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Experimental transmission and tissue tropism of *Macrobrachium rosenbergii* nodavirus (*Mr*NV) and its associated extra small virus (XSV)

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ABSTRACT: White tail disease (WTD) was found to be a serious problem in hatcheries and nursery ponds of *Macrobrachium rosenbergii* in India. The causative organisms have been identified as *M. rosenbergii* nodavirus (*Mr*NV) and its associated extra small virus (XSV). Experimentally transmitted to healthy animals, they caused 100% mortality in post-larvae but failed to cause mortality in adult prawns. The RT-PCR assay revealed the presence of both viruses in moribund post-larvae and in gill tissue, head muscle, stomach, intestine, heart, hemolymph, pleopods, ovaries and tail muscle, but not in eyestalks or the hepatopancreas of experimentally infected adult prawns. The presence of these viruses in ovarian tissue indicates the possibility of vertical transmission. Pleopods have been found to be a suitable organ for detecting these viruses in brooders using the RT-PCR technique.

KEY WORDS: *Macrobrachium rosenbergii* · *Macrobrachium rosenbergii* nodavirus · RT-PCR · Pathogenicity · Tissue tropism

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INTRODUCTION

Macrobrachium rosenbergii is the most important cultured palaemonid in the world, and it is now farmed on a large scale in many countries, including India. There is tremendous scope for development of freshwater prawn culture in India, where total production has increased significantly, reaching an all time high of 20000 metric tons in 2002. Threatening this success, a new disease, similar to white tail disease (WTD) first reported from the French West Indies by Arcier et al. (1999), has been observed in hatcheries and nursery ponds located in Andhra Pradesh and Tamil Nadu. This has caused mortality up to 100% within 2 or 3 d. The loss so far has been estimated to be several million dollars, and it continues to grow (Sahul Hameed et al. 2004). It has caused high economic loss in the West Indies since 1994. WTD has also been reported in Taiwan (Tung et al. 1999) and China (Qian et al. 2003).

Other serious diseases associated with white tails have been reported in freshwater prawns (AQUACOP 1977, Anderson et al. 1990, Cheng & Chen 1998, Tung et al. 1999, Qian et al. 2002). Anderson et al. (1990) reported a parvo-like virus responsible for mass mortality in a freshwater prawn hatchery. *Macrobrachium* muscle virus (MMV) was reported by Tung et al. (1999) and found to be responsible for high mortalities in hatchery-reared *M. rosenbergii*. Cheng & Chen (1998) reported an *Enterococcus*-like bacterium that caused muscle necrosis of *M. rosenbergii* in Taiwan.

The causative agent of WTD has been identified as *Macrobrachium rosenbergi* nodavirus (*Mr*NV). It is a small, icosahedral, non-enveloped particle, 26 to 27 nm in diameter, with a capsid that contains a single polypeptide of 43 kDa (Romestand & Bonami 2003). It is found in the cytoplasm of connective tissue cells and has been placed in the *Nodaviridae* family based on its characteristics and genome sequence (Garzon & Char-

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pentier 1992, van Regenmortel et al. 2000, Romestand & Bonami 2003). The pathogen responsible for WTD in India has also been identified as *Mr*NV, as it has in Taiwan and China, by the RT-PCR technique using primers based on the sequence of *Mr*NV-RNA at GenBank (Arcier et al. 1999, Qian et al. 2003, Sri Widada et al. 2003).

Although the gross sign of whitish tail muscle is the main sign used to diagnose WTD, it is not specific to WTD, as described above. Hence, some sensitive and specific diagnostic methods were urgently needed to detect *Mr*NV in its early stages. A sandwich enzyme-linked immunosorbent assay was first developed using antibodies raised against *Mr*NV (Romestand & Bonami 2003). Three detection methods based on specific nucleic acid probes and primer were subsequently developed (Sri Widada et al. 2003), and RT-PCR has been proven to be the most sensitive method of detection.

Recently, Qian et al. (2003) have reported the occurrence of an additional virus to *Mr*NV that may also be responsible for WTD in prawns from China. This virus is smaller in diameter (14 to 16 nm) than *Mr*NV and appears to be 5 or 6 sided when viewed using transmission electron microscopy (TEM). Because of its unusually small size, it has been designated an extra small virus (XSV) (Qian et al. 2003). Its genome consists of 1 linear, single-stranded RNA fragment that has been fully sequenced, and an RT-PCR method has been developed to detect it (Sri Widada et al. 2004).

In the present study, pathogenicity and tissue and organ distribution of these 2 viruses was examined in experimentally infected post-larvae and adult freshwater prawns using RT-PCR assay.

