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Enterococcus-like infections in *Macrobrachium rosenbergii* are exacerbated by high pH and temperature but reduced by low salinity

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ABSTRACT: *Macrobrachium rosenbergii* (10 to 15 g and 8 to 12 g at intermolt) were challenged with an *Enterococcus*-like bacterium (strain KM002) previously isolated and identified as the causal agent of mortality. Challenge doses and conditions of pH, salinity and temperature were varied to determine the influence of environmental factors on the development of disease and mortality. Survival was 100% for the unchallenged control groups in all trials. In pH tests, the onset of mortality was earlier at pH 8.8 to 9.5 than at pH 4.6 to 5.2 and 7.5 to 7.7. Also, at pH 8.8 to 9.5, all challenged prawns died within 6 d in high dose challenge tests. By contrast, 20% of the prawns challenged at pH 4.6 to 5.2 and 7.5 to 7.7 survived. At low dose challenge (5×10^4 cfu prawn^{-1}), survival increased significantly except at pH 8.8 to 9.5. In salinity tests at 2 challenge doses (1×10^6 and 2×10^7 cfu prawn^{-1}), onset of mortality was earliest at 15 ppt and cumulative mortality was 100% at 15 ppt and 0 ppt. By contrast, survival was 80% at 5 and 10 ppt at the low dose challenge and 40% and 60%, respectively, at the high dose challenge. When the challenge dose was reduced to 5×10^4 cfu prawn^{-1}, survival was not significantly different at different salinity levels. In temperature tests at pH 7.2 to 7.5 and at 2 challenge doses (2×10^7 and 4×10^7 cfu prawn^{-1}), the onset of mortality was earliest at 33 to 34°C and total mortality occurred at 27 to 28°C and 33 to 34°C. By contrast, there were 40% and 20% survivors, respectively, for low and high challenge doses at 30 to 31°C. Reducing the challenge dose to 5×10^4 cfu prawn^{-1} gave higher survival in all groups. However, survival at 33 to 34°C was still lowest. In similar temperature tests but at pH 8.8 to 9.5, onset of mortality was somewhat accelerated and there was 100% death for all the high challenge groups. At low challenge doses, mortality was lower but still highest in the 33 to 34°C group. Results indicated that mortality of *M. rosenbergii* caused by this *Enterococcus*-like bacterium was exacerbated by environmental parameters of temperature and pH different from those known to be optimal for prawn growth. By contrast, low salinity appeared to have a beneficial effect on survival. Further work is needed to determine the mechanisms underlying these effects.

KEY WORDS: *Macrobrachium rosenbergii* · *Enterococcus* · pH · Temperature · Salinity · Defense ability

INTRODUCTION

The giant freshwater prawn *Macrobrachium rosenbergii* is a primarily inland decapod crustacean that is commercially cultured all over the world (New 1995). In Taiwan, culture of *M. rosenbergii* has been intensified, and the farmed production increased from 1315 tons in 1984 to 16196 tons in 1991 (New 1995). However, production of the prawns has declined gradually since that year due to disease outbreaks caused by a yeast in the cool season (Shu 1993) and by an *Enterococcus*-like bacterium in the hot season (Cheng & Chen 1998). Thus, production in 1996 was 7354 tons (Taiwan Fisheries Bureau 1997).

In nature, the physiology of poikilotherms depends on a number of factors acting in synergy. *Macrobrachium rosenbergii* can tolerate a wide range of salinities (0 to 25 ppt) and a wide range of temperatures (14 to 35°C). For growth, the optimal temperature is 29 to 31°C and the optimal pH is 7.0 to 8.5 (New 1995). *M. rosenbergii* inhabits freshwater but the larval and post larval phases are spent in brackish water. Thus, the degree of tolerance towards environmental factors may differ according to phase.

It is recognized that temperature changes, handling, and poor water quality may affect fish health by suppressing the immune system and increasing vulnera-

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bility to invading pathogens (Wedemeyer 1970, Snieszko 1974, Anderson et al. 1984, Zeeman 1986). Physiological stress has also been proposed to cause a diminution in invertebrate defense mechanisms (Cheng & Combes 1990). For example, the activity of granular hemocytes of the American oyster *Crassostrea virginica* differs with salinity level (Fisher & Newell 1986). It has also been reported that tidally related variations affect the immunocompetence of several bivalve species (Conway 1987, Hawkins et al. 1993). Stewart & Arie (1973) reported that salinity has a direct effect on the development of gaffkemia *Aerococcus viridans* in the American lobster *Homarus americanus*. Bray et al. (1994) reported that there was a relationship between infectious hypodermal and hematopoietic necrosis (IHHN) and salinity level in *Penaeus vannamei* and that the growth of IHHN-positive populations was lower at high salinity. Others have also reported effects of environmental variables on the activity of immune parameters of crustaceans (Truscott & White 1990, Hauton et al. 1995).

