Vol. 79: 141–145, 2008
doi: 10.3354/dao01886

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published April 1

NOTE

Natural aquatic insect carriers of *Macrobrachium rosenbergii* nodavirus (*Mr*NV) and extra small virus (XSV)

R. Sudhakaran[1], P. Haribabu[2], S. Rajesh Kumar[1], M. Sarathi[1], V. P. Ishaq Ahmed[1], V. Sarath Babu[1], C. Venkatesan[1], A. S. Sahul Hameed[1,*]

[1]Aquaculture Biotechnology Division, Department of Zoology, C. Abdul Hakeem College, Melvisharam 632 509, Vellore Dt., Tamil Nadu, India

[2]Sri Venkateswara Veterinary University, Department of Aquaculture, College of Fishery Science, Muthukur 524 344, Andhra Pradesh, India

ABSTRACT: Five different species of aquatic insects were collected from nursery ponds containing the freshwater prawn *Macrobrachium rosenbergii* infected with *Macrobrachium rosenbergii* nodavirus (*Mr*NV) and extra small virus (XSV). The insects were screened as potential natural carriers of *Mr*NV and XSV. RT-PCR (reverse transcription polymerase chain reaction) analysis gave positive results for *Mr*NV and XSV in *Belostoma* sp., *Aesohna* sp., *Cybister* sp. and *Notonecta* sp., and negative results for *Nepa* sp. An *Aedes albopictus* mosquito cell line (C6/36) was used for infectivity assays, with viral inoculum prepared from the aquatic insects, since C6/36 cells have recently been shown to be susceptible to infection with *Mr*NV and XSV. The C6/36 cells were harvested 4 d post-challenge for examination by electron microscopy. This revealed aggregation of viral particles throughout the cytoplasm for cells challenged with inocula from all the insect species except *Nepa* sp. Our results indicate that several aquatic insect species may present a risk for *Mr*NV and XSV transmission to *M. rosenbergii*.

KEY WORDS: *Macrobrachium rosenbergii* · Aquatic insects · *Macrobrachium rosenbergii* nodavirus · *Mr*NV · Extra small virus · XSV · Natural hosts · RT-PCR

INTRODUCTION

White tail disease (WTD) is the major cause of high mortality and severe losses in hatchery-reared post-larvae (PL) of the freshwater prawn *Macrobrachium rosenbergii* in the French West Indies, Taiwan, China and India (Arcier et al. 1999, Tung et al. 1999, Qian et al. 2003, Sri Widada et al. 2003, Sahul Hameed et al. 2004a). The cumulative loss in India alone has been estimated to be in the order of several million dollars and this continues to rise. The causative pathogens of WTD have been identified as *Macrobrachium rosenbergii* nodavirus (*Mr*NV) (Arcier et al. 1999) and extra small virus (XSV) (Sri Widada & Bonami 2004). These are the second and third specific viruses reported from *Macrobrachium* since the first report of a parvo-like virus 15 yr ago (Anderson et al. 1990).

The rapid onset of WTD and associated mortality is remarkable. Gross signs of disease include lethargy, opacity of abdominal muscle and degeneration of the telson with mortalities often reaching 100% (Arcier et al. 1999, Sri Widada et al. 2003, Sahul Hameed et al. 2004a). Sensitive 1-step and nested RT-PCR (reverse transcription polymerase chain reaction)-based diag-

*Corresponding author. Email: cah_sahul@hotmail.com

nostic methods have been developed for *Mr*NV and XSV (Sahul Hameed et al. 2004a, Sri Widada & Bonami 2004, Sudhakaran et al. 2006a, 2007a), and they can be used to identify the natural hosts, susceptible carriers and reservoirs of these viruses.

Horizontal transmission of *Mr*NV and XSV may occur via infected prawns or natural hosts from the culture system and the neighbouring ecosystem in the form of live carriers, dead tissues, or free virions (Vijayan et al. 2005). Invertebrate filter feeders such as bivalve molluscs may ingest and accumulate particulate materials as reported in studies on white spot syndrome virus (WSSV) (Canzonier 1971, Hay & Scotti 1986, Mortensen et al. 1993). It is also possible that *Mr*NV and XSV are carried passively in the digestive tracts of invertebrates or other living organisms in the culture system.