MATERIALS AND METHODS

Collection of infected post-larvae. Infected post-larvae (PL) with prominent signs of whitish muscle in the abdominal region were collected from hatcheries located near Nellore, Andhra Pradesh, and Chennai, Tamil Nadu, India. The PL were washed in sterile saline solution, transferred to sterile tubes for transport to the laboratory on dry ice and then stored at –20°C. The physicochemical characteristics of hatchery and nursery pond water and percentage mortality were determined for each sampling of infected animals. Dissolved oxygen, salinity, pH and temperature were measured. Salinity was measured with a salinometer and dissolved oxygen was estimated using the Winkler method (Strickland & Parsons 1968).

Preparation of viral inoculum. Frozen infected 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°C and its supernatant was recentrifuged at $10\,000 \times g$ for 20 min at 4°C before the final supernatant was filtered through a 0.22 µm pore membrane. The filtrate was then stored at –20°C for infectivity studies.

Collection and maintenance of experimental animals. For experimental transmission, healthy PL (10) were colleted 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 PL were washed with sterile freshwater to remove food and other materials adhering to the body. The washed PL were maintained in glass aquaria (25 l) containing aerated freshwater at a temperature of 27 to 30°C and fed twice a day with *Artemia* nauplii. Healthy adult prawns (30 to 50 g body weight) were collected from grow-out ponds located near Nellore and maintained in 1000 l fiberglass tanks with continuous aeration at room temperature (27 to 30°C) in freshwater. The animals were fed with commercial pellet feed (CP shrimp feed, Thailand).

Infectivity experiments. The experimental pathogenicity of *Mr*NV and XSV to healthy PL and adult prawns of *Macrobrachium rosenbergii* was carried out by immersion challenge and intramuscular injection, respectively. In the immersion challenge, the PL were placed (50 ind. l^{-1}) in beakers (5 l) containing freshwater with continuous aeration. The beakers were covered to prevent contamination. The PL were fed with *Artemia* nauplii. The viral inoculum (*Mr*NV and XSV) was added to water at a volume equal to 0.1% of the total rearing medium (1 ml l^{-1}) (Venegas et al. 1999, Chen et al. 2000). Control groups were exposed to tissue filtrates (0.1%) prepared from healthy PL. The experiment was conducted in triplicate.

Adult prawns were infected by intramuscular injection of *Mr*NV and XSV. They were maintained in fiberglass tanks (10 per tank) at room temperature containing freshwater with continuous aeration. The experimental animals were injected intramuscularly in the second abdominal segment with filtrate (50 µl ind.$^{-1}$) prepared from infected PL using 1 ml insulin syringes. Control animals were injected with the filtrate prepared from healthy PL.

The experimental prawns were examined twice per day for gross signs of disease, and the number of deaths was recorded so that cumulative percentage mortality could be calculated.

Total RNA extraction. For extraction of total RNA, 150 mg of whole PL or pieces of different organs (gill tissue, hepatopancreas, heart, stomach, eyestalks, head muscle, abdominal muscle, tail muscle, ovary, intestines and pleopods) or hemolymph (150 µl) from

adult prawns were homogenized in 300 µl of TN buffer (20 mM Tris-HCl, 0.4 M NaCl, pH 7.4). The homogenate was centrifuged at 12 000 × *g* for 15 min at room temperature. The supernatant was collected and referred to as crude tissue extract. Total RNA was extracted using TRIzol reagent (GIBCO-BRL) according to the protocol of the manufacturer. Briefly, 1 ml of TRIzol reagent was added to 150 µl of crude tissue extract and homogenized. After 5 min of incubation at room temperature, 0.2 ml of chloroform was added. The sample was vigorously shaken for 2 to 3 min at room temperature then centrifuged at 12 000 × *g* for 15 min at room temperature. RNA was precipitated from the aqueous phase with isopropanol, washed with 75% ethanol and dissolved in 50 µl 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 of the preparation was checked by measuring the ratio of optical density OD_{260nm}/OD_{280nm}.

RT-PCR for *Mr*NV and XSV. RT-PCR was carried out using the Reverse-IT^{TM} 1-step RT-PCR kit (ABgene), allowing reverse transcription (RT) and amplification to be performed in a single reaction tube. One pair of primers specific to *Mr*NV-RNA2 was designed from sequence data of the *Mr*NV genome (GenBank Accession No. AY222840). The sequences were 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. 2004). The size of the DNA amplicon was 425 bp. Reactions were performed in 50 µl RT-PCR buffer containing 20 pmol of each primer and RNA template, using the following steps: RT at 52°C for 30 min; denaturation at 95°C for 2 min followed by 30 cycles of denaturation at 94°C for 40 s, annealing at 55°C for 40 s and elongation at 68°C for 1 min, ending with an additional elongation step of 10 min at 68°C. For XSV detection, the primers were 5′ GGA GAA CCA TGA GAT CAC G 3′ (forward) and 5′ CTG CTC ATT ACT GTT CGG AGT C 3′ (reverse) (Sri Widada et al. 2004). The amplification product was 500 bp. The reaction conditions were similar to those for *Mr*NV. The RT-PCR products (10 µl) were then analyzed by electrophoresis on a 0.8% agarose gel.

