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Experimental exposure of *Artemia* to Hepatopancreatic parvo-like Virus and Subsequent transmission to post-larvae of *Penaeus monodon*

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ABSTRACT

The different life stages of *Artemia franciscana* were experimentally exposed to Hepatopancreatic parvo-like virus (HPV), in order to evaluate the possibility of *Artemia* acting as reservoir or carrier for HPV. All the five developmental stages of *Artemia* were challenged with HPV both by immersion and oral infection routes. The viral infectivity to *Artemia* was studied by PCR but not much difference in mortality between control and challenge groups were observed. To confirm the vector status of *Artemia* for HPV, the HPV exposed *Artemia* were fed to postlarval forms of *Penaeus monodon*. Post-larvae of *P. monodon* were fed with HPV exposed *Artemia* and could get infected upon feeding on them. Mortality was observed in the post-larvae, which were fed with HPV exposed *Artemia*, and whereas no mortality was observed in post-larvae fed with *Artemia* not exposed to HPV and these post-larvae were PCR negative for HPV, as well. Results of this experiment suggest that *Artemia* might be a possible horizontal transmission pathway for HPV. Further research however is required with histology, immunohistochemistry and transmission electron microcopy to determine whether the *Artemia* are actually infected with this virus or whether they are simply mechanical carriers. This will enable us to understand better whether *Artemia* is a carrier of this virus and if so the mechanism involved.

1. Introduction

Aquaculture is one of the fastest growing food production sectors in the world (Subasinghe et al., 1998). Disease out break in culture ponds and pathogenic infections in hatcheries result in low production and high economic loss in aquaculture. Such infectious diseases can spread through food, water and other exogenous sources. Care is to be taken to produce high quality post-larvae, which are stocked in nurseries and grow out ponds for more sustainable production. For this purpose live feed like *Artemia* is being used as food for postlarval stages in hatcheries due to their high fat content (Leger et al., 1986; Sorgeloss et al., 1986). *Artemia*'s nutritional value is considered to be a better diet than formulated diets for postlarval stages of invertebrates and vertebrates in aquaculture (Gallagher and Brown, 1975; Barahona-Farnades and Girin, 1976; Soejima et al., 1980). *Artemia* nauplius form an important live feed for a variety of finfishes and shellfishes and are given to over 85% of aquaculture species around the world. It has been documented by several authors that feeding nutritious and highly unsaturated fatty acid (HUFA) enriched *Artemia* to post-larvae of *Penaeus monodon* resulted in improved postlarval quality (Tackaert et al., 1992; Rees et al., 1994; Kontava et al., 1995). Use of live feed is also practiced in grow out ponds to increase productivity (Liao, 1985).

Using such live and unprocessed feed in shrimp aquaculture poses great threat as it acts as potential carrier of various diseases including bacterial and viral (Tatani et al., 1985; Colorni et al., 1987; Muroga et al., 1987; Nicolas et al., 1989; Mortensen et al., 1993). Although Artemia is widely used as live feed due to its nutritional and operational advantages, it is considered to be a possible vector for the introduction of pathogens into the rearing systems. *Artemia* nauplii carry a large bacterial load that may be transferred from live prey into the tanks of fish and shellfish larvae. Some bacteria have been reported to be the source of diseases and high mortalities in fish larvae, and live feeds are thought to be responsible (Tatani et al., 1985; Muroga et al., 1987; Nicolas et al., 1989). Therefore, *Artemia* nauplii are often treated in order to reduce the bacteria associated with them prior to feeding them to the larvae. Role of *Artemia* in dissemination of bacterial pathogen in ornamental fish have been reported.

Mortensen et al. (1993) showed the role of *Artemia* as a potential reservoir and vector for infectious pancreatic necrotic virus (IPNV). Sudhakaran et al. (2006) have studied *Artemia* as a vector for MrNV and XSV through pathogenicity experiments. Skliris and Richards (1998) through pathogenecity experiments have also reported that *Artemia* acts as a carrier for nodavirus.

Hepatopancreatic parvo-like virus (HPV) infection have been linked to disease, especially in younger stages of life of prawn (Flegel et al., 1995). HPV is capable of causing mortality ranging from

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50–100% after 4–8 weeks in juveniles of *Penaeus merguiensis* (Lightner and Redman, 1985). The Hepatopancreatic parvo-like virus apart from infecting younger life stages, also causes stunted growth in adult prawns leading to high economic loss world wide (Roubal et al., 1989; Lightner, 1996; Flegel et al., 1999). This virus is known to infect 10 penaeid prawn species around the world and the fresh water prawn, *Macrobrachium rosenbergii* (Lightner and Redman, 1985; Anderson et al., 1990; Lightner, 1996). The transmission of HPV is believed to be both vertical and horizontal (Lightner and Redman, 1992).

As *Artemia* is being used as live feed for postlarval and juveniles of shrimp, there could be a possibility of spread of HPV infection through *Artemia*. Moreover the incidence of HPV is known to be profound in larval stages. In the present investigation the susceptibility of *Artemia* in all the developmental stages to HPV has been studied through pathogenicity experimentation. The possibility of *Artemia* acting as reservoir and vector for HPV that could infect marine shrimp was also studied.