Several investigators have reported that white spot syndrome virus (WSSV) infects primary cultures of lymphoid organs from the giant tiger shrimp *Penaeus monodon* (Kasornchandra et al. 1999, Wang et al. 2000), from the blue shrimp *Litopenaeus stylirostris* (also called *Penaeus stylirostris*) (Tapay et al. 1997) and from the kuruma shrimp *Marsupenaeus japonicus* (also called *Penaeus japonicus*) (Itami et al. 1999). Primary cell cultures of lymphoid organs have also been reported to be susceptible to yellow head virus (YHV) infection (Lu et al. 1995, Assavalapsakul et al. 2003). Although there are no reports on cultivation of *Mr*NV and XSV in crustacean cells, Sudhakaran et al. (2007b) have succeeded in propagating *Mr*NV and XSV in a mosquito cell line (C6/36). Proliferation in an immortal cell line simplifies the process of obtaining large quantities of virus for study.

Shrimp viruses such as WSSV and YHV can infect several crustacean species that can potentially transmit them to cultivated shrimp. For example, natural infections of WSSV have been observed in wild and cultured mud crabs *Scylla serrata*, and infected crabs have been shown to transmit the virus to *Penaeus monodon* (Lo et al. 1996, Flegel 1997, Kanchanaphum et al. 1998, Chen et al. 2000). However, work on potential carriers or natural reservoirs of *Mr*NV/XSV is still lacking. Therefore, the present study was carried out to test whether aquatic insects could act as natural carriers of *Mr*NV/XSV in scampi culture systems.

MATERIALS AND METHODS

Collection of aquatic insects. Various aquatic insects were collected with a fish net from nursery and grow-out ponds containing freshwater prawns experiencing severe WTD outbreaks at Nellore, Andhra Pradesh, India. These included giant water bugs *Belostoma* sp., dragonfly nymphs *Aesohna* sp., diving beetles *Cybister* sp., back swimmers *Notonecta* sp. and water scorpions *Nepa* sp. (Fig. 1). They were kept in separate sterile tubes and transported to the laboratory on dry ice. The insects were identified using standard methods for the examination of water and wastewater (Clesceri et al. 1998). In the laboratory, the specimens were stored at –20°C for RT-PCR analysis and infectivity studies.

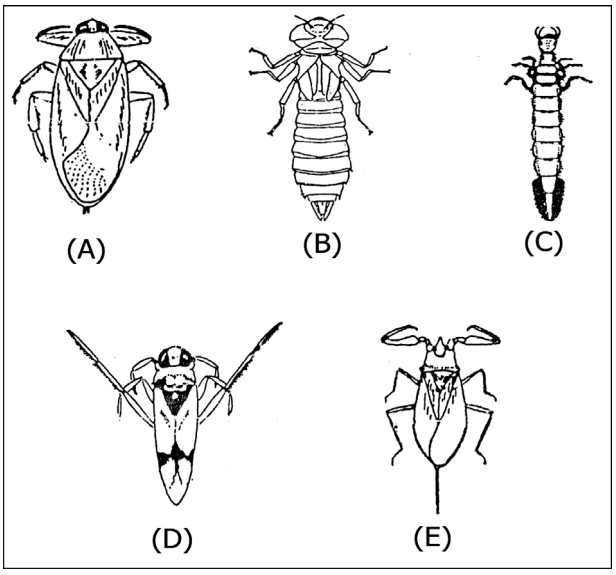

Fig. 1. Line diagrams of aquatic insects collected from prawn cultivation ponds experiencing white tail disease outbreaks: (A) *Belostoma* sp., (B) *Aesohna* sp., (C) *Cybister* sp., (D) *Notonecta* sp. and (E) *Nepa* sp.

