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Polychaete worms — a vector for white spot syndrome virus (WSSV)

K. K. Vijayan*, V. Stalin Raj, C. P. Balasubramanian, S. V. Alavandi, V. Thillai Sekhar, T. C. Santiago

Central Institute of Brackishwater Aquaculture (CIBA-ICAR), 75 Santhome High Road, Chennai-600 028, India

ABSTRACT: The present work provides the first evidence of polychaete worms as passive vectors of white spot syndrome virus (WSSV) in the transmission of white spot disease to *Penaeus monodon* broodstocks. The study was based on live polychaete worms, *Marphysa* spp., obtained from worm suppliers/worm fishers as well as samples collected from 8 stations on the northern coast of Tamilnadu (India). Tiger shrimp *Penaeus monodon* broodstock with undeveloped ovaries were experimentally infected with WSSV by feeding with polychaete worms exposed to WSSV. Fifty percent of polychaete worms obtained from worm suppliers were found to be WSSV positive by 2-step PCR, indicating high prevalence of WSSV in the live polychaetes used as broodstock feed by hatcheries in this area. Of 8 stations surveyed, 5 had WSSV positive worms with prevalence ranging from 16.7 to 75%. Polychaetes collected from areas near shrimp farms showed a higher level of contamination. Laboratory challenge experiments confirmed the field observations, and >60% of worms exposed to WSSV inoculum were proved to be WSSV positive after a 7 d exposure. It was also confirmed that *P. monodon* broodstock could be infected with WSSV by feeding on WSSV contaminated polychaete worms. Though the present study indicates only a low level infectivity in wild polychaetes, laboratory experiments clearly indicated the possibility of WSSV transfer from the live feed to shrimp broodstock, suggesting that polychaete worms could play a role in the epizootiology of WSSV.

KEY WORDS: Broodstock · Live feed · *Penaeus monodon* · Polychaete worms · Vector · White spot syndrome virus · WSSV

INTRODUCTION

During the last decade, white spot syndrome virus (WSSV) has emerged as the major shrimp pathogen causing epizootics and heavy crop failures across the world (Rosenberry 2001). Since its outbreak in 1992 to 2001 in Asia alone, the loss of farmed shrimp production due to WSSV epizootics is estimated to be about US $4 to 6 billion (Lightner 2003). Among all the known crustacean viruses, WSSV has the widest host range including susceptible species, carriers and reservoir hosts (Flegel 1997). Frequent disease outbreaks in the shrimp farms of India and Asia lead to the offloading of dead and decayed shrimps carrying a heavy load of this virus into the coastal ecosystem. Horizontal transmission of WSSV from the affected shrimp farms to the neighboring ecosystem has created a realistic scenario in which the receiving ecosystem carries the WSSV load in the form of live or dead tissues, dead and decomposed tissues and free virions. Invertebrate filter feeders such as bivalve molluscs ingest and accumulate particulate material, including viral particles (Canzonier 1971, Hay & Scotti 1986, Mortensen 1993). WSSV virions can remain infective in the decaying tissues or in detritus up to 4 d, contrary to the common belief that free virus cannot survive in natural waters more than 24 h (Bondad-Reantaso et al. 2001). This virus could be transmitted to benthic crustaceans and other fauna through different feeding pathways such as filter feeding, detritus feeding, and predation. Viruses can also pass into the digestive tracts of other invertebrates, and can persist in the alimentary canal, potentially making the animal a passive carrier or vector of the virus. When these passive carriers are

*Email: kkvijayan@hotmail.com

consumed by the shrimp, they can potentially infect the shrimp with WSSV. Hence, the passage of the viral pathogen to shrimp brood stock in the hatchery through feeding of infected prey items is a realistic possibility.

Polychaetes, form an indispensable component of the maturation diet of penaeid shrimp broodstock in hatcheries all over the world due to their high nutritive value (Bray & Lawrence 1992). In India, almost all penaeid hatcheries use polychaete worms to promote maturation and spawning of wild caught broodstock/spawners of *Penaeus monodon.* Furthermore, polychaetes are reported to be the most prominent zoobenthos in shrimp farming systems and have been recognized as an important prey item of several penaeid species (Nunes et al. 1997). Since polychaete abundance is considered an indication of pond productivity and availability of natural food (Crockett et al. 1988), inoculation of polychaetes in shrimp ponds is a common practice in many countries (Nunes et al. 2000). In India, polychaete collection has emerged as an artisanal fishery in many coastal states and the annual consumption of polychaetes by shrimp hatcheries is estimated to be about 16 to 20 t. As there is no polychaete aquaculture in India, the entire polychaete biomass used in shrimp aquaculture is collected from natural habitats. Though polychaete worms are routinely used as a live broodstock feed in shrimp hatcheries and growout systems, to date there have been no investigations to screen the worms for the presence of potential pathogens such as WSSV. In the present study, we performed studies to answer the following questions: Are natural populations of polychaetes infected with WSSV, and can *P. monodon* broodstock become infected with WSSV through feeding on polychaete worms with ingested WSSV virions? The answers to these questions are essential to an understanding of the epizootiology and management of WSSV.