RESULTS

The viral inoculum used in the experimental infections was prepared from PL with gross signs of whitish tails, and the presence of *Mr*NV and XSV in the inoculum was confirmed by RT-PCR. The cumulative percentage mortality in PL exposed to *Mr*NV and XSV is shown in Fig. 1. Three dilutions of viral suspension were used and mortality was observed at all dilutions.

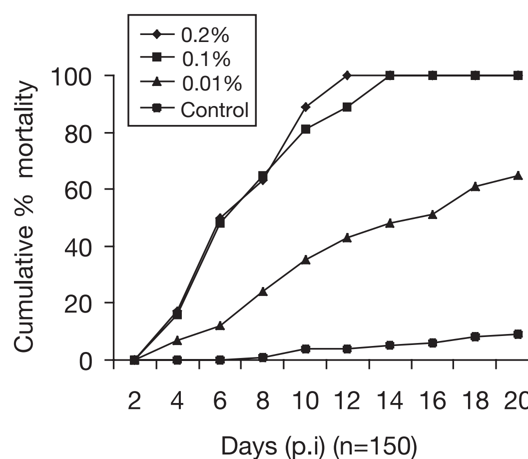

Fig. 1. *Macrobrachium rosenbergii*. Experimentally induced pathogenicity of *Mr*NV and XSV in post-larvae. Cumulative percent mortality was recorded every 2 d post-infection (p.i.) for groups of post-larvae which were immersed in 0.2, 0.1, and 0.01% virus suspension, and for a control group which was immersed in tissue extract prepared from healthy post-larvae

The 0.2 and 0.1% viral suspensions caused 100% mortality at 12 and 14 d post-challenge (dpc), respectively. The 0.01% viral suspension caused 65% mortality at 20 dpc. Clinical signs observed in the experimentally infected animals were similar to those seen in natural infections. These included lethargy, anorexia and opaqueness of abdominal muscle (whitish muscle). This opaqueness gradually expanded on both sides (anterior and posterior) and the telson and uropods degenerated in severe cases. Some live, infected PL without uropods were also observed. The gross sign of whitish muscle was observed in all moribund animals collected after 6 d post-infection but not before. The experimentally infected PL tested positive for *Mr*NV and XSV by RT-PCR from 2 d post-infection onwards (Fig. 2).

These 2 viruses failed to cause mortality or clinical signs of disease in injected adult prawns during the experimental period of 30 d. However, RT-PCR analysis showed the appearance of prominent bands for *Mr*NV (425 bp) and XSV (500 bp) in all the organs except in the hepatopancreas and eyestalks (Fig. 3). The intensity of the band in the case of *Mr*NV differed from organ to organ, whereas that for XSV was very prominent in all positive organs.

DISCUSSION

As with Sri Widada et al. (2003), who reported 2 types of virus particles (*Mr*NV and XSV) associated with WTD from China, we found both viruses using

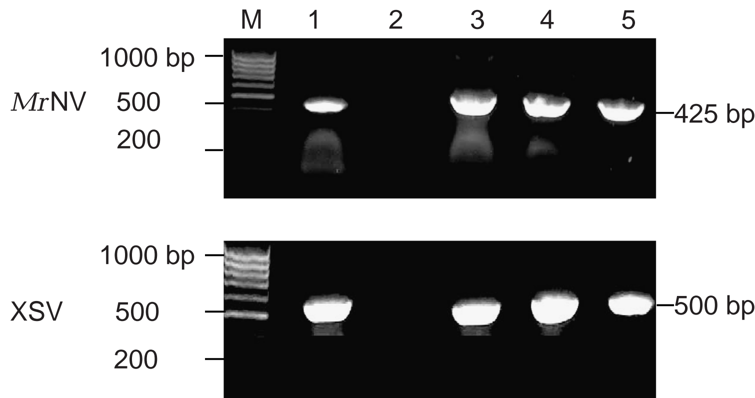

Fig. 2. *Macrobrachium rosenbergii.* RT-PCR detection of *Mr*NV and XSV in experimentally infected post-larvae. M: marker; Lane 1: virus suspention prepared from infected post-larvae; Lane 2: healthy post-larvae; Lanes 3, 4 and 5: different groups of post-larvae exposed to *Mr*NV and XSV