Experimental challenges with C6/36 cells. The collected insects were surface sterilized with 5% chlorex for 5 min, dipped in 70% alcohol, and washed thoroughly with sterile TN buffer (20 mM Tris-HCl and 0.4 M NaCl, pH 7.4) before homogenization. A 10% (w/v) suspension of tissue homogenate was made with TN buffer (20 mM Tris-HCl and 0.4 M NaCl, pH 7.4). The homogenate was centrifuged at 4000 × *g* for 20 min at 4°C, its supernatant was recentrifuged at 10 000 × *g* for 20 min at 4°C, and the final supernatant was filtered through a 0.22 µm pore membrane. The filtrate was stored at –20°C, and was used to inoculate the C6/36 mosquito cell line.

The stock inocula prepared from the aquatic insects was used to challenge a C6/36 mosquito cell line as previously described by Sudhakaran et al. (2007b). At 3 or 4 d post-challenge (dpc) the cell cultures were harvested and centrifuged at 700 × *g* for 5 min at 4°C. One portion of the cell pellet was used for electron microscopy, while the other was stored at –20°C for RT-PCR analysis.

Transmission electron microscopy. C6/36 cell pellets from cultures challenged with stock inocula were suspended in 0.1 M phosphate buffer (pH 7.4) containing 2.5% gluteraldehyde in 0.1 M cocadylate buffer (pH 7.2) and 0.5 M NaCl at 25°C for 1 h. The cells were post-fixed in 1% osmium tetroxide in the same buffer for 1 h at 25°C. The fixed cells were dehydrated in an ethanol series and embedded in epoxy resin. The embedded samples were sectioned with an ultramicrotome, stained with uranyl acetate–lead citrate and examined under an electron microscope (Philips EM 201C) (Sudhakaran et al. 2007b).

RNA isolation. For extraction of total RNA, approximately 50 mg of aquatic insects (3 to 5 individuals) or 0.4 ml C6/36 cell pellet were homogenized in TN buffer. The homogenates were centrifuged at 12 000 × *g* for 15 min at room temperature (27 to 30°C), and the supernatant solution was extracted using TRIzol reagent (GIBCO-BRL) according to the protocol of the manufacturer. The amount of nucleic acid in the sample was quantified by measuring the absorbance at 260 nm, and the purity was checked by measuring the ratio of OD_{260nm}/OD_{280nm}.

RT-PCR and nested RT-PCR for Mr NV and XSV. RT-PCR and nested RT-PCR were 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 used and the protocol followed were according to Sudhakaran et al. (2006a,b). The RT-PCR and nested RT-PCR products (10 µl) were analyzed by electrophoresis on a 1% agarose gel stained with ethidium bromide and visualized by ultraviolet transillumination.

