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Incidence of white muscle disease, a viral like disease associated with mortalities in hatchery-reared postlarvae of the giant freshwater prawn *Macrobrachium rosenbergii* (De Man) from the south-east coast of India

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Abstract

Incidence of post-larval mortalities of 30–100% was reported from commercial freshwater prawn, *Macrobrachium rosenbergii* (De Man) hatcheries in Andhra Pradesh and Tamil Nadu (south-eastern states of India) since 2001. Infected postlarvae (PL) exhibited clinical symptoms with lethargy, anorexia and whitening of abdominal muscles and the disease was identified as white muscle disease (WMD). The waterborne infection of WMD was induced in the laboratory by exposing uninfected and healthy *M. rosenbergii* PL to the filtered muscle homogenates of the naturally infected PL, resulting in mortality that reached 99% within 10 days post infection. Histopathological examination of the infected animals revealed highly necrotic musculature. Degenerated muscle areas showed aggregations of melanized nuclei, many of which looked like inclusion bodies. Bacteriological examination of affected PL showed the presence of *Staphylococcus* spp. as a predominant organism, while laboratory challenge of healthy PL with this bacterial isolate did not reproduce WMD.

Keywords: white muscle disease (WMD), mortality, *Macrobrachium rosenbergii*, postlarvae (PL), muscle opacity, *Staphylococcus*, India

Introduction

The giant freshwater prawn *Macrobrachium rosenbergii* (De Man) is a major commercially important crustacean farmed in the inland waters of India. *Macrobrachium rosenbergii* is often considered as a less susceptible species to disease problems when compared with farmed penaeid shrimp, perhaps because of the generally less intensified culture practices of freshwater prawn farming. A number of parasitic, bacterial and fungal agents have been known to cause infections and mortality in freshwater prawns. Mass mortalities up to 60% in 28-day-old *M. rosenbergii* postlarvae (PL) cultured under intensive conditions showing signs of a milky diffuse white body described as idiopathic muscle necrosis (IMN) was reported by Nash, Chinabut and Limsuwan (1987). Chen, Lin, Liaw and Wang (2001) described the association of a Gram-positive cocci as the causative agent of a white muscle disease in *M. rosenbergii*. Three viruses, one affecting the hepatopancreas (Anderson, Law, Shariff & Nash 1990) and the others affecting muscle tissue (Arcier, Herman, Lightner, Redman, Mari & Bonami 1999; Tung, Wang & Chen 1999; Bonami 2002), have been reported to date in *M. rosenbergii*. There were no reported incidences of any viral infection among the hatchery- or farm-reared *M. rosenbergii* from India, prior to the year 2001. Recently, disease problems have become of serious concern to giant freshwater prawn farming in India, probably because of its expansion, the intensification of culture and the translocation of seed and broodstock.

Since November 2001, *Macrobrachium* hatcheries situated on the south-east coast of India have been facing heavy losses because of an emerging disease,

commonly termed as white muscle disease (WMD). Affected PL showed characteristic whitening of abdominal musculature, associated with anorexia and lethargy. The moribund PL seriously affected with WMD appeared milky white, and the mortalities in hatcheries were reported to be 30–100%.

A 50% production loss in more than 50 freshwater prawn hatcheries situated in the affected states alone has caused an economic loss of about US$15 million annually. Further losses in grow-out farm production because of poor survival of the PL with a low-level asymptomatic infection could result in severe economic losses.

The clinical signs and histopathology of WMD closely resemble the IMN reported in *M. rosenbergii* (Nash *et al.* 1987) from Thailand, and the viral infection reported from Guadeloupe (French West Indies) by Arcier and colleagues (1999), as well as that from Taiwan by Tung and colleagues (1999). These observations suggest a viral aetiology for the WMD. While different types of viruses, such as parvoviridae, picornoviridae and nodaviridae have been described as the cause of the WMD, in the present scenario it is of utmost importance to identify the pathogen causing the disease.

