweat, sputum and feces; it may result in the coloration of soft contact lens. Since it is metabolized in the liver, hepatic functions should be controlled before starting treatment and monitored regularly until the therapy ends. Special care should be taken in patients with pre-existing liver diseases. A moderate increase in alkaline phosphatase can be observed.

18.3.3. Pyrazinamide

Structure and general properties



Pyrazinamide (pyrazinoic acid amide, $C_5H_5N_3O$; MW: 123.1) is a white crystalline powder, soluble in water.

PZA is a bactericidal drug active only against *M. tuberculosis*, having no in vitro activity against other mycobacteria or any other microorganism. Susceptible strains have MICs of 20 mg/L at pH 5.6. It is active against persisting and non-dividing bacilli, even against those residing intracellularly, being almost inactive at neutral pH. The introduction of PZA into treatment regimens for TB allowed reduction of the duration of such regimens to six months. PZA is a pro-drug that requires conversion into pyrazinoic acid to be effective; this is done by mycobacterial pyrazinamidases.

Pharmacokinetics

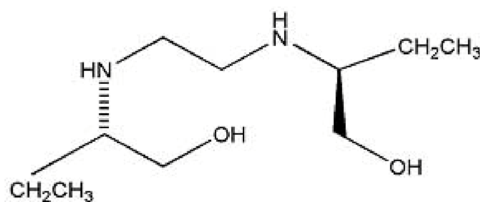
PZA is given orally and is readily absorbed from the gastrointestinal tract. Serum concentrations reach a peak level of about 66 mg/L two hours after administration of a dose of 3 g. It is distributed in all body tissues and fluids, including the cerebrospinal fluid and breast milk. The half-life of PZA is about 9-10 hours. PZA is hydrolyzed in the liver, being converted to pyrazinoic acid, which is further hydroxylated and finally excreted in the urine.

Toxicity

PZA is hepatotoxic in a dose-dependent manner. Following a daily dose of 3 g of PZA, 15 % of patients may develop liver alterations, such as transient increases in liver enzymes, hepatomegaly, splenomegaly and jaundice. Hepatitis has been reported in less than 3 % of cases. It may also produce hyperuricemia, leading to attacks of gout. Therefore, it is contra-indicated in patients with liver damage, and it is advisable to test liver function before and regularly during treatment. It also should not be given to patients having a history of gout or hyperuricemia.

18.3.4. Ethambutol

Structure and general properties

Ethambutol (N,N'-ethylenebis(2-aminobutan-1-ol) dihydrochloride; $C_{10}H_{24}N_2O_2$,2HCl; MW: 277.2) is a white crystalline powder soluble in water and alcohol that must be stored preserved from air.	

This drug is used to treat TB and other opportunistic infections caused by non-tuberculous mycobacteria such as *Mycobacterium kansasii*. The MICs of sensitive *M. tuberculosis* strains range from 0.5 to 8 mg/L.

EMB is only active against dividing mycobacteria, being bacteriostatic. Since EMB affects the biosynthesis of the cell wall, it has been suggested that it contributes towards increasing the susceptibility of *M. tuberculosis* to other drugs.

Pharmacokinetics

EMB is given orally, as it is well absorbed in the gastrointestinal tract (and not affected significantly by food), although a part is excreted in the feces. After absorption, it is distributed in most tissues and diffuses into the cerebrospinal fluid and breast milk; it also crosses the placenta. Following a dose of 25 mg/kg body weight a peak concentration of 5 mg/L in serum is reached after 4 hours. The half-life is about 3 to 4 hours. Only a fraction of EMB is metabolized in the liver; the unchanged drug and its metabolites are excreted in the urine.

Toxicity

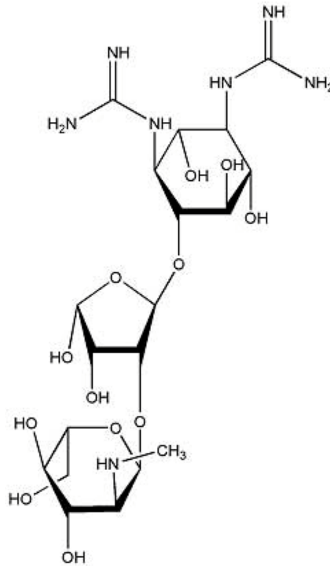
EMB produces retrobulbar neuritis with a reduction in visual acuity, constriction of visual field, central or peripheral scotoma, and green-red color blindness (Fraunfelder 2006). This may affect one or both eyes. The severity of these effects depends on the dose and duration of treatment. Usually, normal vision is recovered a few weeks after the end of the treatment, although in some cases, this recovery may not occur until some months after the completion of treatment. Consequently, EMB is contraindicated in patients with optic neuritis, and should be used with care in patients with visual disorders. Optical examinations are advisable before and during treatment. EMB is not usually given to children under six years of age because of the difficulty in monitoring visual acuity, unless resistance to INH or RIF is highly suspected.

Other adverse effects include a reduction of urate excretion (hence producing gout), gastrointestinal disorders and hypersensitivity skin reactions.

18.3.5. Streptomycin

Structure and general properties

Streptomycin (O-2-deoxy-2-methylamino- α-L-glucopyranosyl-(1-2)-O-5-deoxy- 3-C-formyl-α-L-lyxofuranosyl- (1-4)-N,N-diamidino-D-streptamine; $C_{21}H_{39}N_7O_{12}$; MW: 581.6). It is a white-whitish crystalline powder, highly hygroscopic and soluble in water that must be stored in airtight containers.



SM, an antibiotic produced by some strains of *Streptomyces griseus*, was the first drug with antituberculosis activity to be discovered. It is mainly used in the treatment of TB (most *M. tuberculosis* strains are susceptible to 1-8 mg/L of streptomycin). It can also be used in the treatment of other bacterial infections such as those produced by *Yersinia pestis, Francisella tularensis,* and *Brucella* spp.

Pharmacokinetics

SM, like most aminoglycosides, is poorly absorbed from the gastrointestinal tract, and therefore it must be administered by intramuscular injection. Because of the toxicity of SM (see below) and the introduction of other drugs that can be administered orally for the treatment of TB, the use of SM has decreased, being relegated to the treatment of infections caused by drug-resistant strains. Two hours after an injection of 1 g SM, drug levels in blood may reach up to 50 mg/L, where one third of it circulates bound to plasma proteins. The half-life for SM is about 2.5 hours.

SM and the other aminoglycosides diffuse well into most extracellular fluids, maybe with the exception of the cerebrospinal fluid. They diffuse quite readily into the perilymph of the inner ear, causing ototoxic effects (see below). Aminoglycosides also tend to accumulate in specific body tissues such as the kidneys. Streptomycin does not appear to be metabolized, and is excreted unchanged in the urine.

The concurrence of other diseases may affect the pharmacokinetics of SM and this may become relevant since there is a relatively small difference between the therapeutic and toxic concentrations of aminoglycosides. For example, patients with renal impairment will have increased plasma concentrations of SM, whereas in patients having diseases that cause expanded extracellular fluid volume or increased renal clearance (such as ascites, cirrhosis, heart failure, malnutrition or burns), SM concentrations will be reduced.

Toxicity

Like most aminoglycosides, SM has ototoxic effects affecting vestibular rather than auditory (cochlear) function, which manifest as dizziness and vertigo. It is less nephrotoxic than other aminoglycosides, although it may produce renal failure when administered with other nephrotoxic agents. Regular assessment of both auditory and renal function is recommended. In case of severe adverse effects, SM can be removed by hemodialysis. Paresthesia, neurological symptoms such as peripheral neuropathies, optic neuritis and scotoma, and hypersensitivity skin reactions have also been observed after SM injections.

18.3.6. Other drugs against tuberculosis

Drugs in this group are interesting for one or more of the following features:

- widely used in the past but in our days its use has been relegated by the incorporation of more effective and/or less toxic drugs
- used when resistance to first-line antituberculosis drugs is suspected or confirmed, and are usually denominated second-line drugs
- used when severe adverse effects to other antituberculosis drugs develop
- have been developed recently and, because of their usefulness for the treatment of TB, are potential first-line drugs that could be incorporated soon into standard (and maybe shorter) antituberculosis regimens
- allow intermittent doses, hence facilitating patient's adherence to antituberculosis treatment

Para-aminosalicylic acid

This compound and its salts are active only against *M. tuberculosis*, which can be inhibited by 1 mg/L of this drug. It is bacteriostatic. PAS can be given orally, in a daily dose of 10-12 g divided into two or three doses. It is well absorbed in the gastrointestinal tract and distributes well throughout the body, although it is poorly distributed in the cerebrospinal fluid. It is metabolized in the intestine and in the liver, and it is excreted mainly in the urine. PAS may produce gastrointestinal side-effects such as nausea, vomiting, diarrhea, and hypersensitivity reactions, and should be administered with care in patients with liver or renal impairment. PAS can be used safely during pregnancy but is not recommended because of the gastrointestinal intolerance. The use of PAS has largely decreased since the introduction of RMP and EMB; however, due to its low cost, it is still in use in low-resource countries.

Capreomycin

This polypeptide is bacteriostatic against several mycobacteria including *M. tuberculosis*; susceptible strains are inhibited by 10 mg/L of capreomycin. Doses, usually of 1 g, must be administered by intramuscular or intravenous injection. Capreomycin is excreted in the urine. It must be given with care to patients with renal, hepatic or auditory dysfunction. Commonly, capreomycin affects the frequency of urination or the amount of urine, increases thirst and may produce loss of appetite, nausea and vomiting. Due to its toxic effects, it must not be given in combination with aminoglycosides such as kanamycin or streptomycin.

Cycloserine

This is a broad-spectrum antibiotic that inhibits many microorganisms such as *Escherichia coli, Staphylococcus aureus, Nocardia* spp., *Chlamydia,* and *M. tuberculosis*. Due to its high toxicity, it is only used against bacilli resistant to the main antituberculosis drugs. Doses of 500 mg are given orally twice a day. It is fairly well absorbed in the gastrointestinal tract, being distributed to most tissues and fluids, including cerebrospinal fluid. Cycloserine is metabolized and excreted in the urine. It should be given with care to patients with renal impairment. It may produce diverse adverse reactions involving the central nervous system, from mild headache or restlessness to severe psychosis and seizures, and is therefore contraindicated in patients with epilepsy, depression or anxiety. Hypersensitivity skin reactions have also been described.

Aminoglycosides

Amikacin and kanamycin are active against a range of bacteria including *M. tuberculosis*. Amikacin is also active against atypical mycobacteria that cause opportunistic infections, such as those of the *Mycobacterium avium* complex. Both are considered as second-line antituberculosis drugs; other safer drugs are preferred for the treatment of TB. These antibiotics are often combined with EMB, ciprofloxacin, and macrolides. Like SM (see above) amikacin and kanamycin must be given by intramuscular injections, usually in doses of 0.5-1 g. They are distributed in body tissues and fluids, and cross the placenta but do not reach the cerebrospinal fluid. Like most aminoglycosides, amikacin and kanamycin affect auditory function and must be given with care to patients with auditory dysfunctions. They are also nephrotoxic, producing renal impairment in approximately 8 % of patients. Kanamycin may also produce some gastrointestinal effects, such as nausea, vomiting, and stomatitis, especially when taken by mouth. Both aminoglycosides are excreted unchanged in the urine.

Thioamides

There are two main drugs from the thioamide (or thionamide) family that can be used for the treatment of TB: ETH and prothionamide.

ETH is a structural analogue of INH and in fact some cross-resistance has been observed between both drugs. ETH is active against *M. tuberculosis, M. leprae, M. kansasii*, and some strains of the *M. avium* complex. Susceptible *M. tuberculosis* strains are inhibited by 0.6-2.5 mg/L of ETH. For the treatment of TB, doses of 15-20 mg/kg of body weight are given orally, up to a maximum of 1 g daily. It is well absorbed from the gastrointestinal tract, and diffuses into all body tissues and fluids, including cerebrospinal fluid. Its half-life is 2 hours. ETH is metabolized in the liver and excreted in the urine. Thus, it should not be given to patients with liver dysfunction. Adverse effects associated with ETH administration include dose-related gastrointestinal disorders (such as anorexia, excessive salivation, nausea, vomiting, metallic taste, abdominal pain, and diarrhea), diverse mental disturbances (such as depression, anxiety, psychosis, dizziness, drowsiness, and headache) and hypersensitivity skin reactions have also been described.

Prothionamide is very similar to ETH; complete cross-resistance between these two drugs usually occurs. It can be used orally, at doses similar to those of ETH. It is well absorbed from the gastrointestinal tract, and distributes to all body tissues and fluids, including cerebrospinal fluid. Prothionamide is metabolized in the liver and excreted in the urine.

Fluoroquinolones

Among the fluoroquinolones, there are drugs with several degrees of activity against *M. tuberculosis* (Ginsburg 2003). Whereas norfloxacin has no activity against mycobacteria, ciprofloxacin and ofloxacin have been used for the treatment of TB, especially when caused by drug resistant strains, and also in the treatment of opportunistic mycobacterial infections. Other fluoroquinolones such as sparfloxacin, gatifloxacin, and moxifloxacin are even more active than ciprofloxacin for the treatment of TB, being comparable to INH.

Fluoroquinolones are well absorbed from the gastrointestinal tract (presence of food reduces absorption), and peak plasma concentrations are obtained rapidly, usually after 1-2 hours, where they are partially bound to plasma proteins. Half-life is variable, ranging from 4 hours in the case of ciprofloxacin to 10-13 hours in the case of moxifloxacin. They distribute well into all body tissues, and are finally, eliminated in the urine. Fluoroquinolones are generally well tolerated. Adverse effects include disorders of the gastrointestinal tract, nervous system, and skin. They should not be given to patients having central nervous system disorders such as epilepsy. The use of fluoroquinolones is not recommended in children or during pregnancy. Interactions with other drugs are infrequent.

Rifamycins

The rifamycin family of drugs includes RIF, one of the most potent first-line anti-tuberculosis drugs (see above). Other members of this family include rifabutin and rifapentine, which share their mode of action and spectrum of antibacterial activity with RIF. A high degree of cross-resistance among rifamycins has been found. Rifapentine and rifabutin have, however, some distinct properties in comparison to RIF, which makes them very useful in certain situations.

The MICs of rifabutin for *M. tuberculosis* susceptible strains are usually eight times lower than those for RIF. Rifabutin can be used for the treatment of TB at doses of 150-450 mg daily, combined with other drugs to avoid drug resistance. It is also frequently used for the prophylaxis of *M. avium* infections in immunocompromised patients and for the treatment of other opportunistic infections caused by mycobacteria.

In contrast to RIF, rifabutin is poorly absorbed in the gastrointestinal tract; once it gets into the blood, most of it is bound to plasma proteins, and distributes widely into the body. Rifabutin is metabolized in the liver where it induces microsomal enzymes, although to a lesser extent than RIF. Rifabutin is excreted in the urine.

Rifabutin produces a syndrome of polyarthralgia-arthritis at doses over 1 g daily. Uveitis has been reported in patients also receiving macrolides or azole antifungals. Rifabutin reduces the plasma concentration of several antiretroviral drugs, such as zidovudine. Despite this, rifabutin (at reduced doses) has been recommended in place of RIF in the treatment of TB in HIV/AIDS patients, in order to avoid major interactions of RIF (see below) and the antiretroviral drugs.

Rifapentine is considered a long-acting rifamycin, since it can be given orally at doses of 600 mg twice weekly or even once weekly during the initial phase in the treatment of TB (Temple 1999). It is well absorbed from the gastrointestinal tract. Rifapentine and RIF show cross-resistance. Adverse effects of rifapentine are similar to those of RIF, except for a higher incidence of hyperuricemia. This drug has not been approved for use in children, since the safety and efficacy of this drug has not yet been established for this age group. Also, rifapentine is not recommended for HIV/AIDS patients because of their risk of developing rifamycin resistance.