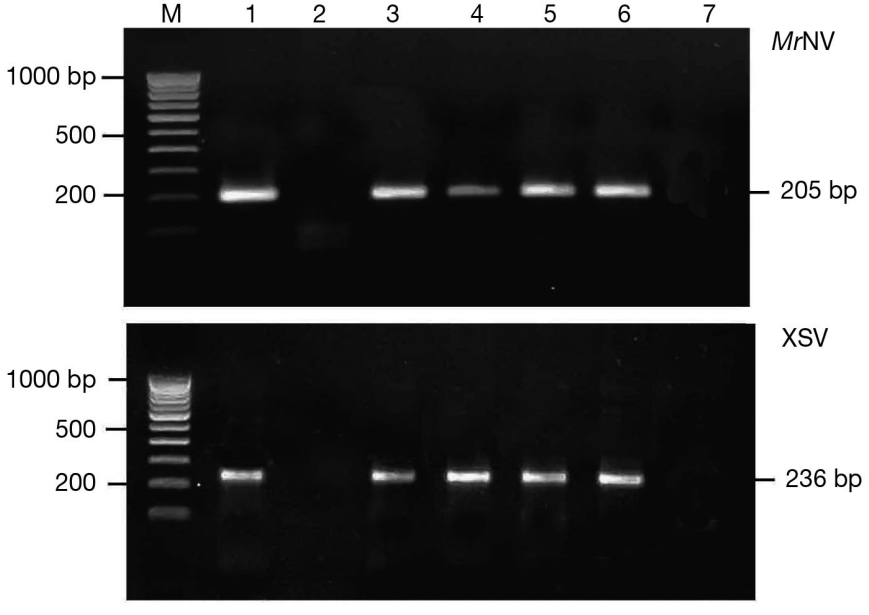

Fig. 2. RT-PCR detection of *Macrobrachium rosenbergii* nodavirus (*Mr*NV) and extra small virus (XSV) in *Aedes albopictus* mosquito cell line (C6/36) cells inoculated with extracts (stock inocula) prepared from wild aquatic insects collected from prawn culture ponds experiencing a severe WTD outbreak. M: DNA marker; 1: virus suspension prepared from infected post-larvae of freshwater prawn; 2: healthy post-larvae; 3: *Belostoma* sp.; 4: *Aesohna* sp.; 5: *Cybister* sp.; 6: *Notonecta* sp.; 7: *Nepa* sp.

RESULTS AND DISCUSSION

Among the 5 species of aquatic insects examined, the giant water bug *Belostoma* sp., dragonfly nymphs *Aesohna* sp., diving beetles *Cybister* sp. and back swimmers *Notonecta* sp. gave positive RT-PCR results for *Mr*NV and XSV. By contrast, results for the water scorpion *Nepa* sp. were negative. RT-PCR results from C6/36 cells challenged with stock inocula from these species gave parallel results (Fig. 2). The positive RT-PCR results for these insects and C6/36 cell cultures were similar to those obtained by Sahul Hameed et al. (2004b) when testing adult *Macrobrachium rosenbergii* infected with *Mr*NV and XSV.

Electron microscopy of C6/36 cells challenged with stock inocula from the 4 RT-PCR positive insect species revealed vacuolation and aggregation of numerous viral particles distributed throughout the cytoplasm (Fig. 3).

*Mr*NV and XSV were found in all positive tissues and organs of both freshwater prawns and marine shrimp (Sudhakaran et al. 2006b), and we detected them both in our RT-PCR positive aquatic insects. This supported the proposal that the 2 viruses are closely associated and that XSV is a satellite virus dependent on *Mr*NV (Qian et al. 2003). Although *Mr*NV and XSV infections can be fatal for larvae of *Macrobrachium rosenbergii*, adult infected prawns do not show signs of disease and can act as unaffected carriers (Sahul Hameed et al. 2004b). The mechanism of tolerance to *Mr*NV and XSV in adult animals is not known, but it demonstrates that the viruses are capable of coexistence with grossly normal carriers. 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 ability of adult prawns to control *Mr*NV and XSV.

Lo et al. (1996) noted that aquatic insect larvae (Ephydridae), copepods, the pest crab *Helice tridens*, and pest