Epizootics of *Enterococcus*-like muscle necrosis of *Macrobrachium rosenbergii* occur only during the summer season in Taiwan, and especially during phytoplankton blooms (Cheng & Chen 1998). These observations suggested that temperature and pH might be important factors in the disease outbreaks. The purpose of the present study was to determine whether pH, salinity and temperature levels could affect the outcome of experimental infections of the *Enterococcus*-like bacterium (strain KM002) in *M. rosenbergii*.

MATERIALS AND METHODS

The bacterial strain (KM002) used in this study was isolated from diseased *Macrobrachium rosenbergii*, where it had been shown to cause opaque and whitish musculature in experimental infections (Cheng & Chen 1998). Stocks were cultured on tryptic soy agar (TSA, Difco) for 24 h at 28°C before being transferred to tryptic soy broth (TSB, Difco) for 24 h at 28°C. The broth cultures were then centrifuged at 7155 × *g* for 15 min at 4°C. The supernatant was removed and the bacterial pellet was re-suspended in saline solution (0.85% NaCl) at 10^9 cfu ml^{-1} as the stock bacterial suspension for injection challenges.

Macrobrachium rosenbergii (10 to 15 g and 8 to 12 g in the intermolt stage) was obtained from 3 commercial farms at Pingtung, Taiwan, and acclimated in the laboratory for 1 wk prior to experimentation. High dose (1×10^6, 2×10^7 and 4×10^7 cfu) challenge tests were conducted twice with test and control groups comprising 5 prawns each. A low dose (5×10^4 cfu) challenge test was conducted in triplicate with test and control groups comprising 10 prawns for each replicate. For experimental infections, 20 µl and 10 µl of bacterial suspension in high dose and low dose challenge tests, respectively, was injected into the ventral cephalothoracic sinus of each prawn. Suspensions were adjusted to give bacterial doses per prawn of 5×10^4, 1×10^6, 2×10^7 and 4×10^7 cfu. Prawns injected with equal volumes of sterile saline solution served as controls.

After injection, prawns were kept in 60 l glass aquaria (5 prawns each) containing 40 l water at various pH, salinity, and temperature levels. They were fed with a formulated prawn feed (Shinta Feed Company, Pingtung, Taiwan) twice daily and observed for up to 7 d. Water parameters (pH, temperature and salinity) were measured and adjusted 3 times daily. The number of moribund prawns and the time of mortality onset were recorded. Four different conditions were tested. They were: (1) different pH levels at 33 to 34°C and 0 ppt, (2) different salinity levels at 33 to 34°C and pH 7.5 to 7.8, (3) different temperature levels at pH 7.2 to 7.5 and 0 ppt, and (4) different temperature levels at pH 8.8 to 9.5 and 0 ppt. A multiple comparison (Tukey) test was conducted to compare the significant difference among treatments using SAS (Statistical Analysis System) computer software (SAS 1988). For statistically significant differences, it was required that $p < 0.05$.

For pH tests, water was adjusted to 4.6 to 5.2 and 8.8 to 9.5 using 1 N HCl or 1 N NaOH as appropriate but no additions were made to water at pH 7.5 to 7.7. Unchallenged control prawns were maintained at pH 8.8 to 9.5. For salinity tests, freshwater was adjusted to 5, 10, and 15 ppt using seawater (35 ppt) and unchallenged control prawns were maintained in freshwater at 33 to 34°C. For temperature tests, water was held at 27 to 28°C, 30 to 31°C and 33 to 34°C and adjusted to either pH 7.2 to 7.5 or 8.8 to 9.5. Unchallenged control prawns were maintained at 33 to 34°C and at pH 7.2 to 7.5 or 8.8 to 9.5.

RESULTS

Results from *Enterococcus*-like challenge tests under various rearing conditions are shown in Tables 1 to 4. As expected, the onset of prawn mortality was earlier with higher challenge doses. In contrast to saline-injected controls, all challenged prawns ceased feeding after injection.