Thiacetazone

This drug, also spelled thioacetazone, is bacteriostatic against *M. tuberculosis*, with susceptible strains being inhibited by 1 mg/L. Cross-resistance with ethionamide and prothionamide can occur. It may be used in anti-tuberculosis regimens, although these may not be as effective as the standard short-course therapy. It is well absorbed in the gastrointestinal tract and peak concentrations of 1-2 mg/L are obtained four hours after administration of a 150 mg dose. It is excreted in the urine. Thiacetazone produces diverse adverse effects such as gastrointestinal disorders, and hypersensitivity reactions (including skin rashes) that may be more frequent in HIV/AIDS patients. Other frequent adverse effects include conjunctivitis, vertigo, toxic epidermal necrolysis, exfoliative dermatitis, hemolytic anemia, and hepatotoxicity with jaundice. It should not be given to patients with liver impairment, or to HIV/AIDS patients because of the risk of increased adverse reactions. Some low-income countries still use thiacetazone because of its low cost.

18.4. Drug resistance mechanisms

18.4.1. Natural drug resistance

The natural drug resistance of *M. tuberculosis* is an important obstacle for the treatment and control of TB. This resistance has traditionally been attributed to the unusual multi-layer cell envelope and active multidrug efflux pumps (De Rossi 2006, Jarlier 1994). Recent insights into mechanisms that neutralize the toxicity of

antibiotics in the cytoplasm have revealed other systems that function in synergy with the permeability barrier and efflux systems to provide natural resistance. Drugs inhibiting these intrinsic systems would enable many antibiotics, which are already available but have not been used for TB, to gain a new potential use against *M. tuberculosis* (Lomovskaya 2006, Nguyen 2006).

18.4.2. Acquired drug resistance

Knowledge of the molecular basis of drug resistance in *M. tuberculosis* increased with the sequencing of the genome and the development of molecular tools (Aínsa 2001, Cole 1998). In other bacterial species, acquired drug resistance is mediated by plasmids or transposons, but in contrast, *M. tuberculosis* acquired drug resistance is caused by mutations in chromosomal genes (Heym 1994). So far, no single pleiotropic mutation has been found in *M. tuberculosis* to cause a MDR phenotype. The MDR phenotype is caused by sequential accumulation of mutations in different genes involved in resistance to individual drugs, due to inappropriate treatment or poor adherence to treatment (Zhang 2000). However, it is important to observe that some resistant strains do not present these classic mutations, suggesting the possibility of the existence of other mechanisms such as efflux pumps and alterations in the permeability of the cell wall.

Isoniazid and ethionamide

INH was synthesized in 1912 by the Czech chemists Hans Meyer and Josef Mally, but it was not until 1952 that it was introduced as an antituberculosis agent. The first indication of the mechanism of action of INH was obtained from the observation that as soon as the treatment with INH began, the acid-fast property of the tubercle bacillus was quickly lost. In 1970, it was demonstrated that INH inhibits mycolic acids synthesis, which explained the microscopic observation of the loss of the acid-fastness (Blanchard 1996).

INH has a simple structure, containing a pyridine ring and a hydrazide group and both molecules are essential for its high activity against *M. tuberculosis*. Despite its sinple structure, the mode of action of INH is very complex (Bernstein 1952).

An important aspect to underline is that INH is a prodrug; its antibiotic action depends on the bacterial activation by the catalase-peroxidase enzyme (KatG) (Zhang 1992) to generate reactive radicals, which attack multiple targets in *M. tuberculosis* (Zhang 2000).

The mechanisms of action of INH and ETH are similar but their activation mechanisms are different, so that strains resistant to INH due to mutations in *kat*G are still susceptible to ETH, indicating that there must be another enzyme responsible for the activation of ETH (Blanchard 1996).

The main target of INH is the pathway synthesizing cell wall mycolic acids (Takayama 1972). Furthermore, at least two enzymes, InhA (enoyl acyl carrier protein reductase) (Banerjee 1994) and KasA (beta-ketoacyl ACP synthase) (Mdluli 1998) have been identified as targets for INH.

Resistance to INH is mostly associated with mutations or deletions in *katG*; other mutations related with INH resistance occur in the coding region of *inhA* gene (or its promoter) and *kas*A. Furthermore, mutations in several other genes have been reported to be associated with INH resistance, but occur less frequently, and their association with INH resistance is less clear (Sreevatsan 1997; Ramaswamy 2003).

The relationship between the overexpression of *ahp*C and INH resistance has been investigated (Rattan 1998), however, it was demonstrated that the increase in the expression of *ahp*C in INH-resistant strains is aimed at compensating the loss or the decrease in catalase activity produced by the alteration of the *kat*G gene; thus, this increased expression of *ahpC* on its own would not be related with INH resistance (Sherman 1999). Other possible resistance mechanisms are being investigated in *M. smegmatis,* which is 300 times more resistant to INH than *M. tuberculosis*, indicating that efflux pumps could be another possible mechanism of INH resistance (Choudhuri 1999, Colangeli 2005).

Rifampicin

RIF, a lipophilic ansamycin, was introduced in 1972 in the treatment of TB. Due to its efficient antimicrobial action, it is considered, together with INH, to be the basis of the short-course treatment regimen (Rattan 1998).

RIF associates with the beta-subunit of the ribonucleic acid (RNA) polymerase, inhibiting the elongation of the messenger RNA (mRNA) (Blanchard 1996). RNA polymerase is an oligomer consisting of a catalytically competent core enzyme formed by four subunits (two alpha subunits, and beta and beta-prime subunits) in association with another subunit, sigma, which is able to specifically initiate transcription (Zhang 2000).

As in *Escherichia coli*, almost all clinical isolates of *M. tuberculosis* resistant to RIF show mutations in *rpoB*, the gene that encodes the beta-subunit of the RNA polymerase, resulting in conformational changes that determine the low affinity of this subunit for RIF and consequently, resistance to the drug (Jin 1988, Williams

1994). Mutations conferring resistance to RIF are clustered in three short regions in the central region of the beta-subunit gene: cluster I (amino acids 512 to 534), cluster II (amino acids 563 to 574) and cluster III (amino acid 687) (Zhang 2000).

Although mutations in *rpoB* usually result in high-level resistance and show cross resistance to other rifamycins, mutations in codons 511, 516, 518, 522, 529, and 533 have been associated with low-level resistance to RIF and/or susceptibility to rifabutin and the new rifamycin KRM1648 (Bodmer 1995, Cavusoglu 2004, Moghazeh 1996, Yang 1998, Williams 1998).

Pyrazinamide

PZA is structurally similar to nicotinamide, and is converted into the acid form (pyrazinoic acid) by the bacterial pyrazinamidase enzyme (PZase) (Konno 1967). PZA is active against bacilli in a semi-dormant state. Its introduction into the primary treatment of TB allowed the reduction of the treatment from nine to six months. This property has been attributed to its ability to inhibit semi-dormant bacilli residing in acidic environments (Mitchison 1985).

The antimicrobial action of PZA is highly specific for *M. tuberculosis*, with little or no activity against other mycobacteria, including *M. bovis*. The reason for the specific activity of PZA against *M. tuberculosis* is because this drug needs to be activated by the PZase enzyme, which is encoded by the *pncA* gene. This gene is altered in many species of mycobacteria, which are resistant to PZA because they lack an efficient PZase. In *M. bovis*, for example, the substitution of the His residue in position 57 for Asp produces a non-effective PZAse (Konno 1967).

In most cases, resistance to PZA is associated with mutations in *pncA*. PZA-resistant strains have shown a wide range of alterations in the 630 bp of the open reading frame or in the 82 bp of the promoter region (Scorpio 1996).

Some PZA-resistant strains do not present any alterations in the coding region or the promoter of the *pncA* gene. For these strains, it has been postulated that PZA resistance could be due to mutations in an unknown *pncA* regulatory gene (Cheng 2000).

Ethambutol

EMB is a synthetic compound used as first-line drug for anti-tuberculosis therapy in combination with other drugs, as recommended by the WHO. It has been demonstrated that EMB acts on enzymes involved in the biosynthesis of arabinogalactan (Takayama 1989), inhibiting the polymerization of cell wall arabinan of arabinogalactan and of lipoarabinomannan (Mikusova 1995). In *M. tuberculosis*, the

emb operon has three contiguous genes: *embC, embA,* and *embB*, which encode mycobacterial arabinosyl transferases (Telenti 1997). These enzymes have been considered the drug targets for EMB, since substitutions of codon 306 in the *M. tuberculosis embB* gene have been shown to be the most frequent and predictive mutations for EMB resistance (Srivastava 2006). For strains with the Met306Leu or Met306Val replacements, EMB MICs were generally higher (40 mg/L) than those for organisms with Met306Ile substitutions (20 mg/L). In *M. tuberculosis*, mutations in genes other than *embB* have been associated with EMB resistance. Often these mutations affect a putative regulatory sequence in the *embC–embA* intergenic region (Ramaswamy 2000).

Streptomycin

SM is an aminocyclitol glycoside antibiotic that was the first antibiotic used for the treatment of TB. SM inhibits the initiation of mRNA translation affecting translation fidelity (Moazed 1987). Mutations associated with SM resistance in *M. tuberculosis* have been identified in the 16S ribosomal RNA (rRNA) gene (*rrs*) and *rpsL* gene encoding ribosomal protein S12 (Finken 1993). The majority of point mutations producing SM resistance occur in *rpsL* and the most common mutation is an AAG->AGG change in codon 43, which results in a Lys->Arg substitution; less frequently, an AAG->ACG (Lys->Thr) substitution is observed (Böttger 1994, Musser, 1995). The second mechanism of SM resistance in *M. tuberculosis* is mutation in *rrs*. Mutations in *rpsL* and *rrs* have been identified in 50 and 20 % of SM-resistant clinical isolates, respectively, resulting in high or intermediate levels of SM resistance respectively. There are some clinical isolates that show low level SM resistance in which no mutation in *rpsL* or *rrs* has been found (Zhang 2000). It has been hypothesized that changes in the cytoplasm concentration of SM due to the action of efflux pumps could be the molecular basis of SM resistance in these strains (Aínsa 1998, Meier 1996, Silva 2001).

Fluoroquinolones

The main targets of the quinolones are the desoxyribonucleic acid (DNA) gyrase, a type-II DNA topoisomerase composed of two A and two B subunits encoded by genes *gyrA* and *gyrB,* respectively (Takiff 1994), and DNA topoisomerase IV (Drlica 2003). High-level resistance to fluoroquinolones in laboratory strains of *M. tuberculosis* and *M. smegmatis* (Takiff 1994) is known to result from amino acid substitutions in the putative fluoroquinolone binding region of the *M. tuberculosis gyrA* or *gyrB* genes (Aubry 2004, Cambau 1994). This is the only type II topoisomerase encoded in the *M. tuberculosis* genome (Cole 1998) and thus, is the unique target for fluoroquinolones in this organism (Aubry 2004).

Fitness and Drug Resistance

The relation between drug resistance and fitness cost has led to the assumption that removal of antibiotic selective pressure would favor the elimination of resistant bacteria, because mutations conferring drug resistance usually affect replication and this is a disadvantage when resistant bacteria have to compete with sensitive bacteria in the absence of antibiotic (Andersson 1999). In fact, antibiotic resistance, caused by target alteration or by other mechanisms, can confer a reduction in fitness expressed as reduced growth, virulence or transmission (Andersson 2006). However, this cost can be compensated, usually without loss of resistance, by second-site mutations during the evolution of the resistant bacteria (Bjorkman 2000). The effects of resistance mutations on the fitness of *M. tuberculosis* could be important in epidemiological predictions of the spread of MDR strains (Cohen 2003).

There are only limited data available on the effect of different drug resistance conferring mutations on the relative fitness of *M. tuberculosis* (Billington 1999, Bottger 1998, Gagneux 2006, Mariam 2004, Pym 2002). The main limitations in some of these studies are the use of *in vitro* models or non-isogenic strains. Host and environmental factors, as well as strain genetic diversity can also influence the transmission dynamics of drug-resistant bacteria, while virulence of strains may reflect other genomic differences uncoupled from drug resistance.

18.5. Drug interactions

In general, when two or more drugs are administered simultaneously to a patient, there is a possibility that the drugs involved may interact between them. This interaction may result in changes (increase or decrease) of the effective concentration of one or more of the drugs involved, which most can usually be solved by adjusting the doses of the affected drug. The interaction may also produce an enhancement in adverse effects produced by any of the drugs, which is frequently solved by using alternative drugs that are not affected by the interaction. Since the antituberculosis treatment itself consists of the administration of two or more drugs, and in some occasions it is given simultaneously with other drug regimes (i.e. the antiretroviral treatment) it is very important to consider those drug interactions affecting the TB drugs.

Few drugs interact to alter the concentration of the antituberculosis drugs (Centers for Disease Control and Prevention 2003a, Martindale 2004, Yew 2002). More frequently, antituberculosis drugs affect the other drugs. Most of the clinically relevant interactions involve the rifamycin drugs (RIF, rifapentine and rifabutin).

Other interactions affecting first-line antituberculosis drugs and the fluoroquinolones will also be described in this section.

18.5.1. Rifamycins

The rifamycins are metabolized mainly in the liver, and to a lesser extent in the intestine wall, where they induce several pathways involving isoenzymes of the cytochrome P450 system, such as the isoenzyme CYP3A4 (Yew 2002). The extent of the induction of the isoenzyme CYP3A4 depends on the particular rifamycin drug that is being used, and so, RIF is the most potent inducer, whereas rifapentine is a moderate inducer and rifabutin is the least potent inducer of the isoenzyme CYP3A4. Rifabutin, but not RIF or rifapentine, is also a substrate of CYP3A4. Then, other drugs that share or interact with the cytochrome P450 system may have significant levels of interaction with the rifamycins.

Drugs affecting the rifamycins

Ritonavir, a protease inhibitor that is combined with inhibitors of reverse transcriptase during anti-HIV therapy, is a potent inhibitor of the isoenzyme CYP3A4, which is the isoenzyme that metabolizes rifabutin. As a consequence, rifabutin levels may increase up to four-fold, and other rifabutin-derived metabolites may also reach higher levels. This produces a higher probability of having leucopoenia and other adverse effects. RIF can be used instead of rifabutin in order to avoid this interaction.

Efavirenz, another antiretroviral drug, is an inducer of the CYP3A4. Its administration may result in a decrease in the concentration of rifabutin to one third of its normal serum concentrations.

Clofazimine, a drug used in the treatment of leprosy, may reduce the absorption of RIF.

Drugs affected by the rifamycins

Since rifamycins induce microsomal liver enzymes, they accelerate the metabolism of some other drugs reducing their half-lives and their concentrations, sometimes to sub-therapeutic levels. This problem can be solved easily by increasing the dosage of the drugs affected, which have to return to normal doses two weeks after completion of the rifamycin treatment. One exception to this general rule can be the case of oral contraceptives in women, and other contraceptive methods should be recommended.

Maybe, the most important family of drugs affected by the rifamycins is the antiretroviral agents, both the protease inhibitors and the non-nucleoside reverse transcriptase inhibitors.

RIF should not be administered simultaneously with anti-HIV drugs such as zidovudine, non-nucleoside reverse transcriptase inhibitors, and HIV protease inhibitors, since it may induce the metabolism of these drugs in the liver. Rifabutin can be used instead of RIF in some situations. The nucleoside reverse transcriptase inhibitors, which are not metabolized by CYP3A4, can be co-administered with rifamycins.

Other drugs, whose concentrations can be decreased by the use of rifamycins include atovaquone, azathioprine, chloramphenicol, cyclosporine, cimetidine, clofibrate, corticosteroids, coumarin anticoagulants, dapsone, diazepam and other benzodiazepines, doxycycline, fluconazole, haloperidol, hexobarbital, itraconazole, ketoconazole, lamotrigine, methadone, ondansetron, oral hypoglycemics, phenytoin, quinine, rofecoxib, statins, sulphasalazine, tacrolimus, the bronchodilator theophylline, thyroid hormones, and several cardiovascular drugs including beta blockers, digitalis alkaloids and antiarrhythmics such as disopyramide, lorcainide, mexiletine, propafenone, quinidine, tocainide, and verapamil and other calcium-channel blockers.

18.5.2. Isoniazid

Drugs affecting isoniazid

Chronic alcoholism may increase liver metabolism of INH. Aluminum-containing antacids reduce the absorption of INH. Food such as cheese and fish, and also red wine may produce INH-associated adverse effects.

