White Spot Disease

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SUMMARY

White spot disease (WSD) is a contagious viral disease of penaeid prawns and is caused by white spot syndrome virus (WSSV), an enveloped, rod-shaped virus containing a double-stranded DNA genome. WSSV is classified as a member of the Whispoviridae. The virus infects a wide range of crustaceans. WSSV was first reported from both Taiwan and the People's Republic of China (PRC) in 1992. Subsequently, it has spread throughout East, Southeast and South Asia, North, South and Central America.

In aquaculture, WSD outbreaks are often characterised by high and rapid mortality of infected populations. Acutely affected prawns are lethargic and anorexic, may have a loose cuticle with numerous white spots and may show a pink to red discolouration. Lesions are associated with systemic destruction of the ectodermal and mesodermal tissues, especially the cuticular epithelium and subcuticular connective tissues. In farmed penaeid prawns, mortality can reach 100% within a few days of the onset of clinical signs. A wide range of decapods is susceptible to infection, but morbidity and mortality as a consequence of infection is highly variable. Sub-clinical infections are also known to occur.

Identification of the agent: There are no pathognomonic clinical signs or lesions associated with WSD. White spots on the cuticle are not observed in all cases and, when they occur, such spots can also be caused by bacterial infection or environmental conditions. A presumptive diagnosis in clinically affected animals may be made on recognition of characteristic microscopic changes in tissues but a confirmed diagnosis of WSD requires the use of polymerase chain reaction, *in situ* hybridisation or transmission electron microscopy.

Status of Australia and New Zealand: Australia and New Zealand are considered free from WSSV.

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INTRODUCTION

Aetiology

White spot disease (WSD) of crustaceans is caused by infection with white spot syndrome virus (WSSV), a member of the Whispoviridae.[1] Clinical disease is more frequently observed in farmed prawns than wild prawns. WSSV is considered exotic to Australia and New Zealand and is notifiable to the World Organisation for Animal Health (OIE).[2]

Australia has extensive populations of wild and farmed penaeid prawns, and detection of WSSV in Australia may have adverse trade implications for the Australian fishing and aquaculture industries as well as environmental and social impacts. Importation of live prawns into Australia is banned, and imported green prawns may be used only for human consumption at this time.

Epidemiology

WSSV was first reported in both Taiwan and People's Republic of China (PRC) in 1992.[3] Subsequently, it has spread throughout the prawn farming areas of Asia from Pakistan in the west to Korea in the north and Indonesia in the southeast. In 1999, the disease was introduced to Central America and has since spread throughout the Americas from the United States of America (USA) to Peru.[2] The spread of WSSV has been predominantly associated with the movement of live infected animals[4]. It has been proven in Australia and elsewhere that frozen commodity prawns can contain viable WSSV.[5,6]

The OIE lists the species susceptible to WSSV infection as all decapod crustaceans from marine and brackish or freshwater sources.[7,8]

Under the conditions of prawn aquaculture used in Asia, WSSV can cause mortality of up to 100%. Modification of husbandry practices over the last 7-8 years has decreased the incidence of disease outbreaks, and successful production of prawns in the presence of the virus is possible. Screening of broodstock for the presence of WSSV has allowed selection of broodstock with no, or a low level of, infection, and the post-larvae produced by these animals have a greater chance of surviving the entire grow-out period.[9,10]

Clinical signs

Clinical signs of disease are not pathognomonic and even white spots, characteristic of WSD, may also be caused by bacterial infection (possibly *Bacillus subtilis*)[11] or environmental conditions, such as high alkalinity.[12] WSD outbreaks in farmed prawns are often characterised by high and rapid mortality. Acutely affected prawns may be lethargic and anorexic, and swim near the surface of ponds. Moribund prawns may have white spots on the carapace and a pink to red discolouration.

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Pathology

Prawns with WSD may have a loose cuticle with numerous white spots (0.5-2.0 mm in diameter) on the inside surface of the carapace and a pink to red discolouration. Lesions are often associated with systemic destruction of ectodermal and mesodermal tissues including, but not restricted to, the gills, cuticular epithelium, antennal gland, lymphoid organ and haemopoietic tissue.[12] Nuclei of infected cells are hypertrophied and when stained with haematoxylin and eosin (H&E) show lightly to deeply basophilic central inclusions surrounded by marginated chromatin. These intranuclear inclusions can be seen in squash mounts of gills or subcuticular tissues.