In the pH tests (Table 1) at a challenge dose of 2×10^7 cfu $prawn^{-1}$ (33 to 34°C, 0 ppt), onset of mortality occurred earliest at pH 8.8 to 9.5 (16 h), next at pH 4.6 to 5.2 (24 h) and then at pH 7.5 to 7.7 (48 h). Cumulative mortality was 100%, 80% and 80%, respectively, over

168 h of observation. Doubling the challenge dose accelerated the onset of mortality. Reducing the challenge dose to 5×10^4 cfu delayed the onset of mortality (16 h for the pH 8.8 to 9.5 group, 48 h for the 7.5 to 7.7 group, and 24 h for the 4.6 to 5.2 group). Cumulative mortality at pH 8.8 to 9.5 (93.3%) was higher than at pH 4.6 to 5.2 (26.7%) and at pH 7.5 to 7.7 (13.3%). There was no mortality in the non-challenged control groups.

In salinity tests (Table 2) at a challenge dose of 2×10^7 cfu prawn^{-1} (pH 7.5 to 7.8, temperature 33 to 34°C), onset of mortality was earliest at 15 ppt (8 h) and the same (24 h) for 0, 5 and 10 ppt. Cumulative mortality was 80% (0 ppt), 60% (5 ppt), 40% (10 ppt) and 100% (15 ppt) over 168 h. Reducing the challenge dose to 1×10^6 cfu delayed the onset of mortality (48 h for the 15 ppt group and 72 h for the others). In the low dose challenge test (5×10^4 cfu), cumulative mortality was not significantly different amongst different salinity levels. There was no mortality in the non-challenged control groups.

In the temperature tests at a challenge dose of 2×10^7 cfu prawn^{-1} (pH 7.2 to 7.5 and salinity 0 ppt) (Table 3),

Table 1. *Macrobrachium rosenbergii*. Susceptibility to *Enterococcus*-like KM002 at different pH levels and at 0 ppt and 33 to 34°C. In the third trial, values in the same column with different superscripts are significantly different ($p < 0.05$)

Bacterial dose (cfu prawn^{-1})	pH	Number dead after various times (h) 8	16	24	48	72	96	120	144	168	Cumulative mortality (%)
First trial											
Control	8.8–9.5										0/5 (0%)
2×10^7	4.6–5.2			1			1	2			4/5 (80%)
2×10^7	7.5–7.7				2	1		1			4/5 (80%)
2×10^7	8.8–9.5		1	1	2				1		5/5 (100%)
Second trial											
Control	8.8–9.5										0/5 (0%)
4×10^7	4.6–5.2		1	2	1						4/5 (80%)
4×10^7	7.5–7.7			1		2	1				4/5 (80%)
4×10^7	8.8–9.5	1	1	2	1						5/5 (100%)
Third trial											
Control	8.8–9.5										0/30 (0%)^a
5×10^4	4.6–5.2			3	1	1	1	1	1		8/30 (27%)^b
5×10^4	7.5–7.7				1	1	1	1			4/30 (13%)^b
5×10^4	8.8–9.5		3	6	6	13					28/30 (93%)^c

Table 2. *Macrobrachium rosenbergii*. Susceptibility to *Enterococcus*-like KM002 at different salinity levels and at pH 7.5 to 7.8 and 33 to 34°C. In the third trial, there were no significant differences in cumulative mortality amongst the groups ($p > 0.05$)

Bacterial dose (cfu prawn^{-1})	Salinity (ppt)	Number dead after various times (h) 8	16	24	48	72	96	120	144	168	Cumulative mortality (%)
First trial											
Control	0										0/5 (0%)
1×10^6	0					1	3	1			5/5 (100%)
1×10^6	5					1					1/5 (20%)
1×10^6	10					1					1/5 (20%)
1×10^6	15				1	1	2		1		5/5 (100%)
Second trial											
Control	0										0/5 (0%)
2×10^7	0			1	1	1		1			4/5 (80%)
2×10^7	5			1		2					3/5 (60%)
2×10^7	10			1	1						2/5 (40%)
2×10^7	15	1	2	1		1					5/5 (100%)
Third trial											
Control	0										0/30 (0%)^a
5×10^4	0				1		1				2/30 (7%)^a
5×10^4	5										0/30 (0%)^a
5×10^4	10		1								1/30 (3%)^a
5×10^4	15			1			1				2/30 (7%)^a