Drugs affected by isoniazid

INH is a potent inhibitor of several cytochrome P450 isoenzymes, and then, it interferes with and inhibits the hepatic metabolism of a large number of drugs (such as, RIF), thus increasing their half-life and therefore their potential toxicity. The main drugs interacting with INH include anti-epileptics such as carbamazepine, ethosuximide and phenytoin, benzodiazepines, and chlorzoxazone.

Combination of isoniazid and rifamycins

In the standard anti-tuberculosis regimes, RIF is administered simultaneously with INH during the complete treatment (initial and continuation phases). Since both drugs are metabolized in the liver, the incidence of hepatotoxicity can be increased

and liver function should be monitored regularly. The risk of hepatotoxicity can also increase when other potentially hepatotoxic drugs are taken.

There is an important number of drugs for which both INH and RIF interact producing opposite effects: INH may increase drug concentrations whereas RIF decreases such concentrations. When both drugs are administered simultaneously, the effect of RIF is more important than that of INH, resulting in a decrease in the concentration of the drugs affected.

18.5.3. Pyrazinamide

Probenecid, a drug used for the treatment of gout, may block the excretion of PZA and co-administration of both drugs also affects excretion of urate. In some cases, patients receiving zidovudine as anti-HIV treatment may have diminished levels of PZA.

18.5.4. Ethambutol

Aluminum hydroxide-containing antacids may reduce the absorption of EMB up to a 20 %. These compounds should be taken at least two hours after the ingestion of EMB to avoid interaction.

18.5.5. Streptomycin

The administration of SM with other nephrotoxic drugs, including other aminoglycosides, vancomycin, and some of the cephalosporins, or potentially ototoxic drugs such as ethacrynic acid or frusemide should be avoided since this could increase the risk of toxicity.

18.5.6. Fluoroquinolones

Several drugs (such as those containing divalent cations, including antacids or vitamin supplements) decrease the absorption of fluoroquinolones (Ginsburg 2003). Taking these medications at least two hours after the dose of fluoroquinolones circumvents this problem.

Some fluoroquinolones can inhibit the metabolism of other drugs, such as the bronchodilator theophylline, therefore enhancing its toxic effects. The most recently developed fluoroquinolones (moxifloxacin, gatifloxacin, etc) lack this effect.

18.6. New drugs for tuberculosis

In the last 40, years no new specific drug, with particular activity against *M. tuberculosis,* has been developed or introduced into the treatment of TB. The available treatment establishes a multidrug regime lasting a minimum of six months, although there is no guarantee that the complete sterilization of the infection will be obtained. Furthermore, the increase in TB cases caused by MDR and XDR strains, and co-infection with HIV have pointed out the urgent need to develop new drugs to treat TB. Research for developing new TB drugs is being conducted using several strategies in different organizations around the world, both in academic institutions and in industrial companies, both financed by private or governmental funds, aimed at researching drugs of either synthetic or natural sources (see World Health Organization at: http://www.who.int/tb/en; TB Alliance at: http://new.tballiance.org/home/home-live.php). An ideal new TB drug should shorten the treatment, kill the persistent bacilli, and be active against resistant strains. Furthermore, the new drug should be specific for *M. tuberculosis*, compatible with existing TB drugs and non-inducer of P-450 enzymes.

In this section, we present some of the main candidates that could be introduced to the therapeutic arsenal of drugs against TB in the near future.

18.6.1. Analogues and derivatives of antituberculosis drugs

The development of new drugs against TB derived from already-known molecules, which have been used in the therapy of TB throughout the years and whose efficacy and safety have been proven, is an attractive strategy from the economic, pharmaceutical and clinical points of view. However, putative cross-resistance with parental molecules could be a negative point. Nevertheless, several analogues and derivatives of the main antituberculosis drugs are being assessed and some preliminary results are promising.

Ethambutol analogues

EMB is one of the main drugs used in the treatment of TB, and in most countries it has replaced SM and thiacetazone. The structure of EMB is favorable to the preparation of analogues by combinatorial chemistry techniques. Some EMB analogues. such as NIH 241 and SQ109 (Figure 18-1), have an efficacy comparable or even better than that of EMB (Protopopova 2005). *In vitro* studies showed that SQ109 interacts synergistically with INH and RIF, and in experimental animal models,

treatments containing SQ109 were 25 % shorter than standard cure of the disease. SQ109 has a narrow spectrum, being active against *M. tuberculosis* and *M. bovis* BCG and less active against *M. smegmatis* and *M. avium*. SQ109 is in Phase I of clinical trials and it could replace one or more of the current first-line anti-tuberculosis drugs, simplify therapy, and shorten the treatment regimen (Jia 2005; Chen 2006).

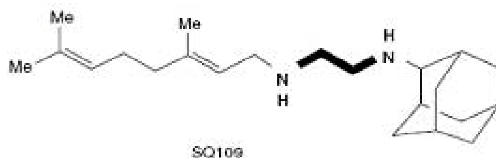


Figure 18-1: SQ109 structure

Isoniazid analogues

Various analogues and derivatives of INH continue to be synthesized. These compounds are likely to be ineffective against INH-resistant strains of TB because of close structural similarities with INH (Hudson 2003). However, since INH is a very important drug of the therapeutic arsenal against TB, efforts are being made to find new INH derivatives with more activity, less toxicity, and fewer side-effects.

Recently, the INH molecule was incorporated into a pyrazoline nucleus, showing activity against strains of *M. tuberculosis,* both susceptible and resistant to INH. Interestingly, other compounds with halogen-substituted phenyl groups showed even more activity (Shaharyar 2006).

In another study, a hydrophobic derivative of INH, 1-isonicotinyl-2-nonanoyl hydrazine, showed enhanced antimycobacterial activity against *M. tuberculosis* H37Rv. It is possible that attachment of chemical groups that help penetration of INH would make *M. tuberculosis* strains more susceptible to this drug (Maccari 2005).

Rifamycin derivatives

RIF is an important drug for the treatment of TB, and its introduction into anti-tuberculosis therapy strongly improved the control of the disease. Some rifamycin derivatives have been developed. Rifabutin shows stronger activity and is used when TB patients are also being treated for HIV infection. This is because rifabutin induces the cytochrome P-450 CYP3A oxidative enzymes at lower levels than other rifamycins (Burman 2001). Rifalazil (KRM1648 or benzoxazinorifamycin)

(Figure 18-2), a new semisynthetic rifamycin with a long half-life, is more active than RIF and rifabutin against *M. tuberculosis* both *in vitro* and *in vivo* in mice. High-level RIF-resistant strains (MIC > 32 mg/L) display cross-resistance to all rifamycins; however, low-level resistant strains (MIC < 32 mg/L) are still susceptible to the new rifamycin derivatives (Zhang 2005).

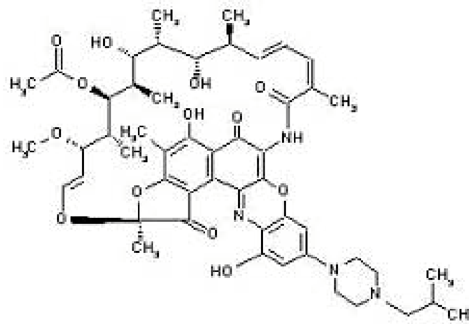
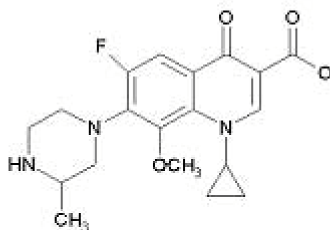


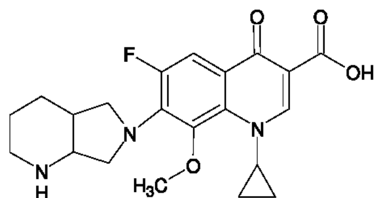
Figure 18-2 Rifalazil structure

Quinolones

Fluoroquinolones have been used sporadically since the late ’80s, primarily for the treatment of TB caused by resistant organisms or because of intolerance to first-line anti-tuberculosis drugs. Two new molecules developed more recently, moxifloxacin and gatifloxacin (Figure 18-3), with longer half-lives, are believed to have the highest *in vitro* activity against *M. tuberculosis*, followed by levofloxacin and ofloxacin (Nahid 2006). Moxifloxacin appeared to kill a subpopulation of tubercle bacilli not killed by RIF (Hu 2003). During Phase II trials, it was found that when gatifloxacin was used instead of ethambutol, the standard six-month regime was shortened to four months (detailed information is available on the internet at the World Health Organization-TDR website: http://www.who.int/tdr/). A recent study showed that moxifloxacin in combination with RIF and PZA was more effective than the classical combination of INH, RIF, and PZA (Nuermberger 2004b). The reason for this could be that moxifloxacin has activity on a subpopulation of microorganisms that is not affected by other drugs, or it could be due to the absence of the antagonism that occurs between INH and PZA (Grosset 1992; Hu 2003). Recently, it was reported that gatifloxacin may cause both hypoglycemia and hyperglycemia in both diabetic and non-diabetic patients (Zvonar 2006; Yamada, 2006), which is a serious obstacle for its use in clinical practice.



Gatifloxacin



Moxifloxacin

Figure 18-3: Structures of gatifloxacin and moxifloxacin

18.6.2. New molecules in clinical trials

DARQ

Diarylquinolines (DARQs) (Figure 18-4) are structurally different from both fluoroquinolones and other quinoline classes. The DARQ R207910 is a promising new drug against TB, because it is bactericidal against both the drug-susceptible and drug-resistant strains of *M. tuberculosis*. Low MICs were also found for other mycobacterial species, including *M. bovis, M. kansasii* and *M. ulcerans*, as well as species naturally resistant to many other anti tuberculosis agents that are involved in opportunistic infections, for example, *M. avium* complex, *M. abscessus*, *M. fortuitum,* and *M. marinum.* The activity of R207910 seems to be specific for myco-

bacteria, having much higher MICs for *Corynebacterium, Helicobacter pylori, Nocardia, Streptococcus pneumoniae, Staphylococcus aureus, Enterococcus faecalis, Escherichia coli* and *Haemophilus influenzae.* Molecular studies identified the C subunit of ATP synthase as a target of the R207910. Inhibition of ATP synthase function may lead to ATP depletion and imbalance in pH homeostasis, both contributing to decreased bacterial survival. Resistant strains of *M. tuberculosis* and *M. smegmatis* showed mutations in the *atpE* gene that encodes AtpE, a part of the F0 subunit of ATP synthase (Andries 2005). The specificity of the R207910 for mycobacteria could be explained because of the low sequence similarity between the AtpE proteins of mycobacteria and other microorganisms. However since the specificity of other antituberculosis drugs such as INH, ETH, and PZA for mycobacteria, is because these are prodrugs requiring activation by a mycobacterial enzyme, it is possible that R207910 could also be a prodrug, although its chemical structure gives no clues to potential activation sites (Cole 2005).

The compound R207910, now designated as TMC207, is being developed in phase IIa trials for the treatment of active TB. In the established murine model of TB, compound R207910 on its own is as active as the standard regimen (RMP, INH and PZA). Furthermore, when added to RIF, INH, and PZA, R207910 can shorten treatment (Lounis 2006).

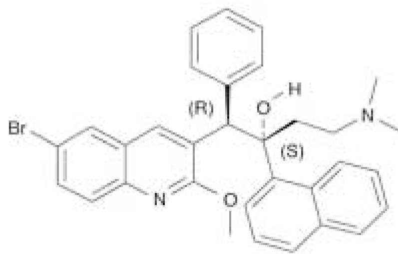


Figure 18-4: Structure of R207910

Nitroimidazoles

A series of bicyclic nitroimidazofurans, originally investigated as radiosensitizers for use in cancer chemotherapy, were found to possess activity against cultures of replicating *M. tuberculosis* and had significant *in vivo* activity in a murine infection model. The lead compound in this series, CGI-17341 was mutagenic, discouraging further investigation of the antibacterial activity of the compound series. These

studies suggested, however, that the bicyclic nitroimidazoles might be potential antituberculosis agents. A series of 328 3-substituted nitroimidazopyrans (NAPs) were synthesized on the basis of the structure of CGI-17341. One NAP compound, PA-824 (Figure 18-5), exhibited a low MIC (0.015 to 0.25 mg/L) against *M. tuberculosis* (Duncan 2003). Multidrug resistant strains exhibited comparable susceptibility to PA-824, indicating that there is no possibly cross-resistance with current antituberculosis drugs. Furthermore, it showed activity against non-replicating *M. tuberculosis* in an anaerobic culture model. In fact, metronidazole, a structurally related antibiotic, used to treat anaerobic infections, possesses activity against static *M. tuberculosis* cells surviving under anaerobic conditions (Stover 2000). In addition, this compound shows no evidence of mutagenicity in a standard battery of genotoxicity studies, no significant cytochrome P-450 interactions, and no significant activity against a broad range of Gram-positive and Gram-negative bacteria (Onyebujoh 2005). Like its progenitors metronidazole and CGI-17341, PA-824 is a prodrug of the nitroimidazole class, requiring bioreductive activation of an aromatic nitro group to exert its antituberculosis effect (Manjunatha 2006). Intriguingly, PA-824 is active under microaerophilic/anaerobic conditions, suggesting that it may have the potential to completely eradicate tissues of *M. tuberculosis.*

Two PA-824 analogues currently under investigation, PA-822 and PA-647, have greater *in vitro* activity than PA-824, but are not as active *in vivo*. Pharmacokinetics may account for the difference between the *in vitro* and *in vivo* activity of the three nitroimidazopyran compounds. Comparative pharmacokinetic studies in rats found that PA-824 had a high degree of tissue penetration and high bioavailability. In contrast, PA-647 and PA-822 have a poor degree of tissue penetration and poor bioavailability. In addition, PA-824 has a longer half-life, and the clearance of PA-822 and PA-647 appears to be faster than that of PA-824. More potent compounds that have better pharmacokinetic parameters hold promise for being more effective *in vivo* than PA-824.

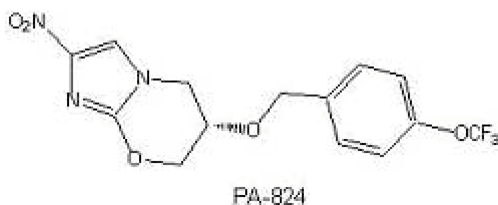


Figure 18-5: Structure of PA-824

Recent studies have demonstrated a diversity of sources and strategies for research on new drugs for the treatment of TB. Natural and synthetic sources, through bioassay-guided or screening methods, have been investigated (Ahmad 2006; Ballell 2005; Biava 2006; De Oliveira 2006; Falzari 2005; Hudson 2003; Okunade 2004; Pauli 2005). Besides, strategies such as the identification of new targets using computational software to investigate vital function (Hasan 2006) or the use of genetic tools such as random mutagenesis can help to identify new targets for new anti-TB drugs (Kana 2004).

18.7. Useful links

- Center for Disease Control and Prevention
  http://www.cdc.gov/nchstp/tb/default.htm
- Global Alliance for Tuberculosis Drug Development
  http://new.tballiance.org/
- Stop TB Partnership http://www.stoptb.org
- World Health Organization http://www.who.int/topics/tuberculosis/en/

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Chapter 19: Drug Resistance and Drug Resistance Detection

Anandi Martin and Françoise Portaels

19.1. Introduction

Drug resistance in tuberculosis (TB) is a matter of great concern for TB control programs since there is no cure for some multidrug-resistant TB (MDR-TB) strains of *M. tuberculosis*. There is concern that these strains could spread around the world, stressing the need for additional control measures, such as new diagnostic methods, better drugs for treatment, and a more effective vaccine. MDR-TB, defined as resistance to at least rifampicin (RIF) and isoniazid (INH), is a compounding factor for the control of the disease, since patients harboring MDR strains of *M. tuberculosis* need to be entered into alternative treatment regimens involving second-line drugs that are more costly, more toxic, and less effective.