Diagnostic Methods

Virus isolation on cell lines is not available for WSSV and therefore detection has focused primarily on molecular methods. In clinical disease, a presumptive diagnosis of WSD may be made by examination of H&E-stained histological sections of tissues from moribund prawns. Confirmation of a diagnosis of WSSV infection requires analysis by polymerase chain reaction (PCR), *in situ* DNA hybridisation (ISH) or transmission electron microscopy (TEM), with PCR being the preferred method. Results should correlate with the pathology of the animal. Sub-clinical infection may occur when an animal does not show pathological signs of WSD but gives a positive result by PCR. All PCR products must be confirmed by sequencing.

Histopathology

Live moribund prawns from a suspected WSSV outbreak should be fixed by injection with Davidson's fixative (see Appendix 1). This highly acidic fixative decalcifies to facilitate tissue processing and sectioning. Conventional fixative solutions, such as 10% neutral buffered formalin, do not decalcify or penetrate the shell, which results in poor fixation and difficulty in cutting sections. Where diagnosis is based on histology, tissues may be fixed for an unlimited time without loss of quality. However, specimens for ISH should be transferred from Davidson's fixative to 70% ethyl alcohol after 24-48 h or they will be unsuitable for ISH. Following fixation, the specimens are embedded in paraffin wax and processed using standard methods. Inclusion bodies in target tissues are suggestive of WSSV infection.

Electron Microscopy

The most suitable tissues for electron microscopy are subcuticular tissues, gills and pleiopods, which have been screened by histology, or rapid-stain tissue squashes that showed signs of hypertrophied nuclei and Cowdry A-type inclusions, or marginated chromatin surrounding a basophilic inclusion body. Tissues are fixed in a 10:1 fixative to tissue volume of 6% glutaraldehyde at 4°C and buffered with sodium cacodylate or phosphate to pH 7.0, for at least 24 h. For long-term storage, glutaraldehyde concentration is reduced to 1%. The tissues are then fixed in 1% osmium tetroxide, and stained with uranyl acetate and lead citrate. WSD virions are rod-shaped to elliptical with a trilaminar envelope and measure 80-120 x 250-380 nm.

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Polymerase Chain Reaction (PCR)

Whenever possible, material for testing should be collected from live or moribund juvenile or adult prawns as degradation of tissue and viral DNA, proceeds rapidly after death. Preferred tissues are gill tissue and/or cuticular epithelium. Whole larvae or post-larvae will also suffice. Cuticular epithelium can be obtained by scraping the membranous epithelium from the carapace, extracting a piece of pleiopod or by dissecting it from the abdominal muscle. A pooled sample of whole post-larvae should be used for the detection of WSSV in post-larvae. Material can be frozen or stored in alcohol preservation solution (see Appendix 2) at ambient temperature until processed. Individual laboratories may employ other acceptable methods for storage of DNA.

The nested PCR amplifies a 1447 base pair (bp) sequence of viral genomic DNA in the first reaction, using primers 146F1 and 146R1 (see Figure 1), and an internal fragment of 941 bp in the nested reaction, using primers 146F2 and 146R2 (see Figure 1). The nested PCR has an overall sensitivity in the range of 18-180 target molecules for non-degraded DNA. Reaction products are visualised by agarose gel electrophoresis. The method is adapted from the OIE *Manual of Diagnostic Tests for Aquatic Animals*.[12] Commercial kits are available for the detection of WSSV by PCR, and it may be preferable for some laboratories to use one of these kits, using the manufacturer's instructions.

As the test may need to be performed on less-than-ideal specimens, the protocol also includes a separate quality control PCR, the decapod PCR (see Appendix 18). This PCR amplifies a specific decapod DNA sequence of 848 bp, using primers 143F and 145R (see Figure 1), but the PCR will work only in the presence of intact host cell DNA. Amplification of decapod DNA from a sample confirms that the host DNA, and, by extension, the WSSV DNA, is suitable for amplification. Non-amplification of host cell DNA with the decapod primers is indicative of a sample too degraded to be useful for WSSV detection.

