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NOTE

Artemia as a possible vector for *Macrobrachium rosenbergii* nodavirus (*Mr*NV) and extra small virus transmission (XSV) to *Macrobrachium rosenbergii* post-larvae

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ABSTRACT: Five developmental stages of *Artemia* were exposed to *Macrobrachium rosenbergii* nodavirus (*Mr*NV) and extra small virus (XSV) by immersion and oral routes in order to investigate the possibility of *Artemia* acting as a reservoir or carrier of these viruses. The second objective was to determine if virus-exposed *Artemia* were capable of transmitting the disease to post-larvae (PL) of *M. rosenbergii*. There was no significant difference in percent mortality between *Artemia* control groups and groups challenged with these viruses. On the other hand, all the developmental stages of *Artemia* were positive for both viruses by nested RT-PCR, regardless of the challenge route. In horizontal transmission experiments, 100% mortality was observed in *M. rosenbergii* PL fed with *Artemia* nauplii exposed to *Mr*NV and XSV by either challenge route. However, no mortality was observed in PL fed with virus-free *Artemia*. RT-PCR analysis of the *M. rosenbergii* PL confirmed the presence of *Mr*NV and XSV in the challenge group and absence in the control group.

KEY WORDS: *Artemia* · *Macrobrachium rosenbergii* nodavirus · Pathogenicity · Carrier · RT-PCR

INTRODUCTION

Live feed organisms play an important role in the dietary regimen of cultivated fish and shellfish, particularly during the larval stages. Among live feed organisms, the brine shrimp *Artemia* is of prime importance because of its nutritional and handling advantages (Sorgeloss et al. 1986). However, *Artemi*a nauplii are also considered possible vectors for viruses and bacteria, either as reservoirs or mechanical carriers. Examples are the bacteria *Bacillus, Erwinia, Micrococcus, Staphylococcus* and *Vibrio* (Austin & Allen 1982, Tatani et al. 1985, Muroga et al. 1989, Nicolas et al. 1989) and the viruses infectious pancreatic necrosis virus and nodavirus (Mortensen et al. 1993, Skliris & Richards 1998). Anecdotal reports from aquarium hobbyists have implicated live brine shrimp as a source of bacterial infection for larval and adult ornamental fish.

Macrobrachium rosenbergii is an economically important crustacean that is cultured on a large scale in many countries including India. A new viral disease similar to white tail disease (WTD) reported by Arcier et al. (1999) 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). Previously, this disease was reported from the French West Indies (Arcier et al. 1999), Taiwan (Tung et al. 1999) and China (Qian et al. 2003). The causative agent of WTD was originally reported to be a virus, subsequently identified as *Macrobrachium rosenbergii* nodavirus (*Mr*NV) (Arcier et al. 1999). *Mr*NV is a small, icosahedral, non-enveloped

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virus 26 to 27 nm in diameter. The genome is formed by 2 pieces of single-stranded RNA (ssRNA) (RNA1 and RNA2) of 2.9 and 1.26 kb, respectively, and there is a single polypeptide of 43 kDa in the capsid. Qian et al. (2003) subsequently reported the occurrence of an additional extra small virus (XSV) in prawns with WTD collected from China. The presence of more than 1 virus in crustacean diseases has previously been reported (Bonami 1980, Mari 1987). Sahul Hameed et al. (2004a) have observed the presence of XSV also in WTD-infected post-larvae (PL) of freshwater prawns in India. Since *Artemia* plays an important role in the feeding regimen of larval and PL stages of freshwater prawns, the present investigation was carried out to assess the pathogenicity of *Mr*NV and XSV for different developmental stages of *Artemia* and to investigate the possibility that it might transmit these viruses to PL of freshwater prawns.

MATERIALS AND METHODS

Preparation of viral inoculum. Naturally WTD-infected *Macrobrachium rosenbergii* PL with prominent signs of whitish muscle in the abdominal region were collected from hatcheries located near Nellore, Andhra Pradesh, India, and used as the source of viral inoculum for infectivity experiments. 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 × *g* for 20 min at 4°C and its supernatant was re-centrifuged at 10 000 × *g* for 20 min at 4°C before the final supernatant (stock viral extract) was filtered through a 0.22 µm pore membrane. The presence of *Mr*NV and XSV in the extract was confirmed by RT-PCR and it was stored at –20°C.

Collection and maintenance of experimental animals. Brine shrimp *Artemia* cysts were obtained from Bonneville Artemia International. Cysts that tested *Mr*NV and XSV-negative by RT-PCR (described below) were hatched in filtered seawater (30 ppt salinity) at 28 to –30°C. After 24 h incubation, hatched instar I nauplii were separated from the unhatched and empty cysts, and stocked in 20 l aquarium tank with fresh filtered seawater and continuous aeration to keep the food particles in suspension and to ensure oxygenation. The nauplii were fed on rice bran and reared to the adult stage. Required developmental stages nauplii, metanauplii, juveniles, sub-adults and adults were collected from this stock for experiments.