Table 3. *Macrobrachium rosenbergii*. Susceptibility to *Enterococcus*-like KM002 at different temperatures and at 0 ppt and pH 7.2 to 7.5. In the third trial, values in the same column with different superscripts are significantly different ($p < 0.05$)

Bacterial dose (cfu prawn^{-1})	Temp. (°C)	Number dead after various times (h) 8	16	24	48	72	96	120	144	168	Cumulative mortality (%)
First trial											
Control	33–34										0/5 (0%)
2×10^7	27–28			1		4					5/5 (100%)
2×10^7	30–31			2				1			3/5 (60%)
2×10^7	33–34		1	2	1		1				5/5 (100%)
Second trial											
Control	33–34										0/5 (0%)
4×10^7	27–28		4	1							5/5 (100%)
4×10^7	30–31		2	1	1						4/5 (80%)
4×10^7	33–34	3		1	1						5/5 (100%)
Third trial											
Control	33–34										0/30 (0%)^a
5×10^4	27–28			1				1			2/30 (7%)^a
5×10^4	30–31				2						2/30 (7%)^a
5×10^4	33–34		1	3	3	1					8/30 (27%)^b

Table 4. *Macrobrachium rosenbergii*. Susceptibility to *Enterococcus*-like KM002 at different temperatures and at 0 ppt and pH 8.8 to 9.5. In the third trial, values in the same column with different superscripts are significantly different ($p < 0.05$)

Bacterial dose (cfu prawn^{-1})	Temp. (°C)	Number dead after various times (h) 8	16	24	48	72	96	120	144	168	Cumulative mortality (%)
First trial											
Control	33–34										0/5 (0%)
2×10^7	27–28			1	3	1					5/5 (100%)
2×10^7	30–31		1	2	2						5/5 (100%)
2×10^7	33–34		2	2	1						5/5 (100%)
Second trial											
Control	33–34										0/5 (0%)
4×10^7	27–28			2	1	2					5/5 (100%)
4×10^7	30–31	1	1	2	1						5/5 (100%)
4×10^7	33–34	2	2	1							5/5 (100%)
Third trial											
Control	33–34										0/30 (0%)^a
5×10^4	27–28				2	1	4	1			8/30 (27%)^b
5×10^4	30–31			1	4	4	2		1		12/30 (40%)^b
5×10^4	33-34		5	2	5	11	2	1	2		28/30 (93%)^c

the onset of mortality occurred first at 33 to 34°C (16 h) followed by the other treatments (27 to 28°C and 30 to 31°C) at 24 h. Cumulative mortality was 100% (33 to 34°C), 60% (30 to 31°C) and 100% (27 to 28°C). Doubling the challenge dose accelerated the onset of mortality by 8 h for each group. Reducing the challenge dose to 5×10^4 cfu delayed the onset of mortality (16 h for the 33 to 34°C group, 48 h for the 30 to 31°C group and 24 h for the 27 to 28°C group). Cumulative mortality at 33 to 34°C (26.7%) was higher than at 27 to 28°C and 30 to 31°C (6.7%). Again, there was no mortality in the non-challenged control groups.

In similar temperature tests also at 3 challenge levels but at pH 8.8 to 9.5 (Table 4), the trends for onset of mortality were similar, but cumulative mortality was 100% for the high dose (2×10^7 and 4×10^7 cfu) challenge groups. At low dose challenge (5×10^4 cfu), cumulative mortality at 33 to 34°C (93.3%) was higher than at 27 to 28°C (26.7%) and at 30 to 31°C (40%). There was no mortality in the control groups.

DISCUSSION

The optimum conditions for rearing juvenile to adult *Macrobrachium rosenbergii* are salinity 0 to 10 ppt, pH 7.0 to 8.5 and temperature 29 to 31°C (New 1995). The present study has shown that, except for salinity, con-

ditions diverging from these values accelerated the time of mortality onset and increased the extent of mortality upon challenge with an *Enterococcus*-like pathogen. The combination of high pH (8.8 to 9.5) and temperature (33 to 34°C) was particularly bad for the prawns and this supported our contention that high summer temperatures combined with phytoplankton blooms may precipitate infections on prawn farms. Although it is not feasible for prawn farmers to control the water temperature, it may be possible for them to manage the pH of the pond water, either by chemical addition or water exchange.