PCR is a highly sensitive method and the slightest contamination of samples with WSSV DNA, or with WSSV amplicons, can cause false positive results. To detect cross-contamination, it is essential to include appropriate positive and negative controls in each set of reactions and to maintain clear separation of samples during collection, processing and analysis. Areas should be segregated and movements of personnel and equipment should be controlled.

1 2 3 4 5 6

Figure 1 PCRs for WSSV detection, electrophoresed on a 1% agarose gel.

Lane 1. 100 bp markers. Lower bright band represents 600 bp, and the bands extending up from this band are in 100 bp increments up to 1500 bp;

Lane 2. Tissue from a WSSV-infected prawn sample tested with the WSSV primers 146F1 & 146R1. A single band of the expected size of 1447 bp is visible;

Lane 3. Tissue from a WSSV-infected prawn tested with decapod primers 143F & 145R. A single band of the expected size of 848 bp is visible;

Lane 4. Tissue from an uninfected prawn sample tested with the WSSV primers 146F1 & 146R1. No reaction products are visible in this reaction;

Lane 5. Tissue from an uninfected prawn sample tested with decapod primers 143F & 145R. A single band of the expected size of 848 bp is visible;

Lane 6. Positive Control: a plasmid DNA clone containing the 941 bp nested fragment of WSSV, tested with primers 146F2 & 146R2. A single band of the expected size of 941 bp is visible.

In Situ *Hybridisation (ISH)*

This procedure uses a digoxigenin (DIG)-labeled RNA probe to detect WSSV in paraffin sections. Commercial WSSV ISH kits are available and may be more suitable for a laboratory that is not doing a lot of WSSV testing and lacks experience, despite the greater cost.

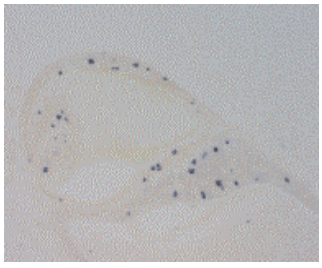

Figure 2 WSSV probe on a WSSV infected tissue section that was supplied by Dr. Supranee Chinabut, Aquatic Animal Health Research Institute, Thailand. The specific positive staining can be seen in this photo as the dark-blue cellular staining, against the light brown prawn tissue. (x 200 magnification)

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If there is WSSV present in the tissue section, the hybridisation process will allow the DIG-labeled RNA probe, developed by *in vitro* transcription, to bind to the complementary WSSV DNA, which will form a stable RNA-DNA hybrid. When anti-DIG antibody conjugated to alkaline phosphatase is applied to the section and a substrate applied, this forms a colour reaction (see Figure 2). For WSSV-positive samples, visible blue-black staining of the riboprobe will be visible by light microscopy. Positive and negative control sections are necessary to demonstrate specific staining, and the absence of background staining due to endogenous alkaline phosphatase.

Interpretation

PCR: Any 941 bp or 1447 bp fragment is indicative of the presence of WSSV, providing that the positive and negative controls are as expected. The decapod PCR should yield an 848 bp product if the DNA has not degraded beyond being amplifiable. If the decapod PCR yields an 848 bp product and the WSSV PCR does not yield a PCR product of the expected size, this is a true negative result for WSSV. If the decapod PCR does not yield a PCR product of the expected size, and the WSSV PCR does not yield a suitable size fragment, then it is likely that the sample has degraded beyond being amplifiable by this method, assuming that the positive control for the decapod PCR has worked as expected. To report a valid WSSV result for a sample, negative or positive, the decapod PCR needs to be amplifying a suitable product for that sample. If the decapod PCR does not amplify a suitable product and the WSSV PCR does amplify a product of the expected size, this must be considered as a false positive result, and the test should be repeated using a non-degraded sample.

If a WSSV commercial kit is used, interpretation of results should be in accordance with the manufacturer's instructions.

Suspected WSSV PCR positives must be sequenced using standard methods to confirm the presence of WSSV.

ISH: Specific dark blue intra-cellular staining is indicative of WSSV infection, provided all controls were included and the results of those controls are as expected.

REFERENCE LABORATORY PROTOCOL

PCR confirmatory diagnosis is available from the Australian Animal Health Laboratory (CSIRO Livestock Industries, Geelong) and OIE reference laboratories.