RT-PCR with WTD samples collected from Indian hatcheries. The presence of >1 virus in crustacean diseases has previously been reported (Bonami 1980, Mari 1987). It has been suggested that the occurrence of viral synergic actions might be due to the lack of an interferon-like reaction in invertebrates (Tanada 1956, Odier 1974). Since XSV is always found associated with the larger virus (nodavirus) and located in muscle and connective cells of infected prawns, it could be an autonomous virus, a helper-type virus or a satellite-like virus (Qian et al. 2003).

The experimental transmission of *Mr*NV and XSV resulting in high mortality and clinical signs of WTD in PL confirmed River's postulate (Iwanowicz & Goodwin 2002) and implicated one or both of these viruses as being responsible for WTD in *Macrobrachium rosenbergii*. However, the fact that the same virus suspension caused mortality in PL but not in adult prawns was curious, especially since most tissues and organs of the adults did become infected with both viruses. It is possible that the adults resisted disease due to better defenses than in PL. Variation in mortality and disease susceptibility with age has been reported. For example, Gacutan et al. (1979) reported decreased susceptibility to *Epheota* infection in larvae of *Penaeus monodon* with age, and Lightner (1975) observed that *P. seliferus* 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 adult resistance to *Mr*NV and XSV is not known. Disease resistance in some invertebrates is related to the production of bactericidins, lysins and agglutinins following exposure to foreign proteins (Bang 1967, McKay & Jenkin 1969). It is possible that similar substances may account for the resistance of adult prawns to *Mr*NV and XSV.

We found *Mr*NV and XSV in all the organs except eyestalks and the hepatopancreas of adult *Macrobrachium rosenbergii*. This was consistent with the results of Sri Widada et al. (2003), but not with those of Arcier et al. (1999), who also observed positive tests for *Mr*NV in the hepatopancreas. The reason for this discrepancy is unknown. Knowing pathogen distribution in tissues and organs can help us to understand issues related to disease susceptibility and transmission and to choose optimal samples for pathogen isolation and detection, especially for potential carriers that may require monitoring for control measures. Tissue tropism of WSSV and yellow head virus has been studied by various workers (Lu et al. 1995, Lo et al. 1997, Sahul

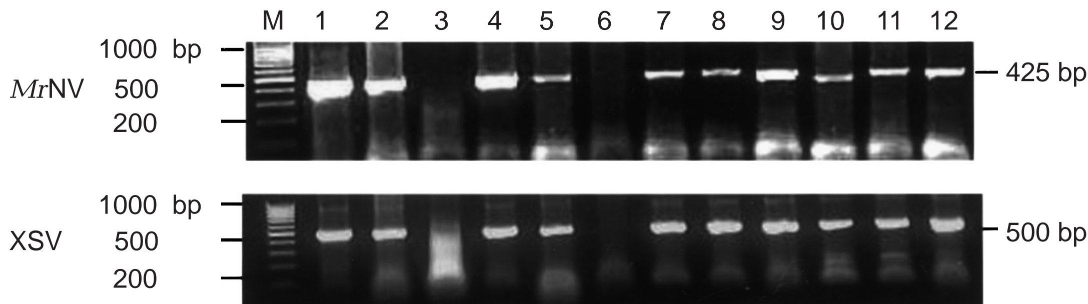

Fig. 3. *Macrobrachium rosenbergii.* RT-PCR detection of *Mr*NV and XSV in different organs of experimentally infected adult prawns. M: marker; Lane 1: hemolymph; Lane 2: gill tissue; Lane 3: hepatopancreas; Lane 4: heart; Lane 5: stomach; Lane 6: eyestalk; Lane 7: head muscle; Lane 8: abdominal muscle; Lane 9: tail muscle; Lane 10: ovarian tissue; Lane 11: intestine; Lane 12: pleopod

Hameed et al. 1998) and has proven useful in discovering and closing transmission routes (Lo et al. 1997).

Finding both *Mr*NV and XSV in all positive tissues and organs indicated that the 2 viruses were closely associated, and suggested that they might be mutually dependent as proposed by Qian et al. (2003). The fact that both viruses were present in ovarian tissue indicates the possibility of vertical transmission of WTD from brooders to larvae and PL. Because of this, we recommend the screening of brooders before they are allowed to spawn in hatcheries. For this purpose, our results on tissue distribution indicate that pleopods would be a convenient source of RNA for non-destructive screening of *Mr*NV and XSV without stress to the prawns.

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