



## Studies on the occurrence of white tail disease (WTD) caused by *MrNV* and XSV in hatchery-reared post-larvae of *Penaeus indicus* and *P. monodon*

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### ABSTRACT

White tail disease (WTD) caused by *Macrobrachium rosenbergii* nodavirus (*MrNV*) and extra small viruses (XSV) is a major problem. It is responsible for severe mortality in post-larvae of *M. rosenbergii* in the hatcheries and nurseries. These viruses have a wide host range including marine shrimp. Recently, WTD has been observed in hatchery reared post-larvae of marine shrimp (*Penaeus monodon* and *P. indicus*). Clinical signs observed in these animals were found to be similar to those found in the post-larvae of *M. rosenbergii*. The infected post-larvae showed positive for *MrNV* and XSV by RT-PCR. The inoculum prepared from these infected post-larvae caused 100% mortality in the post-larvae of freshwater prawn.

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### 1. Introduction

The giant river prawn *Macrobrachium rosenbergii* is a common inhabitant of rivers and estuaries throughout the Indo-Pacific region and an economically important species because of its fast growth in tropical and subtropical regions. One of the major constraints limiting the prawn production all over the world is diseases. Generally, *M. rosenbergii* is considered to be a moderately disease-resistant species when compared to penaeid shrimp. A new viral disease, white tail disease (WTD) has been observed in freshwater prawn hatcheries and nursery ponds in different parts of India, causing high mortalities and huge economic losses (Sahul Hameed et al., 2004a). This disease was first reported in the French West Indies (Arcier et al., 1999), later in China (Qian et al., 2003), India (Sahul Hameed et al., 2004a), recently in Thailand (Yoganandhan et al., 2006) and very recently in Australia (Anonymous, 2008) and Taiwan (Wang et al., 2008). The occurrence of WTD is still being reported from all prawn growing countries. The causative agents of WTD have been identified as *M. rosenbergii* nodavirus (*MrNV*) (Arcier et al., 1999) and extra small virus (XSV) (Sri Widada and Bonami, 2004). *MrNV* is a small, icosahedral, non-enveloped virus 26–27 nm in diameter with the genome of two pieces of ssRNA (RNA1 with size of 2.9 and RNA2 of 1.26 kb) and its capsid contains a single polypeptide of 43 kDa. XSV is a virus-like particle,

icosahedral in shape and 15 nm in diameter, with a linear ssRNA (Qian et al., 2003). Sahul Hameed et al. (2004a) have reported the presence of XSV in addition to *MrNV* in WTD-infected post-larvae of freshwater prawns in India. Recently, Tang et al. (2007) have reported a nodavirus causing whitish muscle in *Penaeus vannamei* with 69% sequence similarity to the capsid gene of *MrNV* of *M. rosenbergii*.

The results of our previous study (Sudhakaran et al., 2006) on marine shrimp with *MrNV* and XSV indicated the possibility of transmitting pathologically significant organisms from native to non-native hosts. This can cause disease in non-native hosts during mixed culture with *M. rosenbergii* either accidentally or by close proximity of the culture environment to each other. Our observation of natural infection of WTD in *P. monodon* and *P. indicus* in one of the hatcheries, where the production of seed of marine shrimp and freshwater prawn was carried out in very close proximity, confirmed the transmission of *MrNV* and XSV from *M. rosenbergii* to marine shrimp. The present study reports the occurrence of natural infection of WTD in marine shrimp and the confirmation of WTD by RT-PCR using *MrNV* and XSV specific primers.

### 2. Materials and methods

#### 2.1. Collection of infected post-larvae

Infected post-larvae of *P. monodon* and *P. indicus* with prominent sign of whitish muscle in the abdominal and tail regions were collected

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from a hatchery located near Chennai, India. The PL were washed in sterile saline, transferred to sterile tubes, transported to the laboratory on dry ice and stored at  $-20^{\circ}\text{C}$  for further study. The physicochemical characteristics of the rearing water and percentage of mortality were determined at the time of sampling of infected animals. Temperature and pH were measured. Salinity was determined with a salinometer and dissolved oxygen by the Winkler method (Strickland and Parsons, 1972).