Moreover, the problem of extensively drug resistant (XDR) strains has recently been introduced. These strains, in addition to being MDR, were initially defined as having resistance to at least three of the six main classes of second-line drugs (aminoglycosides, polypeptides, fluoroquinolones, thioamides, cycloserine, and para-aminosalicylic acid) (CDC 2006). More recently, at a consultation meeting of the World Health Organization (WHO) Global Task Force on XDR-TB, held in Geneva, a revised laboratory case definition was agreed: "XDR-TB is TB showing resistance to at least rifampicin and isoniazid, which is the definition of MDR-TB, in addition to any fluoroquinolone, and to at least 1 of the 3 following injectable drugs used in anti-TB treatment: capreomycin, kanamycin and amikacin." (http://www.who.int/tb/xdr/taskforcereport_oct06.pdf). XDR-TB now constitutes an emerging threat for the control of the disease and the further spread of drug resistance, especially in HIV-infected patients, as was recently reported (Gandhi 2006). For this reason, rapid detection of drug resistance to both first- and second-line anti-tuberculosis drugs has become a key component of TB control programs.

19.2. Drug resistance surveillance

19.2.1. Benefits and recommendations

The surveillance of drug resistance in TB is a critical component of the monitoring system of the disease. The benefits of drug resistance surveillance are numerous

and include the strengthening of laboratory networks, the evaluation of TB control program performance, and the collection of important data for appropriate treatment strategies. Furthermore, global drug resistance surveillance identifies areas of high resistance, warning the health authorities to initiate the appropriate correction measures. To adequately establish drug resistance surveillance at a national level, three recommendations have been provided: the sampled specimens should be representative of the patients from the area under study and the sample size should be statistically determined to allow standard epidemiological analysis; the patient's history should be obtained and medical records carefully reviewed to determine whether the patient has received previous treatment in order to distinguish primary from acquired resistance; and the laboratory techniques used for determining the drug susceptibility to anti-tuberculosis drugs should be selected from those that are internationally recommended (WHO/IUATLD 1998). In 1996, the WHO and the International Union Against Tuberculosis and Lung Disease (IUATLD) launched the Global Project on Drug Resistance Surveillance based on data collected and reported by an international network of laboratories acting as Supranational Reference Laboratories. The network includes twenty-six Supranational Reference Laboratories distributed in the five WHO regions and is coordinated by the Prince Leopold Institute of Tropical Medicine in Antwerp, Belgium.

19.2.2. Global trends in drug resistant tuberculosis

Since the establishment of the WHO/IUATLD Global Project on Anti-tuberculosis Drug Resistance Surveillance, three global reports have been produced (WHO 1997, 2001, 2004). The first two reports covered data from 35 and 58 settings respectively. The main conclusions of those two reports were that drug-resistant TB was present in all settings surveyed, MDR-TB was identified in most settings, and good TB control practices were associated with lower or decreasing levels of resistance.

The third and last report available, published in 2004, covers data from 77 settings and had the main goal of expanding knowledge of the prevalent global patterns of resistance and exploring trends in resistance over time. The data were collected between 1999 and 2002 and represented 20 % of the total global number of new smear-positive TB cases. This third report also contributes to address two issues not thoroughly dealt with in previous reports: the importance of conducting surveillance on re-treatment cases, and stressing the issue of the role of the laboratory in TB control (WHO 2004, Aziz 2006).

The prevalence of drug resistance among new patients is a very important indicator for a TB control program. The prevalence of resistance among previously untreated patients also reflects program performance over a long period of time and indicates the level of transmission within the community. The prevalence of drug resistance among patients with a history of previous treatment, on the other hand, has received less attention, since surveillance of this population is more complex. Retreatment patients are a heterogeneous group composed of chronic patients, those with treatment failure, those who have relapsed, and those who have returned after defaulting. Sometimes this population represents more than 40 % of smear-positive cases. The prevalence of drug resistance varies greatly among subgroups of this population. Chronic cases and treatment failures are at a greater risk of having resistant and MDR-TB. Relapses and default patients are more likely to have drug resistance than new cases, but are almost always at a lower risk for MDR-TB than failures and chronic cases. One of the recommendations of the last report is that all subgroups of re-treatment cases be notified separately and their outcomes reported; furthermore, surveillance of resistance should be conducted on a representative sample of this population.

The second issue stressed in the third resistance report is that of the role of the laboratory. While laboratory services are fundamental for TB control, they are often the weakest components of the system. The importance of the laboratory in the control of TB should be recognized and they should be able to perform sputum smear microscopy, culture, and drug susceptibility testing of a high quality as standard components of TB control. Culture and drug susceptibility testing should be performed by national reference laboratories. Recognizing the pressing need to improve laboratory performance, a Subgroup on Laboratory Capacity Strengthening was established within the DOTS Expansion Working group in 2002 (Portaels 2006). The major objective of the subgroup is to assist high-TB burden and other countries in strengthening TB laboratory capacity and to provide high quality diagnostic services.

In this third report, data were collected through routine or continuous surveillance of all TB cases (in 38 settings) or from specific surveys of sampled patients (in 39 settings). These were reported on a standard reporting form, either annually or on completion of the survey (WHO 2004).

The results show that in new TB cases with data available from 75 settings (55,779 patients) the prevalence of resistance to at least one drug (any resistance) ranged from 0 % in some Western European countries to 57.1 % in Kazakhstan (median = 10.2 %). Median prevalence of resistance to individual drugs was: streptomycin (SM), 6.3 %; INH, 5.9 %; RIF, 1.4 %; and ethambutol (EMB), 0.8 %. Prevalence

of MDR-TB ranged from 0 % in eight countries to 14.2 % in Kazakhstan and Israel (median = 1.1 %). The highest prevalences of MDR-TB were observed in Tomsk Oblast (Russian Federation) (13.7 %), Karakalpakstan (Uzbekistan) (13.2 %), Estonia (12.2 %), Liaoning Province (China) (10.4 %), Lithuania (9.4 %), Latvia (9.3 %), Henan Province (China) (7.8 %), and Ecuador (6.6 %). Trends in drug resistance were determined in 46 settings (20 with two data points and 26 with at least three). Significant increases in prevalence of any resistance were found in Botswana, New Zealand, Poland, and Tomsk Oblast (Russian Federation). Cuba, Hong Kong SAR, and Thailand reported significant decreases over time. Tomsk Oblast (Russian Federation) and Poland reported significantly increased prevalences of MDR-TB. Decreasing trends in MDR-TB were observed in Hong Kong SAR, Thailand, and the USA.

Among previously treated cases with data available from 66 settings (8,405 patients) the median prevalence of resistance to at least one drug (any resistance) was 18.4 %, with the highest prevalence being 82.1 % in Kazakhstan. Median prevalence of resistance to individual drugs was: INH, 14.4 %; SM, 11.4 %; RIF, 8.7 %; and EMB, 3.5 %. The median prevalence of MDR-TB was 7.0 %. The highest prevalence of MDR-TB was reported in Oman (58.3 %) and Kazakhstan (56.4 %). Countries of the former Soviet Union had a median prevalence of resistance to the four drugs of 30 %, compared with 1.3% in all other settings. However, these data should be interpreted with caution given the small number of subjects tested in some settings. Trends in drug resistance in this group were determined in 43 settings (19 with two data points and 24 with at least three data points). A significant increase in the prevalence of any resistance was observed in Botswana. Cuba, Switzerland, and the USA showed significant decreases. The prevalence of MDR-TB significantly increased in Estonia, Lithuania, and Tomsk Oblast (Russian Federation). Decreasing trends were significant in Slovakia and the USA.

The annual incidence of MDR-TB cases was estimated in 69 settings. In most Western and Central European countries, the estimated incidence was fewer than 10 cases each. Estonia, Latvia, Lithuania and two Oblasts in the Russian Federation were estimated to have between 99 and 248 MDR-TB cases. For Henan and Huber Provinces of China, more than 1,000 cases each were estimated, and for Kazakhstan and South Africa, more than 3,000.

The report also evaluated RIF resistance as a predictor of MDR-TB, in order to explore the significance of rapid testing for RIF resistance to identify cases likely to have MDR-TB. The positive predictive value, a function of the sensitivity and specificity of RIF resistance testing and the prevalence of MDR-TB and non-MDR-TB RIF resistance, was highest among previously treated cases in settings with

high MDR-TB prevalence and low non-MDR-TB RIF resistance. The report also confirmed that, globally, more isolates were resistant to INH than to any other drug (range 0–42 %). INH and SM resistance were more prevalent than RIF or EMB resistance. Resistance to INH, SM, RIF and EMB was the most prevalent pattern among previously treated cases and the proportions of isolates resistant to three or four drugs were significantly greater than among new cases, suggesting an amplification of resistance. It appears that monoresistance to either INH or SM is the main gateway to the acquisition of additional resistance.

Tables 19-1 and 19-2 below show a summary of the prevalence of drug resistance and MDR-TB in new TB cases and previously treated patients, respectively, according to the five WHO regions in the world.

Table 19-1: Median prevalence of drug resistance, polyresistance and MDR-TB among new TB cases by region (%)

Region	Any resistance	Polyresistance	MDR-TB
Africa	7.1	1.3	1.4
Americas	9.7	2.1	1.1
Eastern Mediterranean	9.9	2.5	0.4
Europe	8.4	1.1	0.9
South-East Asia	19.8	4.0	1.3
Western Pacific	11.4	2.5	0.9
Overall median	**10.2**	**1.9**	**1.1**

Adapted from Reference WHO, 2006

Table 19-2: Median prevalence of drug resistance, polyresistance and MDR-TB among previously-treated TB cases by region (%)

Region	Resistance	Polyresistance	MDR-TB
Africa	16.7	1.8	5.9
Americas	24.6	3.7	7.0
Eastern Mediterranean	63.3	5.8	48.3
Europe	15.9	2.6	4.7
South-East Asia	39.9	7.3	20.4
Western Pacific	32.8	6.1	15.5
Overall median	**18.4**	**3.2**	**7.0**

Adapted from Reference WHO, 2006

19.3. Methods for detection of drug resistance

Early detection of drug resistance constitutes one of the priorities of TB control programs. It allows initiation of the appropriate treatment in patients and also surveillance of drug resistance. Detection of drug resistance has been performed in the past by so-called ‘conventional methods’ based on detection of growth of *M. tuberculosis* in the presence of the antibiotics. However, due to the laboriousness of some of these methods, and most of all, the long period of time necessary to obtain results, in recent years new technologies and approaches have been proposed. These include both phenotypic and genotypic methods. In many cases, the genotypic methods in particular have been directed towards detection of RIF resistance, since it is considered a good surrogate marker for MDR-TB, especially in settings with a high prevalence of MDR-TB. Genotypic methods have the advantage of a shorter turnaround time, no need for growth of the organism, the possibility of direct application in clinical samples, lower biohazard risks, and the feasibility of automation; however, not all molecular mechanisms of drug resistance are known. Phenotypic methods, on the other hand, are in general simpler to perform and might be closer to implementation on a routine basis in clinical mycobacteriology laboratories. The following section describes the phenotypic and genotypic methods as well as the new methodologies recently proposed for drug resistance detection in TB.

19.3.1. Conventional phenotypic methods

In general, phenotypic methods assess inhibition of *M. tuberculosis* growth in the presence of antibiotics to distinguish between susceptible and resistant strains. This is possible since *M. tuberculosis* isolates from patients never treated before are very uniform in their level of susceptibility, as shown by the narrow ranges of minimal inhibitory concentrations (MIC) of the main anti-tuberculosis drugs (Heifets 1996). The classical definition for a drug resistant *M. tuberculosis* strain is that it displays a degree of susceptibility significantly lower than that of a wild strain that has never been in contact with the drug (Canetti 1963, Canetti 1969).

Phenotypic methods based on cultivation of *M. tuberculosis* in the presence of antibiotics have been most commonly performed on egg-based or agar-based solid media, and can also be performed as a direct or indirect method. For the direct method, antibiotic-containing and control media are inoculated with a decontaminated and concentrated clinical specimen, while for the indirect method the antibiotic-containing and control media are inoculated with a bacterial suspension of the isolated strain. There are three conventional phenotypic methods for drug susceptibility testing based on solid media: the proportion method, the resistance ratio method and the absolute concentration method (Canetti 1963, Canetti 1969, Kent 1985). More recent methods are based on liquid media including the BACTEC radiometric and the Mycobacterial Growth Indicator Tube methods.

The proportion method

The proportion method is the most commonly used method worldwide amongst the three methods mentioned above. It allows the precise determination of the proportion of resistant mutants to a certain drug. Briefly, several 100-fold serial bacilli dilutions are inoculated into drug-containing and drug-free (control) media. One of those dilutions should produce a number of colonies that is easy to be counted. The number of colonies obtained in the drug-containing and control media are enumerated and the proportion of resistant mutants is then calculated. When performed in Löwenstein-Jensen medium tubes, the test is first read after 28 days of incubation at 37°C. If the proportion of resistant bacteria is higher than 1 % for isoniazid, rifampicin and para-aminosalycilic acid, or 10 % for the other drugs, the strain is considered resistant and the results are final; otherwise, the test is read again at 42 days of incubation to assess if the strain is susceptible to a certain drug (Heifets 2000). If the test is performed on agar, a Middlebrook 7H10/11 is used and the medium is incubated in a 10 % CO_2 atmosphere. Results are interpreted after 21 days of incubation or even earlier if they show the strain to be resistant (Kent

1985). The critical concentrations of the main drugs used in the proportion method are shown in Table 19-3.

Table 19-3: Critical concentration of main antibiotics in the proportion method (µg/mL)

Antibiotic	Löwenstein-Jensen	7H10 agar	7H11 agar
Isoniazid	0.2	0.2, 1.0	0.2, 1.0
Rifampicin	40.0	1.0	1.0
Ethambutol	2.0	5.0	7.5
Streptomycin	4.0	2.0	2.0, 10.0
Pyrazinamide	100	-	-
PAS	0.5	2.0	8.0
Kanamycin	20.0	5.0	6.0
Ethionamide	20.0	5.0	10.0
Ofloxacin	2.0	2.0	2.0
Capreomycin	20.0	10.0	10.0
Cycloserine	40.0	-	-

Adapted from: Kent 1985; WHO/CDS/TB/2001.288; and NCCLS 2000

The resistance ratio method

This method is based on the resistance ratio, which corresponds to the MIC of a test strain divided by the MIC of the drug-susceptible reference strain H37Rv tested at the same time. Thus, it compares the resistance of an unknown strain with that of a standard laboratory strain. For the performance of the test, parallel sets of tubes containing two-fold dilutions of the tested drug are then inoculated with a standardized inoculum of both test and reference strain. Reading of the test is performed after 4 weeks of incubation at 37°C. Tubes containing 20 or more colonies are considered as positive for growth and the MIC is defined as the lowest concentration of drug in the presence of which the number of colonies is lower than 20.

An isolate with a resistance ratio value of 2 or less is considered susceptible, while a resistance ratio of 8 or more defines the isolate as resistant (Kent 1985, Heifets 2000).

The absolute concentration method

This method uses a standard inoculum of the test strain grown in a two-fold dilution drug-containing media and drug-free control. The resistance of a strain is expressed in terms of the lowest concentration of a certain drug that inhibits all or almost all the growth of the strain. The critical concentrations included in the medium are similar to the ones used in the proportion method (see Table 19-3) but the drug concentration considered as 'critical' should be determined in each laboratory (Heifets 2000). For the interpretation of the test, the reading is performed after 4 weeks of incubation at 37°C, or at 5-6 weeks if there is not enough growth. A strain is considered to be susceptible if the number of colonies on the drug-containing medium is less than 20 with a 3+ or 4+ (confluent) growth on the drug-free control.