Materials and methods

Source of specimens

Surveys were undertaken in the affected hatcheries during disease outbreaks from September 2001 to December 2002. Nine disease outbreaks in *M. rosenbergii* hatcheries were investigated during this period from the states of Andhra Pradesh (AP) and Tamil Nadu (TN) on the south-east coast of India. Diseased PL were collected from freshwater prawn hatcheries during December 2002, and fresh samples of infected PL were brought frozen to the Central Institute of Brackishwater Aquaculture (CIBA) laboratory, Chennai, India and stored at − 80 °C until further use. Healthy PL for infectivity studies were obtained from a hatchery with no previous record of incidence of WMD. The PL transported to the laboratory by oxygen packing were maintained in 500-L fibreglass tanks with aerated freshwater at ambient temperature (28–30 °C), and fed with *Artemia* nauplii.

Infectivity studies

The samples used for the infectivity studies comprised of a batch of naturally infected moribund PL *M. rosenbergii* (10–15-mg body weight), having abdomens of milky white appearance, collected from one of the hatcheries during a disease outbreak in December 2002, and stored at − 80 °C. An infection trial was performed using the inoculum prepared from the infected PL.

The inoculum was prepared by the filtration of 2-g homogenized PL in 20 mL of TNE buffer (50-mM Tris-HCl, 100-mM NaCl and 1-mM EDTA, pH 7.4). In order to prepare the viral extract, the homogenate was centrifuged at 10 000 rpm (6050g) for 10 min at 4 °C and the resultant supernatant was filtered through a 450-nm Sartorius syringe filter (Sartorius, Goettingen, Germany). The filtrate obtained was diluted 10-fold with sterilized distilled water, following which further dilution to 500 times in freshwater was made to create the waterborne inoculum. Three replicates of 100-PL each (10–15-mg body weight) were immersed in this diluted filtrate for 2 h. Two other populations, which were similarly exposed to the muscle filtrate of healthy *M. rosenbergii* served as controls. After immersion, prawns were kept in aerated glass aquaria and fed with *Artemia* nauplii. The water temperature was 28–30 °C during the experiment. Mortalities were examined daily and 10 moribund PL were sampled for histology from the infected group. Similarly, samples were taken from the controls during the course of experiment. The experiment was terminated after 10 days.

Histopathology

Whole PL of naturally diseased prawns with milky white abdominal muscle, moribund PL from the infectivity study, and from the controls were fixed in Davidson's fixative for 24 h and then transferred to 70% propanol for subsequent histological preparation (Bell & Lightner 1988). Sections of 5–6 μm in thickness were stained with haematoxylin and eosin. Photomicrographs were taken using a WILD MPS 46 microcamera (Leica, Wetzler, Germany) fitted to a Leitz Laborlax S microscope (Leica).

Microbiology and infectivity studies

Samples of healthy and WMD-affected moribund PL of *M. rosenbergii* from four hatcheries brought to the laboratory under oxygen packing in plastic bags were analysed. Postlarvae from each group were washed three times in sterile saline and briefly with 70% ethanol. These PL were collected in sterile Eppendorf tubes (Tarsons, Kolkota, India), homogenized in

sterile PBS, inoculated onto Tryptose Soya Agar (TSA, HiMedia Labs, Mumbai, India) and incubated at 37 °C for 24–48 h. Bacterial colonies were purified on TSA and subjected to detailed physiological and biochemical tests (Smibert & Krieg 1991) and identified (Holt, Krieg, Sneath, Staley & Williams 1994).

Infectivity studies of the predominant bacterial isolate, *Staphylococcus* spp. were carried out by exposing healthy freshwater prawn PL with an inoculum of 10^6 and 10^8 bacterial cells mL^{-1} (final concentration in the experimental tanks). A total of 100 PL were used for these challenge studies in 11 containers. The experiment was conducted in triplicate, with two controls.