## 2.2. Total RNA extraction

For the extraction of total RNA, the infected PL (150 mg) were homogenized in 300  $\mu\text{l}$  of TN buffer (20 mM Tris-HCl, 0.4 M NaCl, pH 7.4). The homogenate was centrifuged at  $12,000 \times g$  for 15 min at  $4^{\circ}\text{C}$  and the supernatant was collected. The RNA was extracted using TRIzol reagent (GIBCO-BRL) according to manufacturer's protocol. Briefly, 1 ml of TRIzol reagent was thoroughly mixed with 200  $\mu\text{l}$  of crude tissue extract and incubated at room temperature for 5 min before addition of 0.2 ml of chloroform. The sample was vigorously shaken for 2–3 min at room temperature and then centrifuged at  $12,000 \times g$  for 15 min at  $4^{\circ}\text{C}$ . The RNA was precipitated from the aqueous phase with isopropanol, washed with 75% ethanol and dissolved in 50  $\mu\text{l}$  of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5). The amount of nucleic acid in the sample was quantified by measuring the absorbance at 260 nm. The purity was checked by measuring the ratio of  $\text{OD}_{260 \text{ nm}}/\text{OD}_{280 \text{ nm}}$ .

## 2.3. Reverse transcriptase-polymerase chain reaction (RT-PCR)

For detection of MrNV and XSV, the RT-PCR was carried out using the Reverse-IT™ 1-step RT-PCR kit (ABgene), allowing reverse transcription (RT) and amplification to be performed in a single reaction tube. The primers specific to MrNV RNA2 were designed from sequence data of the MrNV genome (GenBank Accession No. AY222840). The sequence of the primer is 5' GCG TTA TAG ATG GCA CAA GG 3' (Forward) and 5' AGC TGT GAA ACT TCC ACT GG 3' (Reverse) (Sahul Hameed et al., 2004a). The size of DNA amplified product is 425 bp. Reactions were performed in 50  $\mu\text{l}$  RT-PCR buffer containing 20 pmol of each primer and RNA template, using the following steps: RT at  $52^{\circ}\text{C}$  for 30 min; denaturation at  $95^{\circ}\text{C}$  for 2 min followed by 30 cycles of denaturation at  $94^{\circ}\text{C}$  for 40 s, annealing at  $55^{\circ}\text{C}$  for 40 s and elongation at  $68^{\circ}\text{C}$  for 1 min, ending with an additional elongation step of 10 min at  $68^{\circ}\text{C}$ . For XSV detection, the primers were designed in our laboratory based on sequence data obtained from GenBank (AY247793). The sequence is as follows: 5' CGC GGA TCC GAT GAA TAA GCG ATT AAT AA 3' (Forward) and 5' CGC

GAA TTC CGT TAC TGT TCG GAG TCC CAA 3' (Reverse). The amplification product is 546 bp. The reaction conditions were similar to those for MrNV. The RT-PCR products (10  $\mu\text{l}$ ) were then analyzed by electrophoresis on a 1% agarose gel stained with ethidium bromide and visualized by ultraviolet transillumination.

## 2.4. Preparation of inoculum

The post-larvae (PL) of *P. indicus* and *P. monodon* with prominent sign of whitish muscle in the abdominal region were selected from the samples collected from the hatchery and used as inoculum for infectivity experiments with post-larvae of *M. rosenbergii*. Frozen PL were thawed and homogenized in a sterile homogenizer. A 10% (w/v) suspension was made with TN buffer (20 mM Tris-HCl and 0.4 M NaCl, pH 7.4). The homogenate was centrifuged at  $4000 \times g$  for 20 min at  $4^{\circ}\text{C}$  and its supernatant was recentrifuged at  $10,000 \times g$  for 20 min at  $4^{\circ}\text{C}$ . Then, the final supernatant was filtered through a 0.22- $\mu\text{m}$  pore membrane. The filtrate was stored at  $-20^{\circ}\text{C}$  for infectivity studies.

## 2.5. Collection and maintenance of experimental animals

For experimental transmission, healthy early post-larvae of *M. rosenbergii* were collected from a hatchery in a locality with no record of WTD. They were randomly sampled and screened for WTD by RT-PCR assay prior to challenge experiments. After collection, the post-larvae were washed with sterile freshwater to remove food and other materials adhering to the body. The washed post-larvae were maintained in glass aquaria (25 l) containing aerated freshwater at a temperature of  $27\text{--}30^{\circ}\text{C}$  and fed twice a day with *Artemia* nauplii.