The BACTEC radiometric method

The radiometric method is based on the commercial system BACTEC TB-460 (Becton Dickinson, Sparks, MD), which uses an enriched Middelbrook 7H9 liquid medium containing ^{14}C-labeled palmitic acid as the sole carbon source (12B vial). Growth of the mycobacteria and consumption of the labeled fatty acid will produce $^{14}CO_2$ that is detected inside the 12B vial by the BACTEC apparatus and expressed as a growth index. In the presence of a certain drug, susceptibility can be measured by inhibition of the daily increases in the growth index. For the performance of the test, a test vial containing the drug under study and a drug-free control are inoculated with a standard inoculum and incubated at 37°C. The vials are then read in the BACTEC 460-TB apparatus on a daily basis. Since two control vials are inoculated with a 100-fold serial dilution of the inoculum, results can be interpreted as in the proportion method with the 1 % proportion of growth. The BACTEC radiometric method has been approved by the Food and Drug Administration (FDA) of the United States (US) and is also considered to be the 'gold standard' for drug susceptibility testing to first-line anti-tuberculosis drugs (Roberts 1983, Heifets 1999). More recently, critical concentrations for second-line drugs have also been proposed and tested successfully for most drugs in a multicenter evaluation (Pfyffer 1999). The major advantage of the BACTEC radiometric method is the capacity to detect drug resistance faster than with the solid media-based methods; the major disadvantage is the cost of the system and the need for disposal of the radioactive waste from used vials.

The Mycobacterial Growth Indicator Tube

The Mycobacteria Growth Indicator Tube (MGIT) (Becton Dickinson, Sparks, MD) is part of the 'new generation' of TB diagnostic tools both in its manual version as well as in its more recently introduced automated format (Pfyffer 1997, Idigoras 2000). It is based on fluorescence detection of mycobacterial growth in a tube containing a modified Middlebrook 7H9 medium together with a fluorescence quenching-based oxygen sensor embedded at the bottom of the tube. Consumption of oxygen in the medium produces fluorescence when illuminated by a UV lamp. In the manual system, for the performance of the test a drug-containing tube and a control tube are inoculated with the standardized mycobacterial suspension and incubated at 37°C (day 0). Starting on the third day (day 2), the tubes are controlled daily with an UV lamp. The presence of an orange fluorescence in the drug-containing tube at the same time as in the control tube or within two days of positivity in the control is interpreted as resistance to the drug; otherwise, the strain is considered to be susceptible. The test is valid if the growth control gives a positive signal until the 14th day of incubation (day 12) (Palomino 1999). The MGIT system in its manual version has also been successfully used as a direct method using decontaminated clinical specimens (Goloubeva 2001).

Figure 19-1: MGIT tubes showing a positive and a negative reaction

The MGIT has also been recently introduced as an automated system. The BACTEC MGIT960 (Becton Dickinson, Sparks, MD) is based on the same principle of oxygen consumption and a fluorescence signal, but the tubes are incubated and controlled inside the MGIT960 apparatus. For the performance of the test, drug-containing and drug-free control vials are inoculated with a standardized inoculum of the *M. tuberculosis* isolate and entered into the machine in a special rack-carrier with a printed barcode; this is read by the machine when entering the tubes to identify the test and apply the adequate algorithm for susceptibility or resistance interpretation. All readings are performed inside the machine and the results are printed as susceptible or resistant (Ardito 2001).

Many studies have now been published on the application of the MGIT system for the rapid detection of resistance to first- and second-line antituberculosis drugs (Johansen 2004, Rusch-Gerdes 2006). In all these studies, the MGIT system has shown very good results with a high correlation with the conventional methods on solid media and the BACTEC TB-460 system. The BACTEC MGIT960 system has recently been approved by the US FDA for the detection of drug resistance to first-line drugs.

Other automated systems, such as those already described in Chapter 14, have been used for the rapid detection of drug resistance in *M. tuberculosis,* but they have not been used on a routine basis in the clinical mycobacteriology laboratory (Ängeby 2003, Ruiz 2000). Recent developments of phenotypic formats for rapid drug resistance detection will be presented in section 19.3.3 below.

19.3.2. Genotypic methods

Genotypic methods for drug resistance in TB look for the genetic determinants of resistance rather than the resistance phenotype, and involve two basic steps: nucleic acid amplification such as polymerase chain reaction (PCR), to amplify the sections of the *M. tuberculosis* genome known to be altered in resistant strains; and a second step of assessing the amplified products for specific mutations correlating with drug resistance (García de Viedma 2003, Palomino 2005).

Desoxyribonucleic acid (DNA) sequencing

Sequencing DNA of PCR-amplified products has become the most widely used genotypic method for detecting drug resistance in *M. tuberculosis*; it is accurate and reliable and it has become the reference standard for mutation detection. It was performed several years ago by manual procedures, but in our days, it is performed with automatic sequencers (Victor 2001). DNA sequencing has been widely used

for characterizing mutations in the *rpoB* gene in RIF-resistant strains and to detect mutations responsible for resistance to other anti-tuberculosis drugs (Telenti 1993, García de Viedma 2003, Jalava 2004). Drug resistance detection in *M. tuberculosis* has also been described by pyrosequencing technology (Arnold 2005, Jureen 2006). This technology is a short-read (30–50 bp) sequencing technique, which is based on the quantitative detection of pyrophosphate released following nucleotide incorporation into a growing DNA chain (Ronaghi 1999). However, not all molecular mechanisms of drug resistance for *M. tuberculosis* are known and it would be rather difficult and expensive to implement it routinely for the detection of drug resistance mutations for several drugs (Hazbón 2004).

Solid-phase hybridization techniques

There are currently two commercially available solid-phase hybridization techniques for the rapid detection of drug resistance in TB: the Line Probe Assay (INNO-LiPA Rif TB Assay, Innogenetics, Ghent, Belgium) for the detection of resistance to RIF and the GenoType MTBDR assay (Hain Lifesciences, Nehren, Germany) for the simultaneous detection of resistance to INH and RIF.

The LiPA assay was introduced several years ago and is based on reverse hybridization of amplified DNA from cultured strains or clinical samples to ten probes covering the core region of the *rpoB* gene of *M. tuberculosis*, immobilized on a nitrocellulose strip (De Beenhouwer 1995). From the pattern of hybridization obtained, the presence or absence of mutated or wild regions is visualized by a colorimetric reaction and the strain can be considered as resistant or susceptible to RIF (Rossau 1997). Many studies have been conducted on the application of the LiPA assay for detection of RIF resistance; most of them have been performed on *M. tuberculosis* isolates and just a few have applied the test directly in sputum samples (Jureen 2004, Traore 2006). It has been proposed as a good initial indicator of multidrug resistance with a sensitivity of 98.5 % for detecting RIF resistance (Traore 2000). In a recent systematic review and meta-analysis of studies that applied the LiPA test, 12 of 14 studies performed in isolates had sensitivity greater than 95 % and specificity of 100 %. Four studies that applied LiPA directly to clinical specimens had 100 % specificity, and the sensitivity ranged from 80 % to 100 % (Morgan 2005). In a very recent and large study, not included in the meta-analysis mentioned above, the utility of the LiPA test for detecting RIF resistance was assessed in 420 sputum samples originating from different countries (Traore 2006). There was a 99.6 % concordance between the RIF resistance obtained by culture and by the LiPA test, confirming that with an adequate DNA extraction method, the LiPA test allows rapid detection of resistance to RIF directly from sputum samples.

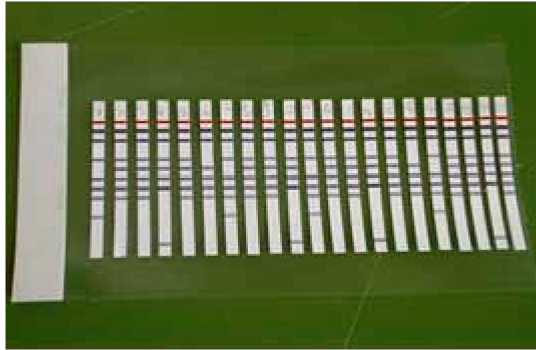

Figure 19-2: LiPA strips showing different mutations

The GenoType MTBDR, on the other hand, detects resistance to INH and RIF in culture samples based on the detection of the most common mutations in the *katG* and *rpoB* genes respectively (Makinen 2006). It also utilizes PCR and reverse hybridization to probes immobilized on a DNA strip. In a recent study that evaluated the GenoType MTBDR assay in 143 *M. tuberculosis* isolates, 99 % of the MDR strains were found to have mutations in the *rpoB* gene and 88.4 % of strains with mutations in the codon 315 of the *katG* gene were also correctly identified (Hillemann 2005). The correlation with DNA sequencing was 100 %, and good sensitivity and specificity was obtained when compared to the conventional tests. As with other genotypic tests, there is interest in the application of these techniques directly to sputum samples. There are only two studies that address this issue. In the study by Hillemann et al., the GenoType MTBDR was tested directly in 42 smear-positive sputum samples obtaining a concordance of 100 % when compared to conventional drug susceptibility testing (Hillemann 2006). In another more recent study, the GenoType MTBDR was evaluated in 143 smear-positive sputum samples and it was able to correctly identify INH resistance in 48 (84.2 %) of the 57 specimens containing strains with resistance to high level of INH (0.4 µg/mL), and RIF resistance in 25 (96.2 %) of the 26 specimens containing RIF-resistant strains (Somoskovi 2006). There is currently interest in expanding these studies to TB-endemic countries to assess the usefulness of this type of assay for the rapid detection of multidrug resistance in TB (http://www.finddiagnostics.org/news/press/hain_oct06.shtml).

Both solid-phase hybridization methods have proven relatively simple to perform; however, basic expertise in molecular biology and PCR techniques is required. As with other genotypic methods, the sensitivity of the test depends on the amount of DNA present in the sample, and the presence of inhibitors could also cause false-negative results (Palomino 2006).

Another solid-phase reverse hybridization test for rapid detection of RIF resistance is rifoligotyping. This is an *in house* low-cost assay for the detection of RIF resistance-associated mutations in the *rpoB* gene of *M. tuberculosis*. The test was developed at the National Institute of Public Health and the Environment (http://www.rivm.nl/en/) in the Netherlands and initially evaluated at the Cetrángolo Hospital in Argentina (Morcillo 2002). It also involves a combination of DNA amplification and reverse-line blot hybridization. DNA of the *rpoB* gene of *M. tuberculosis* is amplified by PCR with specific primers and the PCR products are hybridized to oligonucleotides on a DNA membrane, encoding the wild type *rpoB* sequence, and the most frequent mutations in RIF-resistant strains. Amplified products from RIF-resistant strains will fail to hybridize to one or more of the wild type oligonucleotides, and in most cases, will hybridize to one of the mutant oligonucleotides bound to the membrane. RIF-resistant strains can be detected within a few hours with an enhanced luminescent reaction. In this evaluation, a total of 135 *M. tuberculosis* isolates were tested with the rifoligotyping assay and the results compared with the proportion method and the MGIT960 system. The rifoligotyping assay correctly identified 90 of the 97 RIF-resistant isolates (sensitivity 92.8 %) while all the RIF-susceptible isolates were also correctly identified.

A minor modification of this assay has also been tested in a multicenter study to detect resistance to RIF, INH, SM and EMB in clinical isolates of *M. tuberculosis* (Mokrousov 2004). Oligonucleotides specific for wild type and mutant alleles of selected codons in the genes *rpoB*, *inhA*, *ahpC*, *rpsL*, *rrs*, *embB*, were immobilized on a nylon membrane. For validation of the test, the membranes were sent to seven laboratories in different geographical locations. The reproducibility for *rpoB* mutation detection was performed on a blinded set of reference DNA samples and overall concordant results were obtained. However, when further mutation analysis was performed on local strains, only 132 (85.2 %) of 155 RIF-resistant and 28 (51.0 %) of 55 EMB-resistant isolates were correctly identified. Resistance to INH was successfully identified in 16.9 % and 13.2 % of strains harboring mutations in the *inhA* and *ahpC* promoter region respectively. Likewise, mutations in *rrs* and *rpsL* conferring resistance to SM were identified in 15.1 % and 10.7 % of SM-resistant strains respectively. Nevertheless, the accuracy of this method for RIF resistance detection has recently been confirmed in another study that used a slightly modified

version of the rifoligotyping assay (Senna 2006). This study evaluated 157 isolates of *M. tuberculosis* and when compared to standard drug susceptibility testing had sensitivity and specificity of 93 % and 100 % respectively. Furthermore, high agreement was also obtained with DNA sequencing.

Real-time PCR techniques

Real-time PCR techniques have also been introduced recently for the rapid detection of drug resistance in TB. Different probes have been used for detection, such as the TaqMan probe, Fluorescence Resonance Energy Transfer probes, molecular beacons and biprobes (Shamputa 2004). The main advantages of real-time PCR techniques are the speed of the test and a lower risk of contamination. The main disadvantages would be the requirement for expensive equipment and reagents, and the need for skilled technical personnel. Real-time PCR techniques have been applied to *M. tuberculosis* strains and, more recently, directly to clinical samples (Sajduda 2004, Ruiz 2004, Espasa 2005). Results are generally obtained in an average of 1.5-2.0 hours after DNA extraction. Real-time PCR could eventually be implemented in reference laboratories with the required capacity to properly set up the technique and in settings where it can contribute to the management of TB patients.

Microarrays

Microarrays, also known as biochips or DNA chips, have been proposed as genotypic methods for detecting drug resistance in *M. tuberculosis*. They are based on the hybridization of DNA obtained from clinical samples to oligonucleotides immobilized on a solid support, such as miniaturized glass slides. They have been tested to detect resistance to INH and RIF (Gryadunov 2005). The recently described CombiChip Mycobacteria Drug-Resistance detection DNA chip is an oligonucleotide microchip coupled with PCR for the detection of resistance to INH and RIF. It was compared with sequencing and drug susceptibility testing in 69 INH- and/or RIF-resistant and 27 drug-susceptible *M. tuberculosis* isolates (Kim 2006). It allowed identification of 84.1 % of INH-resistant isolates, based on the *katG* codon 315 and *inhA*15 mutations, and 100 % of RIF-resistant isolates based on seven codons: *rpoB*511, *rpoB*513, *rpoB*516, *rpoB*522, *rpoB*526, *rpoB*531, and *rpoB*533. The overall specificity was 100 % and 95.3 % for detecting INH and RIF resistance respectively. For the time being, and due to the high cost involved, the use of microarrays for detecting drug resistance in *M. tuberculosis* is still beyond the reach of most clinical mycobacteriology laboratories, especially in high-burden countries.

19.3.3. New phenotypic methods

The laboriousness and long time required by conventional methods to give results and, on the other hand, the requirement for expensive equipment and the need for skilled technical personnel for most molecular techniques, continue to stimulate the search for alternative and affordable methods for drug resistance detection in TB. The next section will describe several new developments for *M. tuberculosis* that have already been tested both in culture isolates and directly in clinical sputum samples.

Phage-based methods

There are currently two formats of phage-based assays that have been described for the rapid detection of drug resistance in *M. tuberculosis*. The first one, also known as phage-amplified biologically, was originally described by Wilson *et al*. in *M. tuberculosis* isolates (Wilson 1997); and the second format is based on reporter mycobacteriophages expressing luciferase (Jacobs 1993).

Phage-based methods that rely on the biological amplification of mycobacteriophages have gained wider application in the last years. They are based on the ability of *M. tuberculosis* to support the growth of an infecting mycobacteriophage. The number of endogenous phages, representing the original number of viable *M. tuberculosis* bacilli, is then determined in a rapidly-growing mycobacterium, such as *M. smegmatis* (McNerney 2001). The *in house* phage amplification test and the commercially available *FastPlaque TB* assay have been tested for the detection of RIF resistance both in *M. tuberculosis* isolates and directly on clinical specimens. In a study performed in 129 isolates from a hot-spot area of MDR-TB, the *in house* mycobacteriophage amplification assay showed 100 % sensitivity, 97.7 % specificity, and 95.2 % predictive value for detecting RIF-resistant *M. tuberculosis*; the test was smoothly integrated into the routine work flow of a low-resource reference laboratory (Simboli 2005). The *FastPlaque TB* has been evaluated in a comparative study with the proportion method on Middlebrook 7H11 agar for determining RIF-resistance directly in smear-positive sputum samples (Albert 2004). The study showed 100 % sensitivity and specificity with results available within two days.