For experimental transmission, healthy *Macrobrachium rosenbergii* PL (10) 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 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 with *Artemia* nauplii twice daily.

***Artemia* challenge experiments.** Pathogenicity of *Mr*NV and XSV for different developmental stages of *Artemia* (nauplii, metanauplii, juveniles, sub-adults and adults) was tested by immersion or oral challenge. For immersion challenge, batches of healthy nauplii, metanauplii, juveniles, sub-adults and adults of *Artemia* at 100, 100, 100, 50 and 25 per 400 ml, respectively, were reared separately in beakers containing sterilized aerated seawater and covered to prevent contamination. They were fed rice bran. The stock viral extract was added to the water at 1 ml l^{-1} (Venegas et al. 1999, Chen et al. 2000). Control groups were exposed to a tissue suspension (0.1%) prepared from healthy prawns. Each trial was conducted in triplicate.

For oral challenge, *Artemia* nauplii (100/400 ml) were reared in sterilized 500 ml beakers containing 400 ml of sterilized aerated seawater. The inoculum of *Mr*NV and XSV was prepared by homogenizing WTD-infected prawns as described above followed by ultracentrifugation at 100 000 × *g* at 4°C for 1 h. The supernatant was discarded and the pellet was resuspended in 1 ml of sterile TN buffer. This viral suspension (oral virus stock) was mixed with rice bran for 1 h in an automatic shaker to prepare the viral feeding mixture (VFM). This mixture was tested for the presence of *Mr*NV and XSV by RT-PCR. The experimental *Artemia* were fed VFM for 3 d. After 3 d, the shrimp were fed normal untreated rice bran (URB). In the control group, *Artemia* were fed rice bran mixed with an ultrapellet suspension of muscle homogenate of healthy prawns (MRB) followed by URB. Each experiment was conducted in triplicate. The experimental *Artemia* were examined twice per day for clinical signs of disease. The number of dead shrimp was recorded and the cumulative percentage mortality was calculated. Note that the first larval stage of *Artemia* (instar I) is semi-embryonic and does not feed but that filter feeding begins approximately 12 h later after molting to the second larval stage (instar II), and that further development to the adult stage involves 15 molts over the succeeding 8 d.

For *Macrobrachium rosenbergii* feeding trials, *Artemia* nauplii exposed to *Mr*NV and XSV by immersion and oral challenge for 3 d as described above were collected, washed with sterile seawater and stored at –20°C. These were later thawed and fed to PL of *M. rosenbergi.* The PL (10 per beaker) were divided into 5 groups and maintained separately in beakers (5 l capacity) at 27 to –30°C and starved for 24 h. In Group

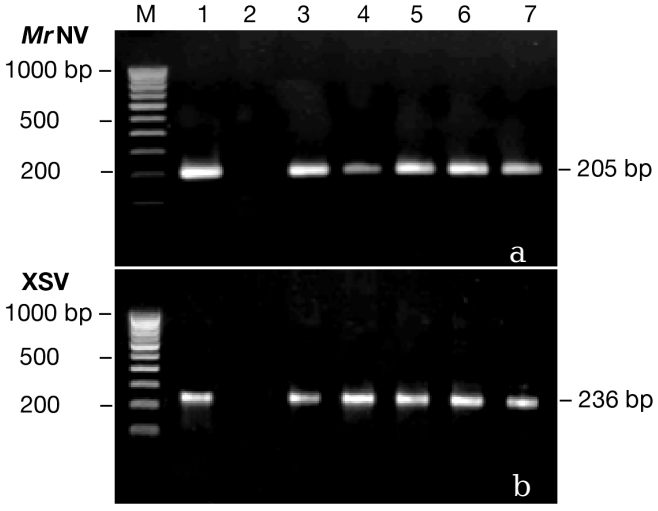

Fig. 1. *Artemia*. Agarose gels showing nested RT-PCR amplicons for detection of (a) *Macrobrachium rosenbergii* nodavirus (*Mr*NV) and (b) extra small virus (XSV) in different developmental stages of experimentally infected *Artemia* by immersion. M: DNA marker; Lane 1: virus suspension prepared from infected post-larvae; Lane 2: uninfected *Artemia*, Lane 3: nauplius; Lane 4: metanauplius; Lane 5: juveniles; Lane 6: sub-adults; Lane 7: adults

I, the PL were fed *Artemia* nauplii challenged with *Mr*NV and XSV by immersion; in Group II, the PL were fed *Artemia* challenged with *Mr*NV and XSV by the oral route; in Group III, the PL were fed *Artemia* not challenged with *Mr*NV and XSV; in Group IV (positive control), the PL were fed on meat of *Mr*NV and XSV-infected prawn PL with prominent signs of whitish abdominal muscle (confirmed by RT-PCR) and in Group V (negative control), the PL were fed with uninfected prawn PL. They were fed the respective feeds for 3 d. After the last feeding, all were fed commercial feed together with normal *Artemia* nauplii. Each experiment was conducted in triplicate. The *M. rosenbergii* individuals were examined twice per day, the number of deaths was recorded, and cumulative mortality was calculated. Moribund PL were collected for confirmation of WTD by RT-PCR (Fig. 1).