OIE REFERENCE LABORATORIES

Aquaculture Pathology Section, Department of Veterinary Science
University of Arizona, Building 90 Room 202,Tucson AZ 85721 USA

TECHNICAL CONTACTS

CSIRO Livestock Industries Australian Animal Health Laboratory PO Bag 24, Geelong VIC 3220	Department of Fisheries 3 Baron-Hay Court South Perth WA 6151

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APPENDICES

Appendix 1

Davidson's Fixative Solution
220 mL 40% formaldehyde
330 mL 95% ethanol
115 mL glacial acetic acid
335 mL distilled water

Appendix 2

Alcohol Preservation Solution
80% v/v ethanol or isopropanol (AR grade)
20% v/v glycerol
0.25% v/v β-mercaptoethanol.

Appendix 3

Preparation of Silanated Slides
1. Soak slides in a 1-2% solution of laboratory detergent for at least 1 h.
2. Wash under running tap water for 10 min.
3. Wash in deionised water followed by absolute ethanol and air dry in a 37°C oven.
4. Immerse slides in a 2% solution of 3-aminopropyltriethoxysilane in fresh acetone, for 2-5 min.
5. Wash quickly in acetone followed by 2 changes of distilled water, and air dry in a 37°C oven.

Appendix 4

Protease Solution
Make up a stock solution of Protease VII at 2.5 mg/mL in sterile distilled water, and store in small amounts at -20°C. For the working solution, dilute the stock solution to a concentration of 375 ng/µL.

Appendix 5

Hybridisation Mixture
(can be stored at ambient temperature)
5 mL formamide
1 mL 50% dextran sulphate in DEPC-water (stock solution: 2 g in 4 mL water; filter with a 0.45 µM filter into a 5 mL vial; store at 4°C)
1 mL 20X SSC

Appendix 6

Salmon Sperm DNA Stock Solution
Dissolve the DNA in sterile distilled water at 10 mg/mL by stirring on a magnetic stirrer for 2-4 h. Shear the DNA by passing it several times through an 18 or 21 gauge hypodermic needle. Boil for 10 min and store at -20°C in 1 mL amounts. Thaw an amount just prior to using and denature the DNA by heating in a boiling water bath for 5 min, then place on ice until ready to use. Remaining material can be frozen again and re-used several times.

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Appendix 7

TE8 Buffer
10 mM Tris-HCl pH 8.0
1 mM EDTA pH 8.0
Sterile distilled water

Appendix 8

Blocking Solution
3% skim milk powder
0.05% Triton-X-100
5 mL Buffer 1 (see Appendix 10)
(150 mg skim milk powder, 2.5 µL Triton-X-100 in 5 mL Buffer 1)

Appendix 9

Buffer 1
100 mM Tris-HCl (pH 7.5)
150 mM NaCl
Sterile distilled water

Appendix 10

Buffer 3
100 mM Tris-HCl (pH 9.5)
100 mM NaCl
50 mM $MgCl_2$
Use previously autoclaved stock solutions of 1 M Tris-HCl (pH 9.5), 1 M NaCl and 1 M $MgCl_2$.

Appendix 11

Neutral Buffered Formalin
90 g NaH_2PO_4
110 g Na_2HPO_4
170 g NaCl
2 L 40% formaldehyde
Make up to 20 L with tap water.

Appendix 12

20X SSC
175.3 g NaCl
88.2 g sodium citrate
800 mL Milli-Q water
Adjust to pH 7.0 with HCl; make up to 1 L and autoclave at 121°C for 15 min.

4X SSC
200 mL of 20X SSC in 1 L sterile water

1X SSC
100 mL of 20X SSC in 2 L sterile water

0.1X SSC
5 mL of 20X SSC in 1 L sterile water

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Appendix 13

CTAB Buffer
2% w/v hexadecyl trimethyl ammonium bromide (CTAB)
1.4 M NaCl
20 mM EDTA
100 mM Tris-HCl pH 7.5
0.25% v/v 2-mercaptoethanol (added just before use).