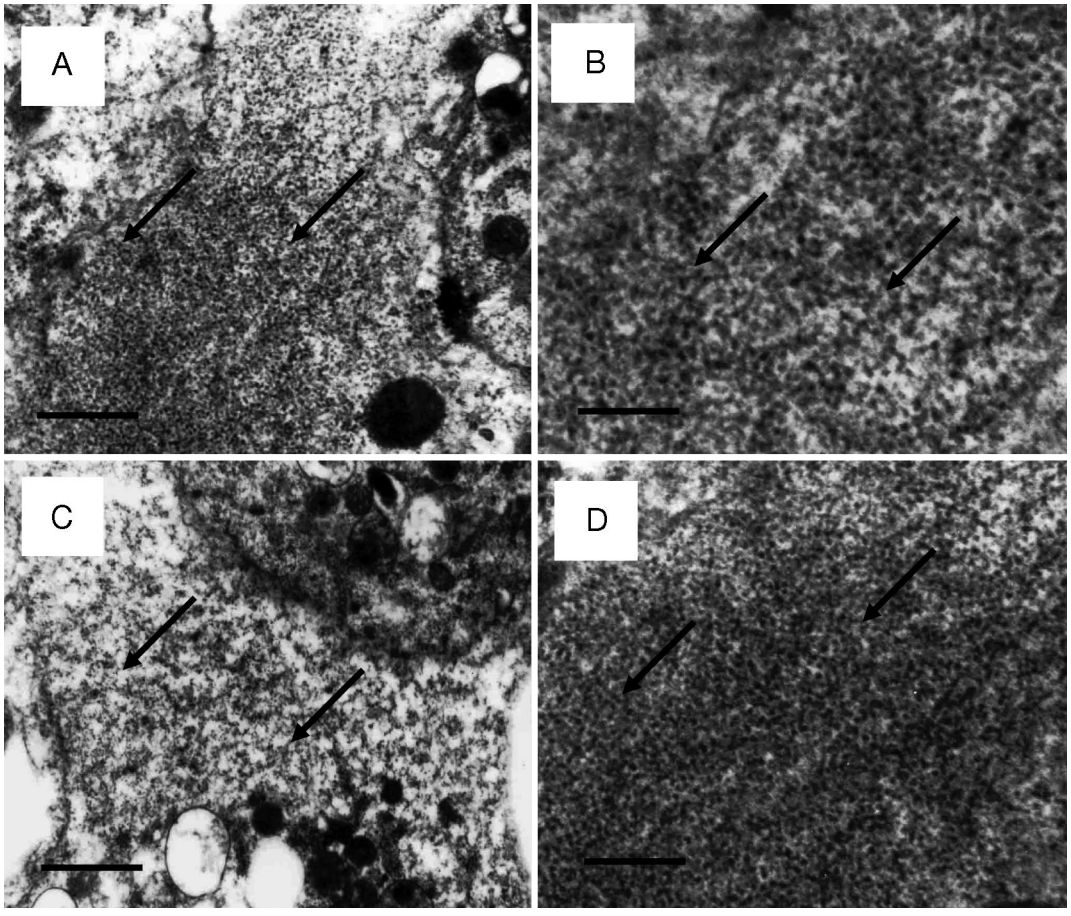

Fig. 3. Electron micrograph of *Mr*NV and XSV C6/36 cells challenged with stock inocula prepared from homogenates of wild aquatic insects collected from prawn culture ponds experiencing a severe WTD outbreak. Arrows indicate vacuolation in infected cells: (A) *Belostoma* sp., (B) *Aesohna* sp., (C) *Cybister* sp. and (D) *Notonecta* sp. Scale bars = 1 µm (A,C) and 5 µm (B,D)

palaemonid shrimp from penaeid shrimp cultivation ponds affected by WSSV can give positive PCR test results for WSSV. However, it is still uncertain whether all of the PCR-positive species and especially the aquatic insects are actually infected with the virus or whether they simply act as mechanical carriers. On the other hand, experimental transmission of WSSV to crabs (Supamattaya et al. 1998) and several other crustacean species has been confirmed histologically (Flegel 2006). In other cases, PCR positive animals are simply mechanical carriers. For example, polychaete worms have been reported to occasionally give PCR positive results from WSSV in gut contents acquired due to their role as active benthic detritivores (Vijayan et al. 2005). This does not diminish the risk of them transmitting WSSV to shrimp (Vijayan et al. 2005). We have established the ability of *Mr*NV and XSV to infect an insect cell line, and this increases the likelihood that they would also be able to infect aquatic insects. On the other hand, we have not carried out *in situ* hybridization tests with our RT-PCR positive insect species, and, until this is done, we cannot confirm their status as mechanical carriers or infected carriers. Since we have shown that *Mr*NV and XSV can be detected in aquatic insects and that the detected viruses are able to infect susceptible mosquito cells in culture, we have established a potential transmission risk to cultivated *Macrobrachium rosenbergii*. Whatever the case, we have clearly demonstrated that the 4 insect species are a potential transmission threat, and we recommend that care be taken to exclude them from freshwater prawn culture systems, especially at the larval production stages.

Acknowledgements. R.S. was a recipient of a Senior Research Fellowship award from the Council for Scientific and Industrial Research, New Delhi. The authors are thankful to the management of C. Abdul Hakeem College, Melvisharam, for providing the facilities to carry out this work. This work was funded by the Indo-French Centre for the Promotion of Advanced Research, New Delhi.