MATERIALS AND METHODS

Polychaete worms. Live polychaete worms with an average body weight of 8 ± 2.3 g were obtained from worm suppliers/worm fishers. Each sample constituted 5 worms, randomly selected and pooled to make 1 sample. The worms were dissected and digestive tracts removed and used for testing. In addition to this, samples were also collected from 8 selected stations on the northeast coast of Tamilnadu, India (Fig. 1). Among the 8 stations, 5 were in areas which directly received shrimp farm discharges (Stns 3, 4, 5, 6 and 7) whereas the remaining stations were in non-shrimp farming areas (1, 2 and 8). The first station in the Adyar estuarine ecosystem was an abandoned shrimp farm and had been isolated from natural water bodies since 1979. Further, there were no shrimp farms within a 30 km radius, and, therefore, this station was taken as a control site. From each station, 12 worms were randomly taken and preserved in 90% ethanol until PCR analysis for WSSV. In addition to this, at least 5 animals were preserved in 4% formaldehyde for taxonomic analysis.

Identification of polychaetes. The animals were identified according to Fauvel (1953). Nearly 95% of samples were dominated by the eunicid polychaete

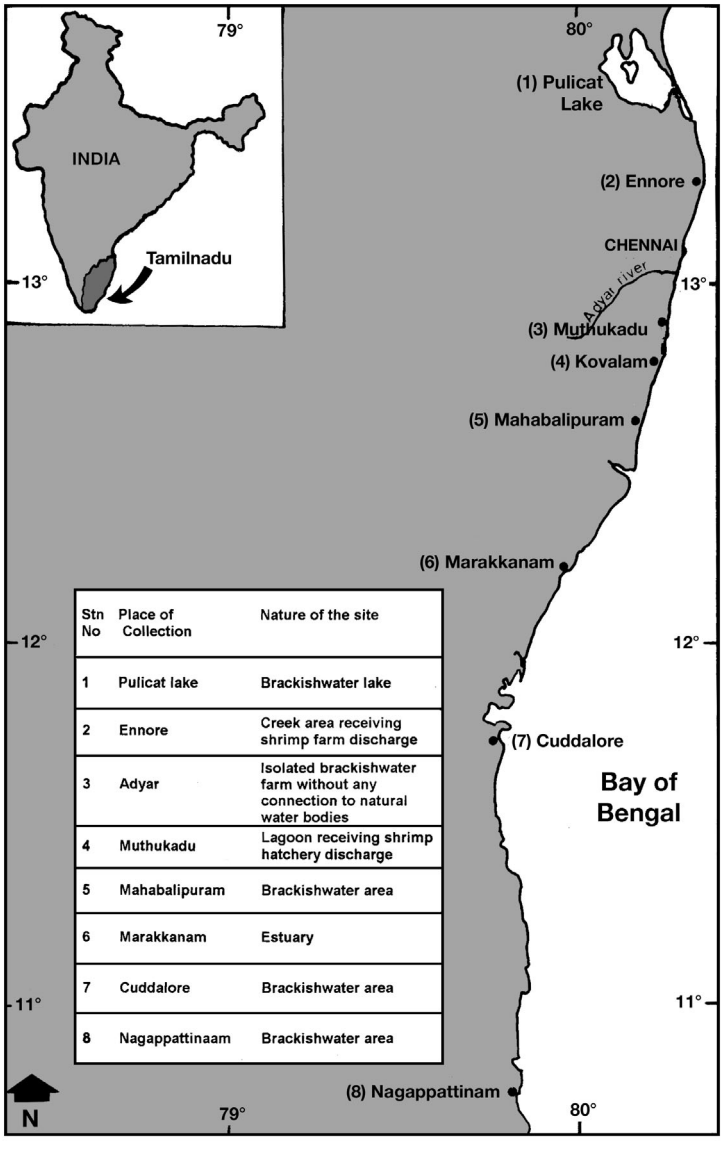

Fig. 1. Location of sampling sites of polychaete worms

Marphysa gravelyi. Therefore, this species was selected and used for the present study