Appendix 14

PCA Solution
Phenol/chloroform/isoamyl alcohol (24:24:1)

Appendix 15

CA Solution
Chloroform/isoamyl alcohol (24:1)

Appendix 16

TE Buffer
10 mM Tris-HCl pH 8.0
1 mM EDTA pH 8.0
Sterile distilled water

Appendix 17

CTAB Method

1. Add approximately 25-50 mg of prawn tissue into a microfuge tube containing 250 µL of CTAB Buffer (see Appendix 13). Frozen tissue is thawed directly in CTAB Buffer. Alcohol-preserved tissue should first be blotted well with absorbent paper to remove as much ethanol as possible, as carryover may interfere with the extraction process. Homogenise with a sterile disposable pestle until the solution is viscous and no large pieces of tissue can be seen.
2. Add a further 650 µL of CTAB Buffer. Incubate the tissue slurry at 25°C for 1 h. (If necessary the tissue slurry can be left overnight at 4°C or for several days at -20°C).
3. Centrifuge at 10,000 *g* for 10 min. Collect the supernatant into a sterile microfuge tube.
4. Add 600 µL PCA solution (Appendix 14) to the supernatant and mix for 30 s, then centrifuge at 10,000 x *g* for 5 min. Remove upper aqueous phase (approximately 800 µL) to a sterile microfuge tube.
5. Add 650 µL of chloroform/isoamyl alcohol (CA) solution (see Appendix 15) to the upper aqueous phase. Mix for 30 s, then centrifuge at 10,000 *g* for 5 min. Remove the upper aqueous phase (approximately 700 µL) to a sterile microfuge tube.
6. Add 630 µL (0.9 volumes) of isopropanol to the upper aqueous phase. Mix gently. Incubate on ice for 15 min, at -20°C overnight or at -70°C for 1 h to precipitate the total nucleic acid (TNA).
7. Centrifuge at 10,000 *g* for 15 min. Discard the supernatant carefully (pellet is usually loose at this step).
8. Add 600 µL of 70% ethanol and gently wash the pellet for 1-2 min. (This sample can now be stored at 4°C or -20°C).
9. Centrifuge at 10,000 *g* for 2 min. Discard the supernatant carefully and air-dry the pellet.
10. Dissolve the pellet in 75 µL of distilled sterile water or 1x TE Buffer (1.5 x original tissue weight) at 50°C for 30 min, at ambient temperature for 1-3 h, or at 4°C overnight. Store at -20°C.
11. Remove 5-10 µL of the nucleic acid and determine the concentration by spectroscopy using standard methods. Adjust the total nucleic acid (TNA) concentration to a working concentration of 300 pg to 300 nag by diluting with sterile distilled water or 1x TE Buffer.

The TNA is now ready for PCR analysis.

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Appendix 18

PCR Method

Unless samples are transported to the laboratory alive, suitable tissue, preferably gill and/or epidermis, (25-50 mg of tissue per 1.5 mL microfuge tube - not per sample as large sampling may require pooling 5 tissue samples into one tube) should be immediately preserved by snap freezing in liquid nitrogen before storage at -80°C. Alternatively, the tissue may be placed into an alcohol preservation solution (see Appendix 2) and stored at ambient temperature. Preserved samples can then be transported to the testing laboratory.

It is important that each laboratory use an extraction method that is suitable for that laboratory. The CTAB method (see Appendix 17) is recommended, however it may be more suitable for certain laboratories to use a commercially available extraction method.

It is recommended that a Master Mix be made, which is at least 10 x the volume of the reagents required for one PCR. This eliminates inaccuracies associated with pipetting very small amounts, and it also standardizes the reagent mix for all reactions in a PCR run. Whilst the PCR Master Mix is being prepared, the PCR reagents should all be thawed, but kept cool to prevent pre-PCR reactions.

The Master Mixes for the WSSV PCR and decapod PCR, per reaction of 25 µL, is as follows:
1X PCR Buffer (use stock 10X concentrate that is supplied with the Thermostable polymerase)
200 µM each dNTP
0.02 U Thermostable polymerase (refer to manufacturer's instructions; may need increasing depending on brand)
1.5 mM $MgCl_2$
1 µM each primer (see Table 1)
Sterile distilled water to make up the volume to a total of 24 µL.