Figure 19-3: *In house* phage amplification method

The luciferase reporter phage method is based on the efficient production of a light signal by viable mycobacteria infected with specific reporter phages expressing the firefly luciferase gene. Light production is dependent on phage infection, expression of the luciferase gene, and the level of cellular ATP (Jacobs 1993). Signals can be detected within minutes after the infection. *M. tuberculosis* isolates susceptible to INH or RIF, result in extinction of light production, while drug-resistant strains continue to produce light. Luciferase reporter tests have now been evaluated against the four first-line antibiotics with an overall agreement of 98.6 % compared with the BACTEC TB-460 system (Banaiee 2003). Furthermore, in a recent study two detection methods, photographic and luminometric, were compared and the sensitivity for detecting INH and RIF resistance was 100% concluding that both methods were appropriate as screening tests for MDR-TB surveillance (Hazbón 2004).

A recent systematic review and meta-analysis summarizes the accuracy of phage-based methods for detecting RIF-resistance in *M. tuberculosis* (Pai 2005). The study concluded that, based on published evidence, phage-based assays performed on *M. tuberculosis* isolates appear to have high sensitivity, but variable and slightly lower specificity. Not enough evidence is available on the accuracy of these assays when performed directly on sputum samples.

Colorimetric methods

Several colorimetric methods have been proposed in the last few years for the rapid detection of drug resistance in *M. tuberculosis*. They use redox indicators or tetrazolium salts to detect mycobacterial growth. The tests are based on the reduction of

the colored redox indicator added to the culture medium after *M. tuberculosis* has been exposed *in vitro* to different antibiotics. Resistance is detected by a change in color of the indicator, which is directly proportional to the number of viable mycobacteria in the medium (Palomino 2004).

Alamar blue (Trek Diagnostics, Ohio, USA) is a proprietary reagent that was the first to be used to detect drug resistance in *M. tuberculosis*. The reagent is blue in the oxidized state but changes to pink when reduced. In a study that evaluated the activity of INH, RIF, EMB, and SM on clinical isolates of *M. tuberculosis*, MICs were obtained after 1-2 weeks of incubation with an overall accuracy of 97 %, compared to the agar proportion method (Yajko 1995). Alamar blue has been tested in several other studies to detect drug resistance in *M. tuberculosis* and to assess the activity of antimycobacterial drugs using a microplate format (Collins 1997, Franzblau 1998, Palomino 1999). In all these studies, Alamar blue has performed very well, especially for the detection of resistance to INH and RIF.

The tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide or MTT is a yellow compound that, when reduced by metabolically active cells, it produces crystals of insoluble purple MTT formazan that can be measured with a spectrophotometer after solubilization (Mosmann 1983). MTT has also been proposed in a colorimetric assay for the rapid detection of resistance to RIF (Mshana 1998, Abate 1998). The test, performed on 92 clinical isolates of *M. tuberculosis,* matched the results obtained with the BACTEC radiometric method used as the gold standard. More recently, the MTT test has also been applied in the detection of resistance to other anti-tuberculosis drugs with good results (Foongladda 2002, Caviedes 2002, Morcillo 2004). With the purpose of speeding up the detection of drug resistance in clinical samples, MTT has also been applied as a direct assay in sputum samples for RIF-resistance detection. The sensitivity and specificity of this direct MTT assay matched those of the standard indirect drug susceptibility testing on 7H10 agar with 98.5 % of the samples giving interpretable results within two weeks (Abate 2004).

As a result of studies identifying resazurin as the main component of the Alamar blue reagent (O'Brien 2000), this redox indicator was also introduced in a rapid test to detect drug resistance in *M. tuberculosis* (Palomino 2002). The resazurin microtiter assay (REMA) allowed rapid detection of multidrug resistance in *M. tuberculosis* isolates with an overall accuracy of 97 % as compared to the proportion method in Löwenstein-Jensen medium. The REMA also showed its usefulness for the detection of resistance to other anti-tuberculosis drugs, including common second-line drugs, quinolones and pyrazinamide (Martin 2003, Lemus 2004, Martin 2005a, Martin 2006).

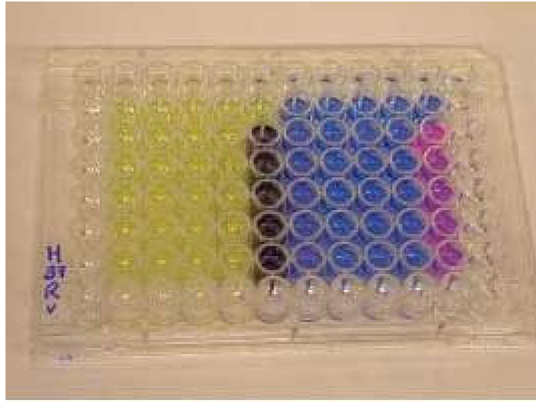

Fig.19-4: Microplate showing MTT and REMA test

In a multicenter study to assess the performance of the REMA and MTT assays in different settings, the resistance of *M. tuberculosis* coded strains to INH, RIF, EMB and SM was determined by REMA, MTT and the proportion method. Excellent results were reported for RIF, INH and EMB, with levels of specificity and sensitivity between 96 % and 99 % (Martin 2005b). Furthermore, a recent systematic review and meta-analysis of colorimetric redox indicator methods to detect multidrug resistance in *M. tuberculosis* found evidence of a high sensitivity and specificity for the rapid detection of MDR-TB (Martin 2007). Colorimetric methods represent a good alternative for the rapid detection of drug resistance in laboratories with limited resources.

The nitrate reductase assay

The nitrate reductase assay (NRA) is a quite simple technique based on the capacity of *M. tuberculosis* to reduce nitrate to nitrite, which is detected by adding a chemical reagent to the culture medium. *M. tuberculosis* is cultivated on Löwenstein-Jensen medium in the presence of an antibiotic and its ability to reduce nitrate is measured after 10 days of incubation. Resistant strains will reduce the nitrate, which is revealed by a pink-red color in the medium, while susceptible strains will lose this capacity as they are inhibited by the antibiotic (Ängeby 2002). The assay has been evaluated in several studies for first-line drugs and ofloxacin with good results (Montoro 2005, Martin 2005a). It has the added advantage of using the same format and culture medium as the standard proportion method. In a recent

multicenter study that evaluated the performance of the NRA for detecting resistance to the first-line drugs, the test performed very well for INH, RIF and EMB with an accuracy of 96.6 % to 98 %. Lower values, were obtained for SM (Martin 2005a). However, the NRA was easily implemented in settings with limited laboratory facilities. Two recent studies applied the NRA directly on sputum samples and produced variable results for sensitivity and specificity; the best results were obtained for INH and RIF resistance detection (Musa 2005, Solís 2005). These two studies have shown the feasibility for implementation of the NRA as a direct test on sputum samples that warrant further evaluations in target populations.

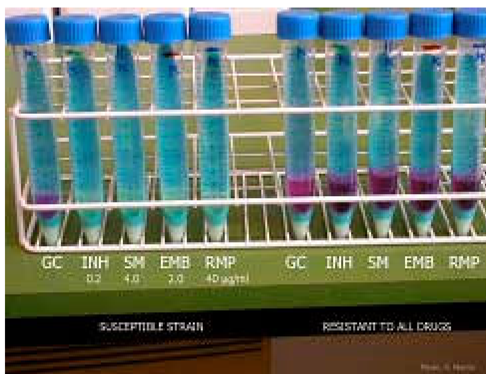

Fig. 19-5: The nitrate reductase assay showing a susceptible and a resistant strain. GC= growth control

The microscopic observation broth-drug susceptibility assay

As already introduced in Chapter 14, the microscopic observation broth-drug susceptibility assay (MODS) has been described for the early detection of growth and rapid drug susceptibility testing method for *M. tuberculosis*. It is based on the observation of the characteristic cord formation of *M. tuberculosis* that is visualized microscopically in liquid medium with the use of an inverted microscope (Caviedes 2000). In this study, TB-positive sputum samples were tested for susceptibility to INH and RIF by MODS. The results compared to those obtained with the colorimetric method using Alamar blue. They obtained 89 % concordance between the two methods with results available in an average of 9.5 days. The method has been proposed as a rapid, inexpensive, sensitive, and specific method for *M. tuberculosis* drug susceptibility testing, appropriate for use in developing countries.

In a recent operational study performed in Peru, the performance of the MODS assay was compared to an automated mycobacterial culture system, and the method of proportion on Löwenstein-Jensen for the direct detection of resistance to INH, RIF, EMB, and SM in sputum samples (Moore 2006). The median time for results was 7, 22, and 68 days for MODS, automated mycobacterial culture, and method of proportion respectively. The agreement between MODS and the reference standard for drug susceptibility testing was 97 % for INH, 100 % for RIF, and 99 % for INH and RIF combined (MDR). Lower values of agreement were obtained for EMB (95 %) and SM (92 %). They concluded that a single MODS culture of sputum provided a more rapid and sensitive detection of MDR-TB. One minor disadvantage of MODS is the requirement for an inverted microscope for observation of the mycobacterial growth.

The thin-layer agar method

The Thin Layer 7H11 agar (TL7H11) method or microcolony method, already described in Chapter 14, has also been adapted for the rapid detection of multidrug resistance directly from sputum samples. The TL7H11/INH/RIF has been shown in preliminary studies to be accurate for the detection of MDR-TB as compared to the reference proportion method, with results available in one week (Robledo 2006). Further evaluation studies are expected in target populations to assess the performance of this method in different settings.

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Chapter 20: New Developments and Perspectives

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20.1. The scenario

"The history of tuberculosis (TB) has been one of scientific, medical and political failure." With this disturbing statement, The Lancet's editors introduced an issue dedicated to TB that was released on the occasion of the World TB Day 2006 (Zumla 2006). According to the Global TB Control Report released one year later by the World Health Organization (WHO), the good news is that *"the worldwide TB epidemic has leveled off for the first time since the disease was declared a public health emergency in 1993."* The bad news is that *"at the current rate of progress, the 1990 prevalence and mortality rates will not be halved worldwide by 2015."* The Global Plan to Stop TB needs to triple investment in order to achieve such a goal (World Health Organization 2007).

TB is the only disease ever declared a global emergency by the WHO. Paradoxically, although we count on effective – and proven cost-effective – interventions for its control, TB continues to cause great mortality and suffering, especially in poor and less-developed countries. Its association with the HIV/AIDS pandemic forms a lethal combination. In addition, multidrug resistant (MDR) TB and the recently-described extensively drug resistant (XDR) TB – with further resistance to key second-line drugs and virtually incurable – severely complicate the management and control of the disease worldwide (Dorman 2007, Shah 2007). As repeatedly stated, one third of the world's population is latently infected with *Mycobacterium tuberculosis* and 10 % of these people will develop active disease at some point in their life. Almost 8.8 million new cases of TB were reported in 2005, and 1.6 million deaths were attributed to the disease. Asia and Sub-Saharan Africa accounted for 7.4 million new cases of TB worldwide (World Health Organization 2007).

Yet, it was not long ago that we envisaged – and proudly announced – the elimination of TB by the end of the last millennium. Indeed, in the late '70s and early '80s, it was thought that TB could be eradicated from most developed and industrialized countries. TB was already regarded as a disease from the past and started to be neglected by medical doctors, scientists and agencies in charge of its control. However, this never became a reality, mainly due to the appearance of antibiotic resistance, and therefore, TB continues to be the big killer it was in the pre-antibiotic

era. This unexpected re-emergence of TB in the ’90s served not only to strengthen control measures but also to fuel research on TB.

Substantial scientific advances were made in knowledge about the agent and the disease in that decade. First, the complete genome sequence of the tubercle bacillus was deciphered. Second, molecular epidemiology came into being, shedding a new light on mechanisms of TB transmission. Third, cellular mechanisms involved in *M. tuberculosis* resistance to several drugs were discovered. Fourth, new tools for speeding TB diagnosis and assessing drug susceptibility were sought while old methods were re-discovered and/or re-formulated. Lastly, research on drug and vaccine development exploded.

As for more recent advances, an analysis published in Nature Medicine highlights the most interesting scientific findings made in TB over the last three years, as identified by TB researchers (Anonymous 2007). The three articles that were considered to have the highest impact in the field were:

- the description – for the first time in 40 years – of a new drug, a diarylquinoline that acts on an entirely new mycobacterial target, the proton pump of adenosine triphosphate (ATP) synthase (Andries 2005)
- the report from a rural South African area of a deadly outbreak of what thereafter became known as XDR-TB (Gandhi 2006)
- the description of a novel recombinant bacille Calmette-Guérin (BCG) vaccine candidate that works by over-expressing not an antigen, but a membrane-perforating enzyme (Grode 2005).

The main topics of these three papers underline the most pressing gaps in the translation of TB research. Other articles in the top list were related to vaccine candidates, virulence factors, genomics, new drugs, bacterial survival, and metabolism.

20.2. Bacillus and disease under the light of molecular epidemiology

The accelerated expansion that TB research underwent during the ’90s is reflected by the way in which molecular epidemiology is regarded nowadays. In fact, molecular strain typing of *M. tuberculosis* is no longer a novelty. What is more, it is taken for granted as if it always had been there. In less than two decades, it has changed our view of TB transmission dynamics, challenging traditional dogmas

and answering unsolved epidemiological questions (Mathema 2006). Nowadays, molecular epidemiology is embedded within almost every aspect of TB research, laboratory diagnosis, clinical management, and control interventions. Indeed, molecular epidemiology could be regarded as a paradigm of TB translational research.

Soon after standardization was agreed on DNA restriction fragment length polymorphism (RFLP) with IS*6110*, its use was generalized worldwide (van Embden 1993). Other DNA strain typing methods were developed, IS*6110* RFLP became the gold standard of *M. tuberculosis* strain genotyping, and a new discipline – molecular epidemiology – came into being. The considerable amount of information gathered in national and international *M. tuberculosis* genotype databases throughout the world enabled the analysis of global TB dissemination and promoted phylogeographical studies (Brudey 2006) that, in turn, incorporated more sophisticated and accurate markers of *M. tuberculosis* evolution (Arnold 2007).

A common conviction of previous times was that the genome of the tubercle bacillus was extremely stable and homogeneous. Phenotypical discrimination between strains – and even between species within the *M. tuberculosis* complex – was limited to phage typing and comparison of drug resistance patterns, which were the only tools available for differentiation. Molecular epidemiology refuted such belief by revealing the existence of wide *M. tuberculosis* polymorphisms in population-based studies (van Soolingen 2001).

Molecular epidemiology tools also enabled the identification, description and differentiation of rare species within the *M. tuberculosis* complex, such as "*M. canettii,*" *M. microti, M. caprae, and M. pinnipedii.* These species had previously been overlooked, mainly because they were difficult to distinguish by conventional biochemical tests (see chapter 8). The differentiation between these *M. tuberculosis* complex taxons contributed to the triggering of evolutionary studies (Gutierrez 2005). In turn, differentiation to the species level by spoligotyping (Kamerbeek 1997) – a user-friendly genotyping tool applied worldwide – turned out to have practical implications on medical management and epidemiology. More recently, basic studies on genomics have been applied for the design of a clinical test – which is already available in the market – for the rapid identification and differentiation of *M. tuberculosis* complex species, including BCG strains, in clinical isolates (see chapter 14).

Genotyping tools have gained a well-deserved place in national TB control programs (see Chapter 9). Indeed, molecular epidemiology studies have a direct impact on the surveillance of TB transmission and serve to adjust control strategies, not only in industrialized countries (van Doorn 2006) but also in medium- and

poor-resource countries (Crampin 2006, Godreuil 2007, Palmero 2003, Prodinger 2007, Ramazanzadeh 2006, Villarino 2006).

DNA strain typing is also a powerful tool for quality control of culture in diagnostic laboratories (Martínez 2006). Its role in national TB programs of medium- and poor-resource countries has not been sufficiently stressed. Still today, the issue is annoying for certain bacteriologists, who still feel that culture is infallible and tend to be reluctant to acknowledge laboratory error. For example in a study performed in Argentina, false positive cultures due to laboratory cross contamination were confirmed by genotyping in 25 out of 26 suspected events investigated in 12 laboratories within the national TB network between 1996 and 2003. The contamination rate of positive cultures was 3 % – similar to rates reported in industrialized countries – in the only network laboratory that performed continuous surveillance of its occurrence (Alonso 2007).