Screening of polychaetes for WSSV using PCR. Samples preserved in 90% ethanol were screened for WSSV using a commercial 2-step PCR kit (Bangalore Genei). A DNA template was prepared from each sample using a modified alkaline lysis method (Vijayan et al. 1998). Briefly, 40 to 50 mg of tissue sample was homogenised in sterile and disposable tissue homogeniser with 500 µl lysis buffer (25 mM Tris HCl, 10 mM EDTA, 50 mM glucose, 0.2 NaOH and 1% SDS at pH 8), and then the suspension was boiled for 10 min, cooled and centrifuged at 12 000 × *g* for 10 min. Fifty microliters of the clear supernatant was removed and 1 µl of the supernatant was used as the DNA template in the PCR reaction. The PCR kit is comprised of an external and internal primer. The amplification was performed in an Eppendorf Master cycler. The first and second step of amplification was expected to amplify a WSSV DNA fragment of 650 and 300 bp respectively. The PCR products were analysed on 1.5% agarose gel and bands were visualized using ethidium bromide staining using a UV transilluminator.

Polychaete worms *Marphysa gravelyi* collected from the control site (Stn 1) were randomly screened for WSSV using nested PCR. The WSSV free worms were brought to the Central Institute of Brackishwater Aquaculture (CIBA) laboratory (Chennai), maintained in 3 l capacity earthen pots with brackishwater of salinity 15‰ and starved for 24 h.

WSSV inoculum. Highly virulent WSSV infected *Penaeus monodon* samples collected and stored at –70°C at CIBA during an epizootic at Nellore, Andhrapradesh (India) in 2001 were used for the preparation of the WSSV inoculum. After removal of the exoskeleton and hepatopancreas, tissues of the cephalothorax of WSSV-infected shrimp were homogenised in sterile water. After centrifugation (1000 × *g* for 10 min at 4°C), the supernatant was filtered through a 0.45 µm membrane and used immediately.

Infection by exposure. Fifty worms were divided into 5 groups of 10 each and introduced into 5 separate polyethylene bottles of 3 l capacity containing 1 kg sterilized substrate (70% sand, 16.5% silt and 13.5% clay) moistened with sterilized brackish water (salinity: 15‰). Water was added until a final depth of 20 mm above the top of the soil was reached. Three groups of worms were exposed by adding 100 ml of WSSV inoculum (experimental) and the remaining groups treated using blank inoculum (control). The top layer of water was exchanged with fresh aerated brackishwater of the same salinity from the third day onwards. At the end of the 7 d culture period, the worms were removed and washed with sterile distilled water. Three worms from each group were selected randomly, washed with phosphate buffer solution and preserved in 95% ethanol for WSSV screening using the 2-step PCR kit. The remaining worms were stored at –70° C to be used in the *Penaeus monodon* infectivity studies.

Infection of *Penaeus monodon* broodstock with WSSV contaminated *Marphysa gravelyi*. Live *P. monodon* broodstocks (60 to 90 g) with undeveloped ovaries were obtained from trawl catches and immediately transported to the CIBA laboratory (Chennai). The animals were individually screened for WSSV using nested PCR, and subsequently 12 WSSV-free individuals were selected for the experiment. Shrimp were divided into 2 groups of 6 (experimental and control) and maintained in aerated seawater (salinity: 28 to 30‰; pH: 7.8 to 8.2; temperature: 28 to 30°C). After starving for 48 h, the experimental group was fed with WSSV exposed worms at the rate of 10% of their body weight, whereas the control group was fed with WSSV-free worms collected from the control site. The experiment was terminated on the 7th d and all the animals were individually tested for WSSV using 2-step PCR.

RESULTS

Fifty percent of samples collected from the worm suppliers were PCR positive, and of these 13% were first step PCR positive indicating the presence of WSSV in the digestive tract of polychaete worms used as broodstock feed in shrimp hatcheries (Figs. 2 & 3). The prevalence of WSSV recorded in the samples from the field stations receiving shrimp farm effluent ranged from 16.7 to 75% (Fig. 2) whereas stations in non-shrimp farm areas (Stns 1, 2 and 8) were free from WSSV. Polychaete sample from Stn 4, which is in close proximity to shrimp farms, was 1-step PCR positive, and the rest were 2-step PCR positive (Fig. 3).