Primer Set	Sequence (5' - 3')	Target Fragment
146F1 146R1	5'-ACTACTAACTTCAGCCTATCTAG-3' 5'-TAATGCGGGTGTAATGTTCTTACGA-3'	WSSV 1447 bp (primary PCR product)
146F2 146R2	5'-GTAACTGCCCCTTCCATCTCCA-3' 5'-TACGGCAGCTGCTGCACCTTGT-3'	WSSV 941 bp (nested PCR product)
143F	5'-TGCCTTATCAGCTNTCGATTGTAG-3'	Decapod 848 bp (control for DNA integrity)
145R	5'-TTCAGNTTTGCAACCATACTTCCC-3'	

Table 1 Sequence of primers used in WSSV PCR and in decapod PCR. Primer sets 146F1/146R1 and 146F2/146R2 target WSSV DNA. Primers 143F and 145R target decapod DNA.

Standard PCR protocol for WSSV and decapod assays

Each sample must undergo testing by the decapod PCR to determine if that sample is suitable for testing by the WSSV PCR. The total nucleic acid (TNA) sample extracted for analysis of WSSV is also used for the analysis of decapod DNA, as a quality control to confirm the integrity of the DNA in each sample.

1. Add 24 µL of Master Mix, prepared according to instructions above and containing the appropriate primer set (see Table 1) to a 0.2 mL thin-walled PCR tube. Add 1 µL of the TNA for the decapod and WSSV primary PCRs, and 0.5 µL of the primary PCR product for the nested WSSV PCR, to the tube. Briefly spin to draw all reagents to the bottom of the tube, and proceed with amplification.

2. PCR amplification for the 1447 bp WSSV fragment is as follows: 95°C for 1 min, then 40 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 90 s, then 1 cycle of 72°C for 10 min, then hold at 14°C. PCR amplification for the 941 bp WSSV fragment and the 848 bp decapod fragment is as follows: 95°C for 1 min, then 40 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 1 min, then 1 cycle of 72°C for 7 min, then hold at 14°C.

3. After thermal cycling, 8-10 µL of the PCR reaction mix is removed, mixed with loading dye, and loaded onto a 1-2% agarose gel and electrophoresed at 100 volts for 45-60 min. Ethidium bromide must be added to the

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molten agarose before the gel is poured, or the gel can be soaked in ethidium bromide following electrophoresis using standard procedures. PCR products are visualised using a UV transilluminator.

The positive control for the WSSV PCRs (primary and nested) should be a plasmid clone of the WSSV 1447 bp fragment, or some other known positive sample. A second positive control consisting of 1 µL of positive control PCR product from the WSSV primary PCR should be included. The positive control for the decapod PCR should be DNA from decapod tissue. A negative control should be included with each PCR assay and should consist of 24 µL of the Master Mix with 1 µL of sterile water instead of DNA.

Appendix 19

in situ Hybridisation Method

Riboprobe Preparation

Precautions need to be taken when handling RNA to avoid contaminants. It is recommended that gloves, filter tips and RNase-free solutions and consumables be used, along with strict asepsis.

1. Perform the nested WSSV PCR as described above on a sample known to contain WSSV, or use M13 primers if using a plasmid that contains WSSV sequence.
2. Use 4 µL of WSSV PCR product made up to 9.5 µL with sterile Milli-Q water in riboprobe reaction.
3. Add reagents from Promega's Riboprobe Synthesis Kit (Catalogue No. P1460), but use Roche's RNA Labeling Mix containing DIG-11-UTP (Catalogue No. 1277073). Reagents should be added at ambient temperature to avoid precipitation.

9.5 µL	Resuspended WSSV DNA
4 µL	5x Transcription Buffer
2 µL	100 mM DTT
3 µL	RNA Labeling Mix
1 µL	T7 or SP6 RNA Polymerase
0.5 µL	RNAsin (or 20 units)

4. Incubate at 37°C for 2 h, and then stop reaction by addition of 2 µL of 0.2 M EDTA (pH 8.0).
5. Precipitate labeled RNA with 2.5 µL of 4 M LiCl and 75 µL of absolute ethanol, and incubate at -80°C for at least 30 min.
6. Centrifuge at 10,000 *g* for 20 min.
7. Wash pellet with 70% ethanol; dry and resuspend in 100 µL sterile water or TE Buffer.
8. Add 1 µL RNAsin and store in 5 µL amounts at -20°C or -80°C.
9. Run 10 µL on a 1.5% agarose gel next to a quantifying ladder (Hyperladder IV, Bioline) to check that the probe has been labeled and to estimate the quantity of the probe. Use standard methods of ethidium bromide staining and UV transillumination to visualise. Labeled RNA should be approximately 100 bp larger than that of the template PCR product used. Adjust the concentration of the probe to approximately 100 ng/µL.