Results

Survey results and clinical signs of WMD

The epizootic of WMD in *M. rosenbergii* in hatchery-reared PL was first observed in November 2001 and was limited to a few hatcheries in AP and TN states. More than 18 cases of WMD in freshwater prawn hatcheries with PL mortalities ranging from 30% to 100% were recorded from November 2001 to December 2002. The first sign related to this disease was the poor feeding and lethargy of the prawns especially during the first 5 days of PL settlement resulting in slow mortality. Few whitish PL with areas of non-transparent abdominal muscles were noticed 2–5 days after metamorphosis. These whitish PL were observed to be cannibalized by healthy PL. Initially, the whitish colour was apparent only against a dark background. Later abdominal muscle opacity increased and the affected PL became milky white in appearance (Fig. 1), followed by mortality. Mortality increased day by day and reached up to 100% within 5–10 days after the appearance of first milky white PL. Since November 2001, the incidence of WMD in freshwater prawns has been recorded throughout the year in the states of AP and TN.

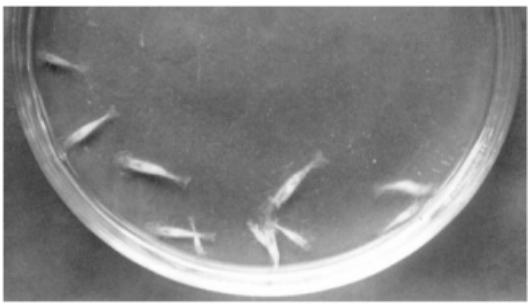

Figure 1 *Macrobrachium rosenbergii* postlarvae affected with white muscle disease showing opaque whitish appearance of the abdomen.

Infectivity studies

In the laboratory challenge using the inoculum prepared from WMD-infected PL, the experimentally infected PL started dying from day 2-post infection (p.i.). The cumulative mortality of the *M. rosenbergii* PL exposed to waterborne infection reached up to 99% on the 10th day of p.i. (Table 1). Mortality recorded among the control groups was $\leq$ 3%, and it was apparently not because of WMD, but because of moulting and related cannibalism. The artificially infected prawns were anorexic, lethargic, firstly developing opaqueness in the abdomen and later turning to a milky white appearance as has been observed in natural infection.

Histopathology and microbiology

Histopathological observation of the naturally infected muscle tissue showed severe necrosis as-

Table 1 Survival of healthy *Macrobrachium rosenbergii* postlarvae (PL) challenged by isolates prepared from prawns infected with white muscle disease

Experimental groups (PL)	Days of post infection										Cumulative mortality (%)
	1	2	3	4	5	6	7	8	9	10	
Tank 1 ($n = 100$)	0	0	2	0	0	7	13	16	47	8	93
Tank 2 ($n = 100$)	0	0	0	0	1	6	10	19	63	0	99
Tank 3 ($n = 100$)	0	2	0	1	0	3	18	21	54	0	99
Control 1 ($n = 100$)	0	1	0	0	0	0	2	0	0	0	3
Control 2 ($n = 100$)	0	0	0	1	0	0	0	0	1	0	2

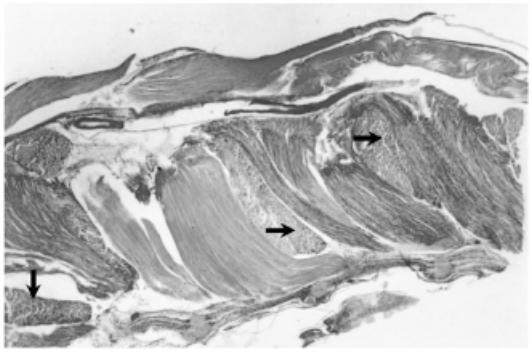

Figure 2 *Macrobrachium rosenbergii* postlarvae longitudinal section (L.S.); necrotized striated muscles (arrow) along with haeamocytic infiltration (arrow head) Haematoxylin and eosin stain, × 100.

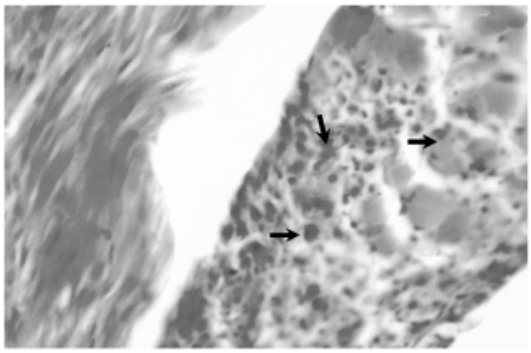

Figure 3 *Macrobrachium rosenbergii* postlarvae L.S.; higher magnification of the necrotic striated muscle showing complete degenerated muscle fibres (arrow) with fragmentation and flocculation. Haematoxylin and eosin stain, × 400.