Total RNA extraction. For extraction of total RNA, the nauplii, metanauplii and juveniles of *Artemi*a were homogenized in pools of 20 to 25 live and 1 to 5 dead individuals, while sub-adults were homogenized as live or dead individuals in 300 µl of TN buffer (20 mM Tris –HCl, 0.4 M NaCl, pH 7.4). For freshwater prawn PL, 50 mg of whole PL was homogenized in TN buffer. The homogenates were centrifuged at 12 000 × *g* for 15 min at room temperature (27 to 30°C). The supernatant of the crude tissue extracts were 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 optical density ratio OD_{260nm}:OD_{280nm}.

RT-PCR and nested RT-PCR for *Mr*NV and XSV. 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. Nested RT-PCR was carried out using the products of RT-PCR wherever it was necessary to confirm infection. Primers used included published primers (Sahul Hameed et al. 2004a) and primers designed in our lab based on sequence data obtained from GenBank (AY222840 for *Mr*NV; AY247793 for XSV). The details of primer sequences, amplified product sizes and annealing temperatures are given in Table 1. 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 for 10 min at 68°C. For nested RT-PCR, reactions were performed in 20 µl reaction containing 2 µl RT-PCR product, 1 µM of each internal primer (*Mr*NV or XSV), 200 µM deoxynucleotide

Table 1. Pairs of primers used to detect *Macrobrachium rosenbergii* nodavirus (*Mr*NV) and extra small virus (XSV) using RT-PCR and nested RT-PCR techniques

Primer name	Annealing temp. (°C)	PCR product size (bp)	Sequences	Primer orientation
*Mr*NV-external	55	425	GCGTTATAGATGGCACAAGG AGCTGTGAAACTTCCACTGG	Upstream Downstream
*Mr*NV-internal	55	205	GATGACCCCAACGTTATCCT GTGTAGTCACTTGCAAGAGG	Upstream Downstream
XSV-external	55	546	CGCGGATCCGATGAATAAGCGCATTAATAA CCGGAATTCCGTTACTGTTCGGAGTCCCAA	Upstream Downstream
XSV-internal	55	236	ACATTGGCGGTTGGGTCATA GTGCCTGTTGCTGAAATACC	Upstream Downstream

triphosphate and 1.25 U *taq* DNA polymerase in PCR buffer supplied with a commercially available kit (Finnzymes, Espoo). The nested RT-PCR protocol for both viruses comprised 35 cycles of 1 min at 95°C, 1 min at 55°C and 1 min at 72°C with final extension of 10 min at 72°C. The RT-PCR and nested RT-PCR products (10 µl) were then analyzed by electrophoresis on a 1% agarose gel stained with ethidium bromide and visualized by ultraviolet transillumination.

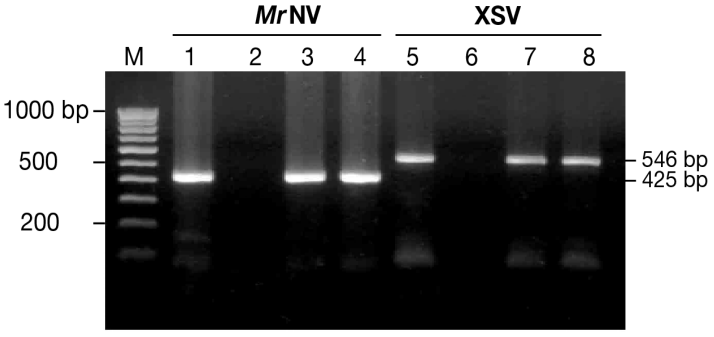

Fig. 2. *Macrobrachium rosenbergii.* RT-PCR detection of *Mr*NV and XSV in post-larvae (PL) fed with different feeds. M: DNA marker; Lanes 1 and 5: PL fed with *Mr*NV- and XSV-infected PL; Lanes 2 and 6: healthy PL; Lanes 3 and 7: PL fed with *Artemia* exposed to *Mr*NV and XSV by immersion; Lanes 4 and 8: PL fed with *Artemia* exposed to *Mr*NV and XSV by oral routes

RESULTS AND DISCUSSION

The cumulative percent mortality for different developmental stages of *Artemia* exposed to *Mr*NV and XSV by immersion and oral routes (Table 2) were not significantly different from those for the negative controls. The percent mortality ranged from 3 to 21 by