Other challenging issues raised by molecular epidemiology studies are related to reinfection (Chiang 2005, Shen 2006, van Rie 2005) and multiple infection (Garcia de Viedma 2005, Shamputa 2006, van Rie 2005), loss of strain fitness associated with drug resistance (Gagneux 2006, Toungoussova 2004, van Doorn 2006), differential virulence and immunopathogenesis (Dormans 2004, Lopez 2003, Manca 2004, Manca 2005, Reed 2004, Reed 2007), tissue or organ affinity (Caws 2006), vaccine development and protection (Abebe 2006, Castanon-Arreola 2005, Grode 2005, see chapter 9). Even the results of genomics rely upon the lineage of the few strains than have so far been sequenced (see chapter 4).

As described in chapter 9, a re-formulated version of the Variable Number of Tandem Repeats (VNTR) typing based on 15 Mycobacterial Interspersed Repetitive Units (MIRU) loci is emerging as the tool of choice – and probably the next gold standard – for *M. tuberculosis* genotyping, at least for the near future (Supply 2006). Unfortunately, this stimulating prospect poses a practical problem for laboratories in medium- and low-resource countries that managed to perform strain typing during the ’90s. The manual VNTR-MIRUs procedure is still too labor-demanding, while high-throughput techniques – entirely or partially automated – are not affordable for most laboratories in such countries, at least in the short or medium term. In addition, these laboratories have little chance of translating the data gathered in existing databases on RFLP and spoligotype information into the new MIRUs language, at a reasonable pace. Thus, as long as science continues to advance, the scientific gap between industrialized and developing countries will widen.

In spite of the impressive advances made in the field with the existing tools, the ideal method for strain typing has not yet been achieved (see chapter 9). Microarray techniques are envisaged as the true future tools for DNA strain typing. Hopefully, VNTR-MIRUs and/or microarray genotyping methods will become standardized and their use generalized so that the increased demand for these techniques will contribute towards lowering their cost to become affordable worldwide.

20.3. New perspectives in diagnosis

It is now 125 years since the tubercle bacillus was described by Robert Koch. Disappointingly, the diagnosis of the disease still relies on the same microscopy technique based on the specific Ziehl-Neelsen staining of the bacillus, which was already available soon after that fundamental discovery. Much more progress needs to be made in obtaining better and faster diagnostic methods. Indeed, in high-burden resource-poor countries, where TB is a major public health problem, the diagnosis of active disease is mainly performed by direct microscopic examination of sputum-smear samples. This technique, although simple and inexpensive, lacks sensitivity in comparison to *M. tuberculosis* culture. Several modifications - mainly based on concentration and centrifugation techniques - have been proposed to improve the sensitivity of sputum-smear microscopy, with varying results. A recent systematic review and meta-analysis has shown that specificity does not vary substantially between different methods, but sensitivity can be improved. By comparison with direct smears, centrifugation and overnight sedimentation are more sensitive when preceded by any of several chemical methods, including the bleach method (Steingart 2006a). No other major improvement has been obtained in the classical staining method based on the Ziehl-Neelsen technique developed many years ago.

Fluorescent microscopy proved to be faster and more sensitive than conventional microscopy based on Ziehl-Neelsen staining, and is the standard diagnostic method in high-income countries (Steingart 2006b). It has the additional advantage of demanding less effort from the laboratorist, thus reducing fatigue and human error. As for low-income countries, the eventual introduction of fluorescent microscopy should be evaluated carefully because it requires a more expensive microscope and a more complex technique. It should be noted, however, that the main burden of fluorescent microscopy lies in the maintenance of the mercury lamp, rather than in the initial cost of the equipment. Lately, an inexpensive device has been released onto the market that can be adapted to any fluorescence microscope. It has a long-life, low power consumption 'Royal Blue' Luxeon light-emitting diode that is used in place of the high-cost, short-lived, and environment-unfriendly mercury vapor

lamp (Anthony 2006). In fact, this lamp lasts 200 times as long, and costs 10 times less than the vapor mercury lamp. This form of illumination is suitable for the detection of auramine O-stained bacilli and may become an affordable alternative for improving diagnostic microscopy in laboratories serving poor-income settings with a high load of smear examinations (Van Hung 2007).

Fluorescein diacetate staining was recently evaluated for assessing bacilli viability in sputum smears. It has been proposed for the early and accurate detection of TB treatment failure in poor-income settings with a high TB burden (Hamid Salim 2006).

Cultivation of *M. tuberculosis* is the gold standard for the diagnosis of active TB in the laboratory. This has been traditionally performed in egg- or agar-based solid media. Although slow and time-consuming, it is relatively simple to perform and rather inexpensive in most settings. Newer alternative methods based on liquid culture media and giving faster results - such as the BACTEC radiometric method, BACTEC MGIT960 and BactT/Alert - have proven useful, especially in medium- and high-income countries with the necessary resources to use them routinely (Scarparo 2002). It is now standard recommendation that the combination of a solid and a liquid culture medium gives the best sensitivity in recovering mycobacteria in primary culture (Tenover 1993).

The development of many new nucleic-acid amplification techniques, such as the polymerase chain reaction (PCR), opened new possibilities for the rapid diagnosis of many infectious diseases (Yang 2004). However, when applied to TB diagnosis, only two methods, the Amplicor *Mycobacterium Tuberculosis* Test (Amplicor) (Roche Diagnostic Systems, Inc., New Jersey) and the Amplified *Mycobacterium Tuberculosis* Direct Test (MTD) (Gen-Probe, California), both commercially available, have received approval by the US Food and Drug Administration (FDA) for direct application in clinical samples (Centers for Disease Control 2000). Compared to culture and clinical status, nucleic-acid amplification tests have high sensitivity and specificity in smear-positive samples. However, lower values are obtained in smear-negative specimens, precluding their use as a screen to rule out the disease. The current recommendation is that molecular tests should always be interpreted in conjunction with the patient's clinical data (Pfyffer 2003). More recent approaches combining PCR amplification and fluorescent probe detection in the same tube, such as real-time PCR technology, are promising to give improved sensitivities and specificities. More evaluations in target populations are needed to assess the real impact on the diagnosis of the disease (Espy 2006, Savelkoul 2006).

Until recently, the tuberculin skin test was the only available test for the diagnosis of latent TB infection. Based on the detection of delayed-type hypersensitivity to purified protein derivative (PPD) obtained from *M. tuberculosis,* it measures the size of the induration produced after sub-cutaneous inoculation of a standardized dose. Since PPD is actually a raw mixture of several antigens shared by *M. tuberculosis*, *M. bovis* BCG and several non-tuberculous mycobacteria (NTM), a positive tuberculin skin test result could be due to latent TB infection, previous BCG vaccination, or previous exposure to NTM. Additionally, it has various disadvantages, such as variability in the interpretation by different readers, the need of some experience to correctly interpret the result, and the requirement for the patient to return after 48-72 hours for test reading.

In order to overcome these disadvantages, immune-based blood tests have been recently introduced to detect latent TB infection (see Chapter 13). Interferon-γ (IFN-γ) assays measure the amount of IFN-γ produced or the actual number of IFN-γ-producing T lymphocytes in response to specific antigens. Both approaches are based on the fact that T cells sensitized with tuberculous antigens will produce IFN-γ when they are re-exposed *ex vivo* to mycobacterial antigens; a high amount of IFN-γ production is then presumed to correlate with TB infection (Pai 2004). The current IFN-γ assays use more specific antigens to *M. tuberculosis* than PPD: the early secretory antigen target 6 (ESAT-6), and the culture filtrate protein 10 (CFP-10). Both proteins are coded by genes located in the region of difference 1 (RD1) of the *M. tuberculosis* genome and are not shared with *M. bovis* BCG or most NTM, with the exception of *M. marinum*, *M. sulzgai* and *M. kansassi* (Andersen 2000). There are many studies evaluating IFN-γ assays in different populations. Based on published evidence, the T-SPOT TB assay seems to be more sensitive than the QuantiFERON-TB Gold test in detecting latent as well as active TB infection. However, the absence of a real gold standard for the diagnosis of latent TB infection prevents a more definitive conclusion. Further studies comparing these two assays are needed, especially in immunosuppressed patients (Richeldi 2006).

It should be remarked that TB diagnosis in endemic countries has so far gained little benefit from scientific progress, namely biotechnological developments (Perkins 2006a, Perkins 2006b). There is an urgent demand for a field-friendly test, ideally, a point-of-care one able to diagnose the disease on the spot in order to avoid delays in diagnosis, thus, preventing further transmission and reducing complications. This type of test is particularly useful when patients do not return for care and would greatly benefit people in settings such as prisons, homeless shelters, and clinics for migrant workers who have no ready access to, or do not seek, public health service assistance. The ideal diagnostics for TB should be available in one

hour and should require no electricity, refrigeration, fresh water supply or highly trained personnel (Keeler 2006).

Serological tests - aimed at the detection of either antigens specific to, or antibodies directed against *M. tuberculosis* - would in principle provide a choice platform for a point-of-care diagnostic tool. However, as commented in chapter 13, there are still many unsolved hindrances to this approach. In particular, in the development of tests for antibody detection, careful attention should be paid to the selection of the target group and the control population groups for performance evaluation. Inclusion and exclusion criteria should be quite stringent regarding age range, geographical location, previous exposure to *M. tuberculosis* and environmental mycobacteria, tuberculin skin test status, previous BCG vaccination, and unrelated pathologies for differential diagnosis. Also, the sensitivity of the test should be evaluated in the actual target population, namely patients with (pulmonary and/or extrapulmonary) paucibacillary TB rather than in acid fast bacilli smear-positive pulmonary cases. Unfortunately, some serological tests are being marketed in developing countries without a proper on-site assessment. Another issue to consider is the genetic diversity, not only between hosts but also between *M. tuberculosis* lineages, which might render a promising test only suitable for a restricted geographical region (Lopez 2003). In order to improve performance, a comprehensive set of purified, well-characterized antigens should be investigated, searching for differences in patterns of response rather than comparing responses to individual candidate antigens.

A quite different approach that utterly fulfills the requirements of the point-of-care diagnosis is based on the electronic nose technology, which is able to detect and identify tiny amounts of virtually every substance in a few minutes. The device can be assembled as the sensory part of a portable artificial intelligence system, able to detect several microbes simultaneously through their specific “odors.″ Such a system could be used to investigate the agent either directly in the patient’s breath or in a swab of a specimen obtained from any bodily site. This highly suited technology has already been reported for other bacterial human pathogens but is still awaiting development for TB diagnosis (Dutta 2006).

Lastly, diagnostic methods currently under development and expected to be available in two to four years include a dipstick PCR, the detection of mycobacterial proteins in urine, and a blood antibody test, among others (Marris 2007).

20.4. The problem of drug resistance detection

Traditionally, drug resistance in TB was assessed by culturing *M. tuberculosis* on solid media in the presence of antibiotics and measuring growth by enumeration of colonies. This methodology, although simple to perform and rather inexpensive, is quite slow and laborious, requiring several weeks to give the final results (Heifets 1999). Many alternative approaches and methods have been proposed, some of which have already been presented in Chapter 19 (Palomino 2007, Piersimoni 2006). The most important consideration before they can be implemented in the routine diagnostic laboratory is that they are better and faster than the currently available methods and that they have been properly evaluated and have shown high accuracy in target populations.

Several molecular tools have also been developed and proposed as rapid methods to detect drug resistance (Garcia de Viedma 2003). They search for genetic determinants of resistance rather than for the resistance phenotype, and involve molecular nucleic acid amplification by PCR and detection of amplified products for specific mutations correlating with drug resistance. Molecular methods have several advantages over culture-based techniques: shorter turnaround time, no need for growth of the organism, the possibility for direct application in clinical samples, less biohazard risks, and feasibility for automation. However, not all molecular mechanisms of drug resistance are known. In most cases, molecular methods have been directed towards detecting resistance to rifampicin for two major reasons. First, rifampicin resistance is a good surrogate marker for treatment failure and, in settings with a high prevalence of drug resistance, for multidrug resistance. Second, the associated mutations are well defined, restricted to a short chromosomal segment, and their prevalence is sufficiently known worldwide (see Chapter 19). Current developments aim at the simultaneous identification of *M. tuberculosis* and detection of resistance to two or more key drugs. The desideratum would be to achieve identification and multiple drug resistance detection directly on clinical specimens, thus avoiding the delay implied in culturing the bacilli (Cavusoglu 2006, Kim 2006, Marin 2004, Park 2006, Sekiguchi 2007, Somoskovi 2006, Yang 2005). Desoxyribonucleic acid (DNA) sequencing of amplified products remains the reference standard to which new molecular tools are compared.

20.5. On drug development

Associated with the problem of drug resistance is the search for new anti-tuberculosis drugs. As mentioned in previous chapters of the book, almost no new anti-tuberculosis drug classes have been developed over the last 40 years. In fact, once the industrialized countries felt confident in accomplishing TB control, the leading pharmaceutical industries lost interest in the development of anti-tuberculosis drugs. This lack of investment became evident when the HIV/AIDS pandemic emerged, soon followed by MDR-TB and the unavoidable interactions between anti-tuberculosis and anti-retroviral drugs (see Chapter 17). In view of this, interest in the discovery of new drugs against TB was awakened towards the end of the millennium. Many candidate compounds have been considered in the last decade, but very few of them have entered into further evaluations. These potentially useful anti-tuberculosis drugs are currently in different stages of the evaluation pipeline.

The Global Alliance for TB Drug Development (http://new.tballiance.org) was established in 2000 to promote the development of new anti-tuberculosis compounds. Its goal is to bring a new anti-tuberculosis drug onto the market by 2010. The most advanced program on new drugs is examining moxifloxacin, and is about to enter phase III clinical trials in multiple centers. The program aims at using this fluoroquinolone instead of ethambutol or isoniazid in the first-line drug scheme of anti-tuberculosis treatment, in order to shorten the current 6-month duration of the treatment (Burman 2006). A similar program is being carried out in Africa, where gatifloxacin, another fluoroquinolone, is also substituted for ethambutol. This combination treatment is currently in Phase III clinical trials aimed at shortening the standard regimen to four months (Anonymous 2006). PA-824 is another pro-drug of the nitroimidazole class that has already passed animal model testing in combined therapy, and is currently undergoing phase I clinical trials (Nuermberger 2006). Interestingly, a protein was described in *M. tuberculosis* that is involved in both intracellular drug activation and resistance to this drug candidate (Manjunatha 2006). Other promising drugs are in the pre-clinical phase of evaluation. OPC-67683 is a nitro-dihydro-imidazooxazole derivative that inhibits mycolic acid biosynthesis, is free of mutagenicity and is highly active against TB *in vitro* and in mice (Matsumoto 2006). The diarylquinoline known as R207910, TMC207 or compound J is a new compound exhibiting a completely novel mode of action - inhibition of ATP synthase - and very high activity against *M. tuberculosis*, *M. leprae* and *M. ulcerans*. This promising compound is being tested in phase IIa trials on TB treatment (Andries 2005, Lounis 2006).

The availability of such a spectrum of new drug candidates offers great promise but also entails a great challenge. The role played by each drug must be explored within the frame of a multidrug treatment regimen. In the immediate future, a complete series of clinical trials will be needed to find the optimal treatment scheme of ultra-short duration, i.e. 2 months or even shorter. Innovative strategies must be designed in order to meet this challenge (Spigelman 2006). Glickman *et al.* have calculated the probability of developing a new anti-tuberculosis drug by 2010 (Glickmann 2005). Applying a Monte Carlo simulation model, they evaluated drug development from the perspective of a public-private partnership, taking into account several factors such as the expected number of successful compounds, the expected costs of each stage of development and the development costs for successful and unsuccessful compounds. As for the currently-available candidate drugs in all stages of development, the probability of at least one successful compound being generated was less than 5 %. Obviously, many more efforts and funding are required to reach the objectives of developing new and successful anti-tuberculosis drugs in the near future.

Research and development is also needed on innovative drug formulations and drug delivery systems aimed at increasing compliance and achieving a high local drug concentration while minimizing systemic side effects. In this respect, the growing field of inhalation therapy offers a very promising new prospect (Chow 2007, Shoyele 2006). A technology based on porous particles for pulmonary drug delivery is already in use for insulin. This technology – presented as a simple, low-cost, disposable, dry-powder inhaler – can be applied to the delivery of anti-tuberculosis drugs (Edwards 2006).