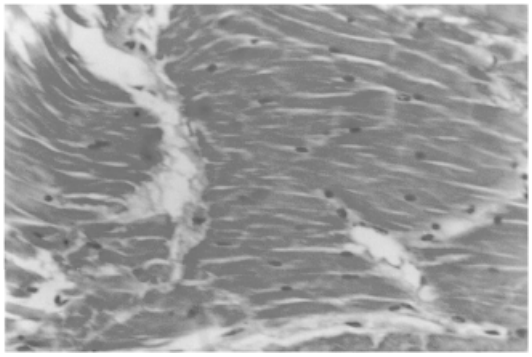

Figure 4 *Macrobrachium rosenbergii* postlarvae L.S.; abdominal muscle tissue of uninfected control. Haematoxylin and eosin stain, × 400.

sociated with conditions such as fragmentation, flocculation, granulation and heavy haemocytic infiltration (Figs 2 and 3). Histopathology of the artificially infected PL was similar to the natural infection of WMD. Uninfected PL showed normal muscle organization (Fig. 4).

Twenty-three bacterial isolates, 13 from infected moribund PL samples and 10 from healthy PL were recovered. *Staphylococcus* spp. was found to be predominant in the WMD-affected PL, being recovered from all the four samples screened, while *Staphylococcus* spp. was recovered as scanty growth on primary isolation medium from healthy PL samples. Members of the family Enterobacteriaceae and *Bacillus* spp. were also recovered from both groups of PL samples (Table 2). Laboratory challenges with the dominant isolate of *Staphylococcus* spp. did not produce WMD, suggesting that it was a secondary bacterial infection (Table 3).

Discussion

Compared with penaeids, where more than 14 viral pathogens have been recorded so far (Fulks & Main 1992; Bower, McGladdery & Price 1994; Lightner 1996), only three diseases with viral aetiology have been recorded in *M. rosenbergii* (Anderson *et al.* 1990; Arcier *et al.* 1999; Tung *et al.* 1999; Bonami 2002). Though a fourth virus, white spot syndrome virus, has been reported in *M. rosenbergii* (Peng, Lo, Ho, Chang & Kou 1998), generally it does not cause any immediate mortality; instead, the prawn is primarily a reservoir host for the virus (Rajendran, Vijayan, Santiago & Krol 1999). However, since 2001 the WMD described here is emerging as a major epizootic in Indian prawn hatcheries causing recurrent mortalities reaching 100% in a short span of time.

Morphological and microbiological examination as well as the infectivity studies in the present study revealed no parasitic, fungal or bacterial pathogens. Infectivity studies using bacterial isolates in the present case did not produce typical WMD in *M. rosenbergii,* in contradiction to the experience of Chen and colleagues (2001). As the morphological and clinical signs of the disease reported by Chen and colleagues (2001) were similar to the WMD recorded in this study, it is likely that the involvement of the Gram-positive cocci, *Lactococcus garvieae* in WMD found by those workers could possibly have been a secondary infection. In our studies, *Staphylococcus* spp. isolates did not produce WMD, and *L. garvieae* could not be isolated from the PL samples analysed. The viral infection reported by Anderson and colleagues (1990), infected only hepatopancreas and was non-pathogenic.

The morphological, clinical and histopathological observations in the present study showed close resemblance with the *Macrobrachium* muscle virus reported by Tung and colleagues (1999) and the nature and spread of infection suggest the involvement of a viral pathogen. Laboratory bioassay using infected tissue showed that the disease could be reproduced in uninfected *M. rosenbergii* by the immersion in an aqueous inoculum prepared from affected muscle tissue. The disease progressively destroyed the abdominal muscular organization of the prawns, especially the striated muscles, finally leading to mortality.