Section Preparation

1. Section paraffin-embedded tissues at 5 µm thickness onto silanated glass slides (see Appendix 3) and allow to dry.
2. Heat sections to 65-75°C in a hot oven, and deparaffinise in 2 stages of xylene (10 min each if slides are not pre-heated; 5 min each if slides are heated in oven).
3. Rehydrate by placing slides in each of absolute and 70% ethanol for 5 min and then distilled water.
4. Apply 300 µL of protease VII enzyme working solution (see Appendix 4) to each section and incubate in a humidified chamber at 37°C for 15-20 min.
5. Wash slides in distilled water, soak in absolute ethanol for 2 min, and allow to air dry.

Hybridisation Procedure

6. Just before use, denature the stock solution of salmon sperm DNA at 10 mg/mL by boiling in a water bath or heating block for 5 min.
7. Make 100 µL of hybridisation solution for each section as follows:

0.5 µL riboprobe (approximately 50 ng/µL)

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70 µL hybridisation mixture (see Appendix 5)
4 µL salmon sperm DNA at 10 mg/mL freshly denatured (see Appendix 6)
25.5 µL TE8 buffer (see Appendix 7)
8. Mix, then boil hybridisation solution for 10 min to denature probe, and place immediately on ice until ready for use.
9. Apply 100 µL hybridisation solution to each section, coverslip, and apply rubber bands as stoppers at each end of the slide, to prevent the coverslip from moving during heating. Place over boiling water in a steamer for at least 8 min for denaturation of nucleic acid in the section.
10. Quickly place slides in a humidified (sealed) chamber pre-warmed to 50°C and moistened with 4X SSC (see Appendix 12), and place at 50°C in an oven for overnight hybridisation. Avoid cooling of the slides during the transfer to the oven, as this increases background staining.

Post-Hybridisation Washing and Blocking

Perform all washes in a suitable vessel on a very slow moving rocker or shaker.
11. Remove rubber bands from slides and wash off coverslips gently with a washer bottle containing 4X SSC. Wash slides in 4X SSC for 2 x 5 min washes.
12. Wash slides in 1X SSC (see Appendix 12) for 2 x 5 min washes.
13. Wash slides in 0.1X SSC (see Appendix 12) heated to approximately 80°C, for 15 min.
14. Apply freshly made Blocking Solution (see Appendix 8) at ambient temperature in a humidified chamber for 30 min.
15. Apply 200 - 300 µL per section of sheep anti-DIG antibody conjugated to alkaline phosphatase (Roche Catalogue No. 1093274), diluted 1:500 in the Blocking Solution above. Incubate at ambient temperature in a humidified chamber for 30 min.
16. Wash in Buffer 1 (see Appendix 9) for 2 x 5 min.
17. Equilibrate in Buffer 3 (see Appendix 10) for 2 min at ambient temperature.

Colour Development and Staining

18. Apply NBT/BCIP colour development solution (Roche Catalogue No. 168451) made up according to manufacturer's instructions, in darkness for no more than 1.5 h. The slides should be completely covered and not allowed to dry out. Monitor the colour development using a light microscope if available.
19. Stop reaction by rinsing in Milli-Q water.
20. Wash in 2 changes of Milli-Q water for 2 min.
21. Counterstain with filtered 0.5% aqueous Bismark Brown for 60 s. Rinse with Milli-Q water.
22. Immerse in acetone for 20 s to remove non-specific staining.
23. Dehydrate by immersing in 2 changes of propanol, then 2 changes of xylene, and mount immediately in a permanent mounting medium.
24. Each section is examined by light microscopy for the presence of hybridised blue-black probe, indicating the presence of viral DNA, against a light brown background of counterstained tissue.

A positive control slide consisting of a section of known WSSV-infected tissue should be included. Several negative control slides should be included to test that the probe is specific for WSSV. A control slide with uninfected prawn tissue on it should be included, with the WSSV probe applied as for the test slides. A known WSSV positive slide should also be included with a non-WSSV probe, to test for non-specific reactions. If it is thought that the probe is reacting with endogenous alkaline phosphatase, a negative control consisting of the same tissue and following the same procedure, except no probe in the hybridisation mixture, should be included.

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