LITERATURE CITED

Anderson IG, Law AT, Shariff M, Nash G (1990) A parvo-like virus in the giant freshwater prawn, *Macrobrachium rosenbergii*. J Invertebr Pathol 55:447–449

Arcier JM, Herman F, Lightner DV, Redman RM, Mari J, Bonami JR (1999) A viral disease associated with mortalities in hatchery-reared postlarvae of the giant freshwater prawn *Macrobrachium rosenbergii.* Dis Aquat Org 38: 177–181

Assavalapsakul W, Smith DR, Panyim S (2003) Propagation of infectious yellow head virus particles prior to cytopathic effect in primary lymphoid cell cultures of *Penaeus monodon.* Dis Aquat Org 55:253–258

Bang FB (1967) Serological responses among invertebrates other than insects. Fed Proc 267:1680–1684

Canzonier WJ (1971) Accumulation and elimination of coliphage S-13 by the hard clam *Mercenaria mercenaria.* Appl Microbiol 21:1024–1031

Chen LL, Lo CF, Chiu YL, Chang CF, Kou GH (2000) Natural and experimental infection of white spot syndrome virus (WSSV) in benthic larvae of mud crab *Scylla serrat*a. Dis Aquat Org 40:157–161

Clesceri LS, Greenberg AE, Eaton AD (1998) Standard methods for the examination of water and wastewater, 20th edn. The American Public Health Association, the American Water Works Association and the Water Environment Federation, Washington, DC

Flegel TW (1997) A special topic review: major viral diseases of the black tiger prawn (*Penaeus monodon*) in Thailand. World J Microbiol Biotechnol 13:433–442

Flegel TW (2006) Detection of major penaeid shrimp viruses in Asia, a historical perspective with emphasis on Thailand. Aquaculture 258:1–33

Hay B, Scotti P (1986) Evidence for intracellular absorption of virus by the Pacific oyster, *Crassostrea gigas.* NZ J Mar Freshw Res 20:655–659

Itami T, Maeda M, Kondo M, Takahashi Y (1999) Primary culture of lymphoid organ cells and haemocytes of kuruma shrimp, *Penaeus japonicus.* Methods Cell Sci 21:237–244

Kanchanaphum P, Wongteerasupaya C, Sitidilokratana N, Boonsaeng V and others (1998) Experimental transmission of white spot syndrome virus (WSSV) from crabs to shrimp *Penaeus monodon.* Dis Aquat Org 34:1–7

Kasornchandra J, Khongpradit R, Ekpanithanpong U, Boonyaratpalin S (1999) Progress in the development of shrimp cell cultures in Thailand. Methods Cell Sci 21:231–235

Lo CF, Leu JH, Ho CH, Chen CH and others (1996) Detection of baculovirus associated with white spot syndrome virus (WSBV) in penaeid shrimp using polymerase chain reaction. Dis Aquat Org 25:133–141

Lu Y, Tapay LM, Loh PC, Brock JA (1994) Infection of the yellow head baculo-like virus in two species of penaeid shrimp, *P. stylirostris* and *P. vannamei.* J Fish Dis 17: 649–656

McKay D, Jenkin CR (1969) Immunity in invertebrates. II. Adaptive immunity in the crayfish (*Parachaeraps bicarinatu*). Immunology 17:127–137

Mortensen S, Evensen Ø, Rødseth O, Hjeltnes B (1993) The relevance of infectious pancreatic necrosis virus (IPNV) in farmed Norwegian turbot (*Scophthalmus maximus*). Aquaculture 115:243–252

Qian D, Shi Z, Zhang S, Cao Z and others (2003) Extra small virus-like particles (XSV) and nodavirus associated with whitish muscle disease in the giant freshwater prawn, *Macrobrachium rosenbergii.* J Fish Dis 26:521–527