## 2.6. Infectivity experiments

The inoculum prepared from naturally infected PL of *P. indicus* and *P. monodon* was tested for its virulence to infect healthy PL of *M. rosenbergii*. The infectivity experiment was carried out by immersion challenge. In the immersion challenge, the post-larvae (30 Nos.) of freshwater prawn were placed in a 5 l beakers containing freshwater with continuous aeration. The beakers were covered to prevent contamination. The post-larvae were fed with *Artemia* nauplii. The inoculum prepared as mentioned above was added to the water at a volume equal to 0.1% of the total rearing medium ( $1 \text{ ml l}^{-1}$ ) (Venegas et al., 1999; Chen et al., 2000). Control groups were exposed to tissue filtrates (0.1%) prepared from healthy PL of marine shrimp collected from a marine shrimp hatchery located near Chennai where there was no report of WTD. The experiment was conducted in triplicate. The animals were examined twice a day for clinical sign of WTD. The



Fig. 1. Post-larvae of *Penaeus indicus* with whitish muscle (arrow).

number of deaths was recorded and the cumulative mortality levels were calculated. The PL at moribund stage were collected and RT-PCR was carried out to confirm the presence of MrNV and XSV.

### 3. Results and discussion

The post-larvae of *P. indicus* and *P. monodon* with symptom of whitish abdominal muscle were collected along with water samples from a hatchery where the seed of marine shrimp and freshwater prawn was produced in very close proximity. In the hatchery, temperature, pH, salinity and dissolved oxygen ranged from 27 to 30 °C, 8.0 to 8.2, 10 to 12 ppt and 5.5 to 6.0 mg/l, respectively. The clinical signs of infected post-larvae were lethargy and opaqueness (whitish appearance) of abdominal muscle (Fig. 1). Initially the whitish muscle appeared in the second abdominal segment and gradually extended to anterior and posterior regions as the infection progressed. The mortality reached 100% after appearance of whitish muscle.

The post-larvae of marine shrimp showing whitish muscle were analysed by RT-PCR using MrNV and XSV specific primers. It showed the presence of prominent bands for MrNV (425 bp) and XSV (546 bp) (Fig. 2).

To confirm the viability and virulent nature of inoculum prepared from PL of marine shrimp with whitish abdominal muscle, the PL of *M. rosenbergii* were exposed to this inoculum by immersion challenge. The inoculum prepared from PL of marine shrimp caused 100% mortality within 7 days post infection (Fig. 3). Moribund post-larvae collected at 5 days p.i. showed positive for MrNV and XSV by RT-PCR (Fig. 4).

The clinical signs, mortality data and RT-PCR analysis confirmed the occurrence of WTD caused by MrNV and XSV in PL of marine shrimp as observed in *M. rosenbergii*. This is the first report on the occurrence of WTD in PL of marine shrimp. Our previous work on experimental infection in marine shrimp with MrNV and XSV indicated that these viruses are capable of utilizing tissue of marine shrimp as a proliferating system for their propagation and also for maintaining their virulence. Producing the seeds of marine shrimp and freshwater prawn in close proximity invites the possibility of

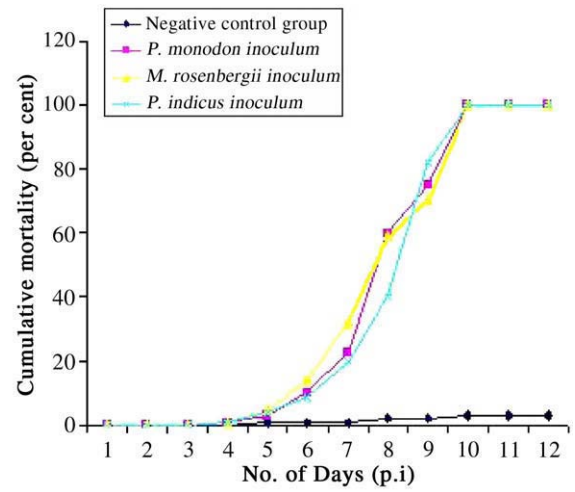


Fig. 3. Cumulative percent mortality of post-larvae of *Macrobrachium rosenbergii* infected with inoculum prepared from MrNV and XSV infected *P. monodon*, *P. indicus* and *M. rosenbergii* by immersion challenge. Normal tissue extract prepared from healthy post-larvae was used as negative control.

transmitting pathologically significant organisms from native to non-native hosts as observed in the present study.