2. Materials and methods

2.1. Experimental animals

Brine shrimp *Artemia franciscana* cysts were obtained from Bonneville *Artemia* International USA. The cysts were incubated at 28–30 °C in filtered seawater for 24 h. After 24 h incubation, hatched instars I nauplii were separated from the unhatched and empty cysts by filtering through a 160 nm synthetic fiber net filter. They were separated twice from hatching debris and thoroughly rinsed with tap water. The separated instars I nauplii were stocked in a 20–1 aquarium tank containing fresh filtered seawater with continuous aeration to keep the food particles in suspension and to ensure adequate oxygenation. The nauplii were fed on rice bran and reared to the adult stages. The required stages (nauplius, metanauplius, juveniles, sub-adults and adults) of *Artemia* were taken from this stock for experimental purpose.

2.2. Preparation of viral inoculum

Samples of *P. monodon* post-larvae suspected to be HPV infected were collected from hatcheries in Kakinada, Andhra Pradesh, India. The post-larvae were kept in seawater-filled, aerated aquaria and maintained at 23–28 °C. They were fed with commercially available shrimp food. From this batch, 20 post-larvae were randomly selected and checked for HPV infection by microscopic examination of malachite green-stained squash mounts, while another sample of post-larvae was used for PCR assay following a previously described protocol of Phromjai et al., 2002 that yielded HPV-specific amplicons of 441 bp. The positive PLs were collected and frozen for use as the source of viral inoculum for infectivity experiments. Frozen, infected post-larvae 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 fluid was recentrifuged at 10,000*g* for 20 min at 4 °C before filtering the final supernatant fluid (stock viral extract) through a 0.22 μm pore membrane. The presence of HPV in the extract was confirmed by PCR. It was stored at −20 °C until further use. The homogenate was also tested for viruses like MBV and WSSV by PCR, as multiple viral infections are common in India. A similar concentration of HPV, MBV and WSSV free *P. monodon* post-larvae was also homogenized for use as a negative control.

2.3. Experimental design for Artemia challenge

Artemia developmental stages were carefully aspirated with the aid of a Pasteur pipette and placed in a petridish where the individual micro crustaceans were counted with the aid of a magnifying hand lens. The nauplii, metanauplii and juveniles thus selected were stocked at densities of 400 while sub-adults and adults were stocked at densities of 100 each in a 500 ml beaker containing filtered and sterilized seawater. All the different life stages of brine shrimp were screened for HPV by PCR before stocking. Each stage was maintained in triplicate of which one served as control. All the developmental stages were infected by both oral and immersion routes for a period of one week.

In immersion challenge, batches of healthy nauplii, metanauplii, juveniles, sub-adults and adults of *Artemia* were reared separately in sterilized, aerated seawater. The beakers were covered to prevent contamination. The inoculum of HPV prepared as described above was introduced to the water only once at a volume equal to 0.1% of the total rearing medium (1 ml/l) (Paynter et al., 1992; Venegas et al., 1999; Chen et al., 2000). The control groups were exposed to healthy PL meat homogenate free from viruses like HPV, MBV, WSSV (tested by PCR). Each trial was conducted in triplicate. After a period of 7 days post infection to virus the samples were collected, washed with sterile seawater thoroughly and total DNA extracted for PCR analysis of HPV.

For infection through oral route, batches of healthy nauplii, metanauplii, juveniles, sub-adults and adults of *Artemia* were reared in sterilized 500 ml beakers containing 400 ml of sterilized aerated seawater. The inoculum of HPV was prepared by homogenizing HPV infected *P. monodon* post-larvae as described above followed by ultracentrifugation at 100,000*g* at 4 °C for an hour. The supernatant fluid 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 an hour in an automatic shaker to prepare the viral feeding mixture (VFM). This mixture was tested for the presence of HPV by PCR. The experimental animals were fed with this viral feed mixture (VFM) for 3 days. After 3 days the animals were fed on unexposed rice bran. In control group the animals were fed on rice bran mixed with ultra pellet suspension of PL meat of healthy *P. monodon* post-larvae followed by unexposed rice bran. The experimental animals were examined twice a day for clinical signs of disease. The percentage survival of larvae after treatment was determined by counting the live larvae using a magnifying hand lens. The numbers of dead animals were calculated by subtracting the number of live animals from the total animals taken for the experimentation.

The adult *Artemia* were collected from the rearing tank and exposed to HPV by both the methods (immersion and oral) as described above for one week. After the exposure the animals were collected, washed with sterile seawater and stored at −20 °C. These were then used to feed post-larvae of *P. monodon*.

2.4. Infectivity study of HPV treated Artemia to P. monodon

Healthy postlarval stages of *P. monodon* were grouped into five batches (50 per batch) and maintained in 1-L beakers filled with filtered and sterilized sea water at ambient temperature. The post-larvae were starved for 24 h prior to experimentation and the feeding procedures were followed for 3 days. The group I, post-larvae were fed with *Artemia* exposed to HPV by immersion method. The group II post-larvae were fed with *Artemia* exposed to HPV by oral route. In-group III the post-larvae were fed with *Artemia* not exposed to HPV. The group IV post-larvae were fed with HPV infected post-larvae meat and served as positive control group and were subsequently fed with commercial post-larvae feed. The group V post-larvae were fed with uninfected postlarval meat and