20.6. On vaccine development

Vaccine development is a problematic issue for many reasons. It is not appealing for the industry, because it demands a huge investment, takes a long time and the risk of failure is high. Besides, even in the best scenario, profit margins are meager and the risk of legal prosecution in the event of side effects is high (Rosenthal 2006).

Moreover, major obstacles also lie in the initial vaccine design itself, such as the difficulty in inducing a potent and long-lasting cellular immune response in humans, due to our poor understanding of host-parasite interactions. Vaccines that promise to be more potent than BCG were designed following a rational approach: they are recombinant BCGs over-expressing major *M. tuberculosis* antigens. Grode *et al.* used an entirely novel approach, as commented on thoroughly in Chapter 10.

Instead of using an antigen, these authors selected the membrane-perforating listeriolysin to construct a recombinant BCG. This hemolytic enzyme, produced by *Listeria monocytogenes,* allows the agent to escape from the phagosomes of infected host cells. This improves the access of mycobacterial antigens to the major histocompatibility complex Type I pathway, thus resulting in better CD8+ T cell stimulation. At least in the mouse model, recombinant BCG secreting listeriolysin elicited an enhanced protection against a strain of the *M. tuberculosis* Beijing/W genotype family, while parental BCG failed to do so consistently (Grode 2005). This latter finding should be highlighted because the selection of strains used for challenging any vaccine candidate is not a minor issue. Future vaccines must prove able to protect against the most prevalent, transmissible and/or virulent lineages worldwide, not merely against laboratory-domesticated strains (Lopez 2003).

Another promising approach has gone even further in proving efficacy. The design is based on the fact that viral vectors, such as poxviruses, are powerful at boosting previously primed T-cell responses against intracellular pathogens. McShane *et al.* successfully applied such an approach by using BCG as the priming immunization – eventually maintaining the beneficial protective effects of BCG against disseminated disease – and a recombinant modified vaccinia virus Ankara expressing antigen 85A (MVA85A) for boosting (McShane 2004). Both CD4+ and CD8+ T cells were successfully boosted in preclinical experiments with MVA85A and also in human volunteers. The vaccine candidate is now in clinical trials in the United Kingdom and Africa. Results from one of these trials showed that the recombinant viral vector vaccine is a strong booster of BCG-primed and naturally acquired antimycobacterial immunity. In fact, this is the first clinical trial showing successful results with a novel subunit TB vaccine. Besides, the strategy is feasible and practical in low-resource high-burden countries (McShane 2005). Most importantly, this pioneer study also raises highly sensitive protocol issues and, in particular, ethical issues (Ibanga 2006).

A provocative finding was reported by researchers at the Institut Pasteur, where the BCG vaccine was first developed in 1924. They gathered a large body of evidence on the evolutionary sequence of events leading to modern vaccine variants. By comparing various genetic markers, the authors were able to construct an evolutionary tree, showing that distinct variants of the original BCG strain underwent different deletions during *in vitro* passages, mainly in the process of adaptation to the broth medium used for vaccine production. These mutations might have been responsible for a gradual loss in immunogenicity and protection ability. Oddly enough, their findings suggest that the early BCG Japan strain may even be superior to the later ones that are currently much more widely used, such as the Danish

BCG strain. It is speculated that, in general, vaccine manufacturers tended to prefer BCG strains that cause less serious local reactions over those provoking more intense inflammation at the site of injection. As a result, less protective strains for vaccine production might have unintentionally been selected through time (Brosch 2007).

According to Kaufmann, a single vaccine is not expected to cover the entire spectrum of persons susceptible to developing active TB (Kaufmann 2006), namely HIV-infected and non-infected, *M. tuberculosis*-infected and non-exposed, BCG-vaccinated and non-vaccinated, with high- and low-risk of exposure to non-tuberculous mycobacteria. Kaufmann envisages the application of customized vaccination schemes for every specific scenario. Strategies based on combined vaccination schemes with a prime-boost approach would produce various optimal vaccination regimens that will be ready for combined – simultaneous and successive – phase II clinical trials by 2010. The fulfillment of this chronology is highly dependent upon the availability of reliable biomarkers of effective vaccination. Some preliminary studies on biomarkers to differentiate between persons with active TB, *M. tuberculosis* infection, and healthy individuals, are already on the way (Jacobsen 2007). New TB vaccine regimens are not expected to be introduced into national TB programs before 2020.

20.7. Global management of research & development resources

Last but not least, the emphasis placed by international organisms on fostering the synergy of efforts in TB research and development deserves to be mentioned. Several public-private partnerships have been established to combat the global burden of TB (Kaufmann 2007). A major outcome is the formation of a conglomerate of official and non-governmental agencies, in which several WHO branches are represented together with leaders of the pharmaceutical industry, as well as powerful sponsors such as the World Bank and the Bill & Melinda Gates Foundation. This unprecedented initiative provides a robust platform for driving the global research agenda toward the most compelling gaps in knowledge, and readily translating scientific findings into patient care and public health improvement. The International Union against Tuberculosis and Lung Disease is a key non-governmental organization that has been promoting TB control worldwide since 1920. The Union publishes a renowned peer-reviewed scientific journal on tuberculosis and lung disease, and together with the WHO, has been leading the Global Project on Anti-Tuberculosis Drug Resistance Surveillance since 1994.

Similarly, the European Commission is undertaking a major effort through its successive Framework Programmes to foster basic research and development in TB, and, at the same time, to strengthen TB research capacities in developing countries. Altogether, these merging initiatives have started to awaken the concern of public health authorities and research agencies at the national level in medium- and low-resource countries. Yet, much remains to be done.

The tubercle bacillus is both an amazing creature and a formidable enemy that has proven hard to conquer. It is pushing science to its limits. Medical research itself has developed into a complex and engaging living creature whose evolution is driven by selective pressure. Synergic efforts, interdisciplinary approaches, and translational research are expressions of its adaptive response. The greater the challenge the more eager the endeavor must be. We just can not afford to lose this battle.

20.8. Useful links

- Aeras Global TB Vaccine Foundation www.aeras.org/
- Damien Foundation http://www.damienfoundation.org/
- European and Developing Countries Clinical Trials Partnership Programme (EDCTP)
  http://ec.europa.eu/research/info/conferences/edctp/edctp_en.html
- European Union Framework Programme 7
  http://cordis.europa.eu/fp7/home_en.html
- Foundation for Innovative New Diagnostics (FIND)
  http://www.finddiagnostics.org/
- Global Alliance for Tuberculosis Drug Development
  http://new.tballiance.org/
- International Union against Tuberculosis and Lung Disease
  http://www.iuatld.org
- Partners in Health http://www.pih.org/
- Stop TB Partnership http://www.stoptb.org
- WHO Special Programme on Research and Training in Tropical Diseases (TDR) http://www.who.int/tdr/

- WHO/IUATLD Global Project on Drug Resistance Surveillance
  http://www.who.int/tb/dots/dotsplus/surveillance/en/index.html

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Index

A

absolute concentration method 643
acid fast bacilli (AFB) 401
actinomycetes 93
adenosine deaminase (ADA) 409, 564
adverse reactions 576
*ahp*C 614
Alamar blue 652
amikacin 610
aminoglycosides 610
anhidrotic ectodermal dysplasia with immunodeficiency 220
animal model 173, 347
antiretroviral therapy 579
apoptosis 177
ATP synthase 625
attenuated *M. tuberculosis* 352
atypical presentations 564

B

bacillus Calmette-Guérin (BCG) 94, 290, 342, 552
BACTEC MGIT960 414, 645
BACTEC TB-460 414, 643
Balb/c 134, 173
BCG vaccination 371
Beijing 73, 182, 325
biological safety cabinets 388
biosafety levels 376
bovine TB 283
brain abscesses 566

C

capreomycin 609
caseous necrosis 490
catalase-peroxidase 106
CD4+ T cells 170
CD8+ T cells 170
CDC1551 121
cell envelope 97, 144
cellular immune response 170, 430
Central-Asian 74
cetylpyridinium chloride (CPC) 404
chemoprophylaxis 546, 599
chest X-ray 512, 543, 561
chronic granulomatous disease 220
ciprofloxacin 611
colorimetric methods 651
comparative genomics 79
complex of Ranke 488
computerized tomography 513, 544, 566
congenital TB 536
consumption 38
contact tracing 320, 518
containment 373
contingency plan 392
cord factor 101
culture of *M. tuberculosis* 539
cycloserine 609

D

default 571
defensins 165
delayed type hypersensitivity (DTH) 426
dendritic cells 162, 233
diabetes mellitus 506
diarylquinolines (DARQs) 624
direct observation of therapy (DOT) 47
Directly-Observed Treatment, Short-course (DOTS) 47, 267, 272, 574
disinfectants 382
DNA fingerprinting 331

DOTS-Plus 274
double-barred cross 41
DR locus 58
droplet nuclei 487, 526
drug susceptibility testing 641

E

East African-Indian 72
efflux pumps 612
emb operon 616
emergency procedures 394
endobronchial TB 531
environmental mycobacteria 343
erythema nodosum 530
ESAT-6 120, 296, 348, 436
ethambutol (EMB) 605
ethionamide (ETH) 610
extensively drug resistant TB (XDR-TB) 47, 569, 573, 635
extra-pulmonary TB 533, 564

F

fever 508
fitness 323, 617
fluoroquinolones 611

G

G+C content 115
gamma/delta T cells 170
gastric lavage 538
gatifloxacin 611
genome sequence 115
GenoType MTBDR 646
GenoType Mycobacterium 466
Gohn's nodule 488
Great White Plague 30
gyrA 303, 616
gyrB 294, 298, 616

H

H37Rv 115
Haarlem 75
heat-shock protein 65 (Hsp65) 186
hemoptysis 509
High efficiency particulate air (HEPA) filters 367
high-burden countries 267
highly active anti-retroviral treatment (HAART) 574
HIV/AIDS 46, 270, 271, 549
human leukocyte antigens (HLA) 225, 429
humoral response 168, 428
hyper-IgE syndrome 219

I

Immune reconstitution inflammatory syndrome (IRIS) 579
immunotherapy 184
induced sputum 402
infectivity 44
inhA 614
INNO LiPA Mycobacteria 465
INNO-LiPA Rif TB 646
Insertion Sequences 116
interferon-gamma (IFN-γ) 162, 435
International Union Against Tuberculosis and Lung Disease (IUATLD) 41
intracraneal tuberculoma 566
IS*1081* 116
IS*6110* 116
IS*6110* RFLP 57, 326
isoniazid (INH) 43, 601

K

kanamycin 610
*kas*A 614
katG 303, 324, 570, 614
Koch phenomenon 171

L

laboratory hazards 378
laboratory-acquired TB 374
Large Sequence Polymorphism (LSP) 58
latent TB 183, 517, 599
Latin American and Mediterranean 76
Löwenstein-Jensen 413

M

macrophage 160, 233
magnetic resonance imaging 544
major histocompatibility complex (MHC) 225
mast cell 159
Mendelian susceptibility to mycobacterial diseases (MSMD). 221
meningitis 534, 566
microarray 121, 125, 128, 130, 132, 296, 301, 649
microscopic observation broth-drug susceptibility assay (MODS) 654
Middlebrook 7H10 414
Middlebrook 7H9 442, 446
miliary TB 497
milk pasteurization 289
Millennium Development Goals 266
milliary TB 534
molecular epidemiology 317
monoresistant TB 568
moxifloxacin 611
mtp40 302
MTT 652
multidrug-resistant TB (MDR-TB) 46, 274, 323, 568, 635
Mycobacteria Growth Indicator Tube (MGIT) 414, 444, 644
Mycobacterial Interspersed Repetitive Units (MIRU) 58, 329, 664
mycobacterial lipids 144
mycobacterial phagosome 161
mycobacteriophage 472
Mycobacterium 93
Mycobacterium africanum 55, 93, 293
Mycobacterium bovis 94, 283
Mycobacterium canettii 55, 93, 182
Mycobacterium caprae 94
Mycobacterium microti 94, 295
Mycobacterium pinnipedii 94, 299
Mycobacterium tuberculosis 93
Mycobacterium tuberculosis complex 54, 93, 301, 416
mycolic acids 93, 144, 601

N

natural killer cell 164
neutrophil 157
niacin accumulation 106
nitrate reductase 106, 107, 653
nitroimidazofurans 625
non-tuberculous mycobacteria (NTM) 419, 581

O

ofloxacin 611
Ogawa 413
opportunistic infections 580
osteoarticular TB 536
outbreak 46, 325, 571
oxyR 300, 302

P

para-aminosalicylic acid (PAS) 42, 107, 609
pathogenicity 102
patient isolation 365
PCR restriction-enzyme analysis (PRA) 462
PE family 117
persistence 102
Phage-based methods 650

phlyctenular conjunctivitis 530
phthisis 29
pleural TB 498
pncA 300, 302, 615
polymerase chain reaction (PCR) 450, 540
polyresistant TB 568, 639
polyserositis 567
post-primary TB 493
Pott's disease 26, 504, 536, 565
primary TB 492, 533
prophages 116
proportion method 641
prostaglandin 178
protection 343
prothionamide 610
purified protein derivative (PPD) 426, 434, 541
pyrazinamidase enzyme (PZase) 615
pyrazinamide (PZA) 298, 604

R

Real-time PCR 461, 649
recombinant BCG (rBCG) 350
recommended doses 548, 596
regions of difference (RDs) 63, 120, 294, 296, 301, 302, 304
relapse 571
renal TB 501, 539
repetitive DNA sequences 63
resazurin microtiter assay (REMA) 652
resistance ratio method 642
resistance to anti-tuberculosis drugs 570
respiratory isolation area 365
rifabutin 611
rifampicin (RIF) 44, 545, 603, 611
rifamycin 611
rifapentine 611
rifoligotyping 648
risk group 376
RNA polymerase 603
Robert Koch 32
rpoB 614
rpsL 616
rrs 616
RUTI 187

S

safety guidelines 378
sanatorium 34
scrofula 29, 533
second-line anti-tuberculosis drugs 597
serology 425
severe combined immunodeficiency (SCID) 219
sigma (σ) factors 128
Simon foci 492
Single Nucleotide Polymorphism (SNP) 58, 229
sparfloxacin 611
species identification 416
spinal TB 565
spoligotyping 28, 57, 68, 293, 295, 298, 300, 302
sputum smear microscopic examination 511, 539
Stop TB Partnership 47, 266
Stop TB Strategy 277
strand displacement amplification 456
streptomycin (SM) 42, 607
subunit vaccines 348
symptoms 175

T

T families 77
T helper 1 (Th1) 172, 185, 345, 430
T helper 2 (Th2) 173, 187, 430
TB control committee 365
TB infection 490
TB pleurisy 564
TbD1 123, 303
Thin Layer 7H11 agar (TL7H11) 473, 655

thioacetazone 612
Toll-like receptors (TLR) 233
transfer factors 185
transforming growth factor beta (TGF-β) 188
treatment 548
treatment failure 571
trehalose 6, 6'-dimycolate 101
tubercles 31
tuberculin 426
tuberculin skin testing (TST) 371, 434, 513, 527
tumor necrosis factor alfa (TNF-α) 175

U

ultrasonography 568
urease 106, 107
UV-light 388

V

vaccination 552
Variable Number of Tandem Repeats (VNTR) 58, 329, 664

W

Wayne's model 141
weight loss 508
World Health Organization 264

X

X family 76

Z

Ziehl-Neelsen 406
zoonotic TB 283

Phthysis
Consumption
White Plague

Tuberculosis has been called by many names and has haunted humanity since primordial times.
Still today it is a leading cause of death from a single infectious disease. What do we really know on this host-pathogen interaction? What have we learned about the uneven spread of different strains of the tubercle bacillus in different populations and geographic regions? Which resources are available to control tuberculosis transmission? Which tools do we have to manage tuberculosis in adults, children and HIV/AIDS patients? What has still to be learned? Which developments can be envisaged for its control in the near future? When will better diagnostic tests, new drugs and more effective vaccines be available for routine use?

In this book, the reader will find the answers to these questions.