These viruses have been reported to infect larvae and post-larvae of *M. rosenbergii* but not adult prawn (Qjan et al., 2003; Sahul Hameed et al., 2004b) as observed in the case of marine shrimp. These viruses caused 100% mortality in post-larval stage but not in the adult stage. Our previous studies revealed that three species of marine shrimp were not susceptible to these viruses since they failed to produce mortality in them (Sudhakaran et al., 2006). Our observation agrees with the observation of Tang et al. (2007) in the case of *P. vannamei* nodavirus (PvNV) which did not cause mortality in laboratory bioassays. It is possible that the adults showed resistance to the disease because they have better defence mechanism compared to post-larvae. Variation in mortality and disease susceptibility with age has been reported. For example, Gacutan et al. (1979) reported decreased susceptibility to *Ephelota* infection in larvae of *P. monodon*

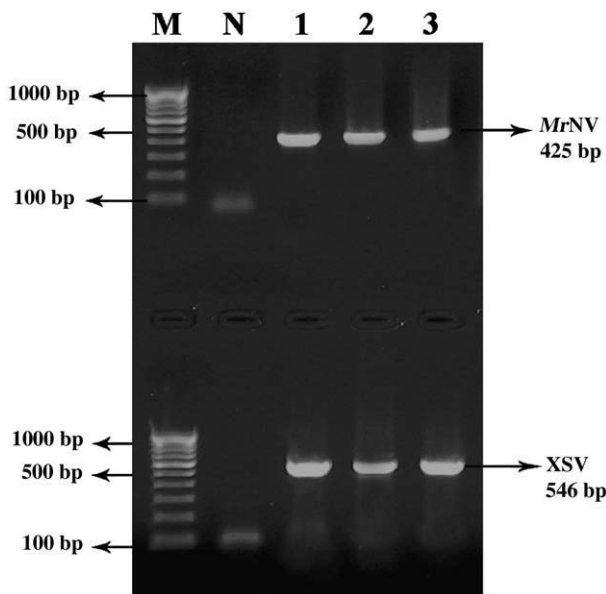


Fig. 2. Amplification of the RT-PCR products of MrNV and XSV in naturally infected post-larvae of marine shrimp collected from a hatchery. Lane M: marker; Lane N: negative control; Lane 1: positive control; Lane 2: *P. monodon*; Lane 3: *P. indicus*.

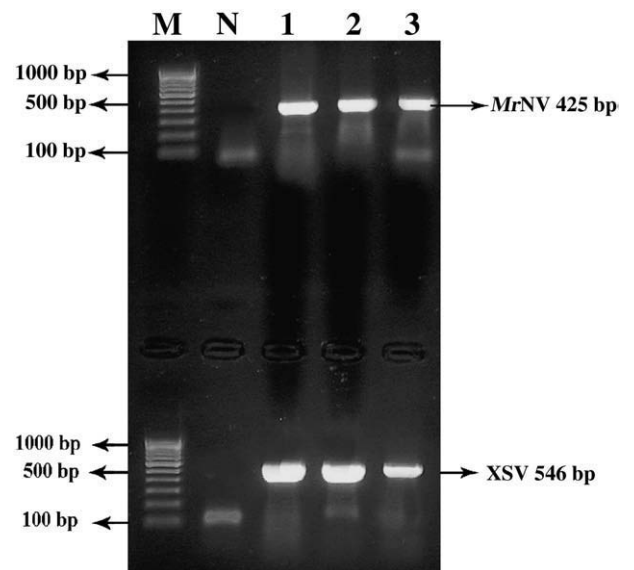


Fig. 4. Amplification of the RT-PCR products of MrNV and XSV in experimentally-infected post-larvae of *M. rosenbergii* infected with inoculum prepared from infected post-larvae of marine shrimp. Lane M: marker; Lane N: negative control; Lane 1: positive control; Lane 2: Post-larvae of *M. rosenbergii* infected with inoculum of *P. monodon*; Lane 3: Post-larvae of *M. rosenbergii* infected with inoculum of *P. indicus*.

with age, and Lightner (1975) observed that *P. setiferus* seemed to be resistant to *Lagenidium callinectes* infection from the mysis stage onwards. The rapid propagation of larval necrosis caused by bacteria in zoea of penaeids and the young stages of *M. rosenbergii* showed that age is certainly an important factor in sensitivity to disease (AQUACOP, 1977). A similar type of resistance against white spot syndrome virus (WSSV) has been observed in adult *M. rosenbergii* (Sahul Hameed et al., 2000), although the larvae can suffer mortality from it (Peng et al., 1998). The mechanism of resistance to MrNV and XSV is not known in adult marine shrimp and freshwater prawn. Therefore studies need to be carried out on the defence mechanism in shrimp and prawn against MrNV and XSV.

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