Table 2. *Artemia.* Cumulative percent mortality levels in different developmental stages of *Artemia* exposed to *Mr*NV or XSV by immersion and oral routes. –: negative RT-PCR reaction; +: *Mr*NV or XSV positive by nested RT-PCR; ++: *Mr*NV or XSV positive by 1-step RT-PCR. WTD: white tail disease

Stages	Method of challenge	No. of ind. used	Cumulative mortality (%)	WTD detection by RT-PCR *Mr*NV	XSV
Nauplius	Control	300	19.6	–	–
	Immersion	300	21.0	+	++
	Oral	300	20.3	+	++
Metanauplius	Control	300	19.3	–	–
	Immersion	300	21.4	+	++
	Oral	300	22.6	+	++
Juveniles	Control	300	16.1	–	–
	Immersion	300	17.3	+	++
	Oral	300	16.8	+	++
Sub-adults	Control	150	7.1	–	–
	Immersion	150	6.9	+	++
	Oral	150	7.6	+	++
Adults	Control	75	3.6	–	–
	Immersion	75	2.8	+	++
	Oral	75	3.1	+	++

Table 3. *Macrobrachium rosenbergii.* Details of experiments (carried out in triplicate) on rearing of post-larvae (PL) *M. rosenbergii* fed healthy *Artemia* or *Artemia* exposed to white tail disease (WTD). ++: *Mr*NV- or XSV-positive by 1-step RT-PCR

Group	No. *Artemia* used	Feeding regimen	Cumulative mortality (%) on Day 1	3	5	6	7	8	9	WTD detection by RT-PCR *Mr*NV	XSV
I	30	Fed with *Artemia* exposed to *Mr*NV/XSV by immersion	0	0	0	40	70	90	100	++	++
II	30	Fed with *Artemia* exposed to *Mr*NV/XSV orally	0	0	40	65	80	100	0	++	++
III	30	Fed with *Artemia* not exposed to *Mr*NV/XSV	0	0	0	0	0	0	0	–	–
IV	30	Fed with *Mr*NV/XSV-infected PL	0	40	100	0	0	0	0	++	++
V	30	Fed with uninfected PL	0	0	0	0	0	0	0	–	–

immersion and 3 to 23 by oral challenge. None of the challenged *Artemia* showed any signs of disease, despite the fact that they were positive for both viruses (*Mr*NV and XSV) by RT-PCR.

Experiments where *Macrobrachium rosenbergii* PL were fed with *Artemia* exposed to *Mr*NV and XSV (Table 3) resulted in 100% mortality in Groups I, II and IV at 9, 8 and 5 d post challenge, respectively, whereas no mortality occurred in Groups III and V (negative controls). RT-PCR was positive for both viruses for PL collected from Groups I, II and IV but negative for Groups III and V (Fig. 2). Typical clinical signs of WTD were observed in most of the PL collected from Groups I, II and IV.

Although the developmental stages of *Artemia* showed no gross signs of WTD upon challenge with *Mr*NV and XSV, the nested RT-PCR tests were positive for both viruses after challenge, suggesting that they would act as reservoirs or carriers for these viruses. This was confirmed by transmission of *Mr*NV and XSV to *Macrobrachium rosenbergii* PL, satisfying River's postulate (Iwanowicz & Goodwin 2002) and implicating one or both of these viruses as responsible for WTD in *M. rosenbergii* (Sahul Hameed et al. 2004b).

Mortensen et al. (1993) detected infectious pancreatic necrosis virus (IPNV) in *Artemia* and suggested that it might be an IPNV reservoir and a vector when eaten by fish. Skliris & Richards (1998) have also suggested that *Artemia* and rotifers might act as nodavirus carriers. In addition, virus-like particles have been reported in the shell gland of *Artemia* (Criel 1980) and *Artemia* has been used as a mechanical carrier in an infectivity bioassay with *Baculovirus penaei* (Overstreet et al. 1988). On the other hand, Sahul Hameed et al. (2002) carried out pathogenicity experiments on *Artemia* challenged with white spot syndrome virus (WSSV) and showed that WSSV failed to infect any of its developmental stages. Nor did mortalities occur in juvenile *Penaeus indicus* fed with these challenged *Artemia.* Similar results were obtained by Chang et al. (2002) with WSSV PCR-positive *Artemia* cysts.

In contrast to the work with WSSV, our observations clearly indicate that *Artemia* may act as a reservoir or mechanical carrier for *Mr*NV and XSV and transmit it horizontally to *Macrobrachium rosenbergii* in a hatchery. Further work with histology and/or transmission electron microscopy is needed to determine whether the *Artemia* are actually infected with these viruses or whether they are simply mechanical carriers.

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