Sahul Hameed AS, Yoganandhan K, Sri Widada J, Bonami JR (2004a) Studies on the occurrence and RT-PCR detection of *Macrobrachium rosenbergii* nodavirus and extra small virus-like particles associated with white tail disease of *Macrobrachium rosenbergii* in India. Aquaculture 238: 127–133

Sahul Hameed AS, Yoganandhan K, Sri Widada J, Bonami JR (2004b) Experimental transmission and tissue tropism of *Macrobrachium rosenbergii* nodavirus (*Mr*NV) and its associated extra small virus (XSV). Dis Aquat Org 62: 191–196

Sri Widada J, Bonami JR (2004) Characteristics of the monocistronic genome of extra small virus, a virus-like particle associated with *Macrobrachium rosenbergii* nodavirus: possible candidate for a new species of satellite virus. J Gen Virol 85:643–646

Sri Widada J, Durand S, Cambournac I, Qian D and others (2003) Genome-based detection methods of *Macrobrachium rosenbergii* nodavirus, a pathogen of the giant freshwater prawn, *Macrobrachium rosenbergii*: dot-blot, *in situ* hybridization and RT-PCR. J Fish Dis 26:583–590

Sudhakaran R, Yoganandhan K, Ishaq Ahmed VP, Sahul Hameed AS (2006a) *Artemia* as a possible vector for *Macrobrachium rosenbergii* nodavirus (*Mr*NV) and extra small virus transmission (XSV) to *Macrobrachium rosenbergii* post-larvae. Dis Aquat Org 70:161–166

Sudhakaran R, Syed Musthaq S, Haribabu P, Mukherjee SC, Gopal C, Sahul Hameed AS (2006b) Experimental transmission of *Macrobrachium rosenbergii* nodavirus (*Mr*NV) and extra small virus (XSV) in three species of marine shrimp (*Penaeus indicus*, *Penaeus japonicus* and *Penaeus monodon*). Aquaculture 257:136–141

Sudhakaran R, Ishaq Ahmed VP, Haribabu P, Mukherjee SC, Sri Widada J, Bonami JR, Sahul Hameed AS (2007a) Experimental vertical transmission of *Macrobrachium rosenbergii* nodavirus (*Mr*NV) and extra small virus (XSV) from brooders to progeny in *Macrobrachium rosenbergii* and *Artemia.* J Fish Dis 29:1–9

Sudhakaran R, Parameswaran V, Sahul Hameed AS (2007b) *In vitro* replication of *Macrobrachium rosenbergii* nodavirus (*Mr*NV) and extra small virus (XSV) in C6/36 cell line. J Virol Methods 146:112–118

Supamattaya K, Hoffmann RW, Boonyaratpalin S, Kanchanaphum P (1998) Experimental transmission of white spot syndrome virus (WSSV) from black tiger shrimp *Penaeus monodon* to the sand crab *Portunus pelagicus*, mud crab *Scylla serrata* and krill *Acetes* sp. Dis Aquat Org 32:79–85

Tapay LM, Lu Y, Gose RB, Nadara ECB Jr, Brock JA, Loh PC (1997) Development of an *in vitro* quantal assay in primary cell cultures for a non-occluded baculo-like virus of penaeid shrimp. J Virol Methods 64:37–41

Tung CW, Wang CS, Chen SN (1999) Histological and electron microscopic study on *Macrobrachium* muscle virus (MMV) infection in the giant freshwater prawn, *Macrobrachium rosenbergii* (de Man), cultured in Taiwan. J Fish Dis 22:319–323

Vijayan KK, Stalin Raj V, Balasubramanian CP, Alavandi SV, Thillai Sekhar V, Santiago TC (2005) Polychaete worms — a vector for white spot syndrome virus (WSSV). Dis Aquat Org 63:107–111

Wang CH, Yang HN, Tang CY, Lu CH, Kou GH, Lo CF (2000) Ultrastructure of white spot syndrome virus development in primary lymphoid organ cell cultures. Dis Aquat Org 41:91–104

Editorial responsibility: Timothy Flegel, Bangkok, Thailand

Submitted: April 4, 2007; Accepted: November 15, 2007
Proofs received from author(s): March 6, 2008