In nature, *Macrobrachium rosenbergii* inhabits a wide range of environmental salinities during its life cycle (0 to 18 ppt). During the reproductive season, adults migrate from freshwater habitats to estuarine regions where the eggs hatch and where the larval development occurs (Nelson et al. 1977). The isosmotic point of *M. rosenbergii* is 18 ppt (Sandifer et al. 1975). In the salinity tests, prawns which were challenged with the *Enterococcus*-like bacterium and reared at salinities of 0, 5, 10 and 15 ppt were hyperosmotic to their ambient environments. Hyperosmoregulation in dilute media relies on the ability to absorb ions against a concentration gradient.

In the present study, virulence of KM002 to *Macrobrachium rosenbergii* adults was higher at 0 ppt than at 5 and 10 ppt and highest at 15 ppt. It was surprising to find that salinity at 5 and 10 ppt had some beneficial effects in increasing the time to disease onset and prawn survival upon bacterial challenge. The reason for this is presently unclear. It may relate simply to the osmolarity of the rearing water or to the concentration of one or more of the ionic constituents of seawater. In this regard, it has been reported that magnesium salts can be beneficial for the treatment of fungal diseases in crayfish *Astacus astacus* (Rantamaeki et al. 1992). An imbalance in divalent cations such as Ca^{2+} and Mg^{2+} in the ambient water is important in the genesis of idiopathic muscle necrosis in *Penaeus aztecus* (Lakshmi et al. 1978). It has been reported that application of calcium oxide can be beneficial for the treatment of rickettsial disease in *M. rosenbergii* (Cohen & Issar 1990), and that application of sodium chloride can be beneficial for the treatment of crayfish plague fungus *Aphanomyces astaci* (Lilley & Inglis 1997). It is possible that appropriate addition of salt could be used as a preventative measure for the development of white disease caused by *Enterococcus*-like bacteria in prawn farms, although negative impacts on the freshwater environment may make it unfeasible.

The results showed that *Macrobrachium rosenbergii* was most susceptible to KM002 when reared at pH 8.8 to 9.5 and at 33 to 34°C. These conditions would be most likely to occur naturally during summer phytoplankton blooms. Therefore, maintaining a lower temperature (30 to 31°C) and a lower pH (pH 7.5 to 7.7) may have beneficial effects in avoiding disease outbreaks by *Enterococcus*-like bacteria.

It is generally considered that prevention of disease is better than therapy. Aquaculturists use disease information and data from challenge tests to decide whether immunization, therapy or environmental management is the most cost-effective method to control disease outbreaks (Anderson 1990). In terms of long-term costs, development of drug resistance due to bioaccumulation in aquatic organisms and the environment must be considered as a factor in the decision to use antibiotic therapy.

For crustaceans, Browdy et al. (1993) observed that there was a trend toward decreasing incidence of IHHN virus associated inclusion bodies with increased water exchange. Bray et al. (1994) reported that there is an apparent interaction between high salinity and IHHN virus infection in white shrimp *Penaeus vannamei*. Prayitno & Latchford (1995) observed that low salinity and high pH increased virulence of *Vibrio harveyi* to *Penaeus monodon*. These environmental parameters are known to affect the metabolism and defense mechanisms of decapod crustaceans (Claybrook 1983, Truscott & White 1990).

Several scientists have investigated the effects of environmental parameters on crustacean defense mechanisms. Dean & Vernberg (1966) reported that temperature affects hemolymph clotting time, hemocyte counts and serum protein concentration in the hermit crab *Uca pugilator*. Truscott & White (1990) found tide-associated rhythms in the total hemocyte count for freshly captured shore crab *Carcinus maenas*, with peak count occurring at high tide. Increased hemocyte numbers provide an enhanced immune capability during periods of high activity. Hauton et al. (1995) reported a significant negative correlation between phenoloxidase activity and tidal height in *C. maenas*, and this indicated cyclical changes in immunocompetence. An increased prevalence in the shell disease of marine decapod crustaceans has been reported to result from polluted environments, also suggesting a decrease in immunocompetence (Gopalan & Young 1975, Young & Pearce 1975). The present study has clearly shown that susceptibility of *M. rosenbergii* to *Enterococcus*-like (KM002) could be influenced by environmental conditions. Further research is needed to understand the mechanisms and to determine whether they are related to changes in the prawn immune system.

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