Epizootiological surveys revealed that the WMD has been spreading rapidly, leading to mass mortal-

Table 2 Biochemical characteristics of bacteria isolated from white muscle disease affected and clinically unaffected *Macrobrachium rosenbergii* postlarvae (PL)

	Isolates from infected PL													Isolates from healthy PL									
Characteristic	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**
Gram reaction	GPB	GPC	GPC	GPC	GNB	GPC	GNB	GPC	GPC	GNB	GPB	GPC	GPC	GPB	GPB	GPC	GNB	GNB	GPB	GPC	GNB	GNB	GNB
Morphology																							
Cocci	–	+	+	+	–	+	–	+	+	–	–	+	+	–	–	+	–	–	–	+	–	–	–
Coccobacilli	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Bacillus	+	–	–	–	+	–	+	–	–	+	+	–	–	+	+	–	+	+	+	–	+	+	+
Arrangement																							
Chains	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Pairs	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	+	–	–	–	–	–	–	–
Clusters	–	+	+	+	–	–	–	+	+	–	–	+	+	–	–	+	–	–	–	+	–	–	–
Acid from glucose	–	+	+	+	–	–	+	+	–	+	+	–	+	+	+	–	+	–	+	–	–	–	–
Gas from glucose	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Catalase	+	+	+	–	–	+	+	+	+	–	+	+	+	+	+	+	+	–	+	+	+	+	+
Oxidase	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	+	–v
Growth in 0% NaCl	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+	+
Growth in 6.5% NaCl	+	+	–	+w	–	+	–	+	+	–	+	+	+	+	–	–	–	–	–	–	+	–	–
Growth in 7.5% NaCl	+	+	–	–	–	+	–	+	+	–	–	+	+	–	–	–	–	–	–	–	+	–	+
Motility	+	–	–	–	+	–	+	–	–	–	+	–	–	+	+	–	–	+	+	–	–	+	–
Nitrate Reduction	–	+	+	+	+	+	+	+	+	+	+	–	+	–	+	–	+	+	+	–	+	+	+
Growth at 45 °C	–	–	+	+w	–	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Growth at 10 °C	–	–	–	+w	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Organism identified as	*Bacillus* spp.	*Staphylococcus* spp.	*Staphylococcus* spp.	*Enterococcus* spp.	Enterobacteriaceae	*Staphylococcus* spp.	Enterobacteriaceae	*Staphylococcus* spp.	*Staphylococcus* spp.	Enterobacteriaceae	*Bacillus* spp.	*Staphylococcus* spp.	*Staphylococcus* spp.	*Bacillus* spp.	*Bacillus* spp.	*Staphylococcus* spp.	*Vibrio* spp.	Enterobacteriaceae	*Bacillus* spp.	*Staphylococcus* spp.	Enterobacteriaceae	*Vibrio* spp.	Enterobacteriaceae

GPB, gram positive bacilli, GPC, gram positive cocci; GNB, gram negative bacilli.

Table 3 Laboratory challenge of healthy prawn postlarvae with *Staphylococcus* spp. isolated from prawns infected with white muscle disease

	Percentage cumulative mortality (days post infection)																			
Bacteria	**10^6 cells mL^{-1} in tank**										**10^8 cells mL^{-1} in tank**									
Days after exposure	1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9	10
Macrobrachium rosenbergii postlarvae ($n = 100$) challenged	2	6	7	7	8	8	8	11	11	11	3	5	8	8	9	9	9	11	11	11
M. rosenbergii postlarvae ($n = 100$) control	1	3	4	5	7	7	8	10	10	10	4	5	5	5	7	7	8	10	10	10

ities and the termination of production in many prawn hatcheries in India. The mode of transmission needs to be studied further, although it is known that the pathogen is spreading through the transport of infected PL and the movement of infected/carrier broodstock, etc. Control of WMD using therapeutic agents appears impossible because of the probable involvement of a viral pathogen; prevention through improvements in handling and transporting prawns is presently the only available option. More studies on the ultrastructural, molecular, geographical and diagnostic aspects of this emerging viral infection in giant freshwater prawns in India could provide valuable information, which would be useful in its control.

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