Challenge of polychaete worms with WSSV

More than 60% of worms exposed to WSSV inoculum were proved to be PCR-positive for WSSV after the 7 d experiment (Table 1). Of the 19 out of 30 that were PCR-positive, only 1 was 1-step positive, indicating a low level of contamination.

Challenge of *Penaeus monodon* broodstock with WSSV contaminated polychaete worms

A high level of infection (up to 83%) could be observed in *Penaeus monodon* broodstock fed with WSSV infected polychaete worms (Table 1). Out of

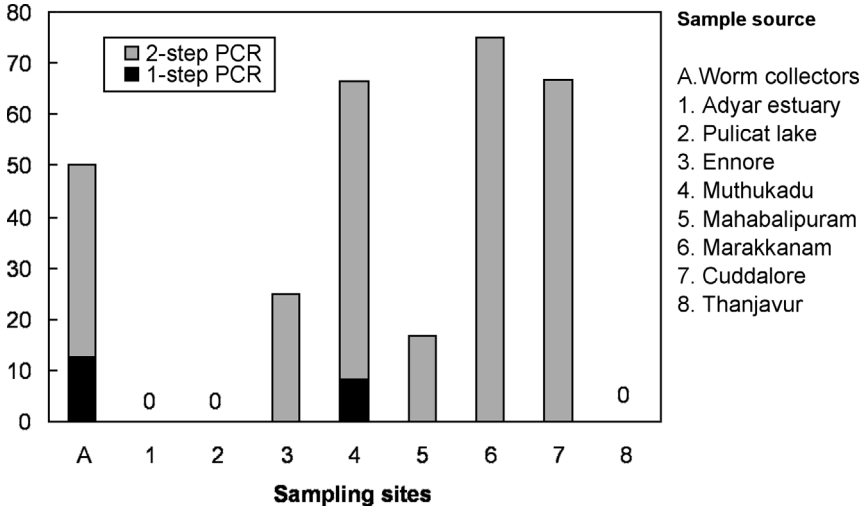

Fig. 2. *Marphysa gravely*. Percentages of wild polychaete worms infected with white spot syndrome virus

6 broodstock fed with infected worms, 5 were proven to be 2-step PCR positive whereas all the 6 control individuals were negative.

DISCUSSION

At present, little is known about the role of benthic invertebrates such as polychaetes as vectors for viruses. The present study indicated that the shrimp pathogen WSSV, considered to be the most serious virus disease of farmed shrimps, could be transmitted through polychaete worms to infect *Penaeus monodon* broodstock. Polychaete worms accumulated the viral pathogen in their digestive tract through feeding. Though the virus itself is not infecting the worms, worms remain potent in their digestive tracts and acts as a passive vector of WSSV in aquatic systems. When these worms are fed to broodstock shrimp in the hatchery, the shrimp ingest the virus in the worms causing patent WSSV infection. This observation is similar to earlier reports on the mode of virus transmission in fishes (Mortensen 1993) and in molluscs (Canzonier 1971, Hay & Scotti 1986). Polychaete worms, being detritivore and active benthic feeders (Fauchald et al. 1979) ingest the pathogen and serve as a passive vector of WSSV, transmitting the virus to shrimp.

Our results indicate that infectious WSSV virus particles in brackishwater sediments can be passed via the alimentary canal of polychaetes and infect shrimp, when the worms are fed to broodstock in shrimp hatcheries. The likelihood of infection by this route is much higher in hatchery tanks compared to natural conditions, as polychaetes are a routine broodstock feed for shrimp maturation (Ogle 1992, Cahu et al. 1994, Louis & Passos 1995, Sudaryono et al. 1995). The practice of feeding unscreened worms increases the risk of pathogen transmission, especially when the worms are collected from shrimp farming areas where WSSV is prevalent. The present study recorded WSSV prevalence up to 75% in areas where WSSV epizootics were common. Though the viral load is low (2-step PCR positive), this level of pre-patent infection in spawners may be sufficient to transmit the virus to the progeny. Further, it has been reported that excision of pereiopods, eyestalk ablation or spawning stress, can be sufficient to trigger viral multiplication (Peng et al. 1998), further increasing the risk of vertical transmission to the offspring or horizontal transmission to other broodstock.

The present study highlights the urgent need to adopt management measures such as screening of polychaete worms for WSSV using 2-step PCR, before use as a broodstock feed. Horizontal infection of WSSV through polychaetes could be prevented by exposing the worms to temperatures of approximate 70°C for 5 min (Chang et al. 1998). However, this may affect

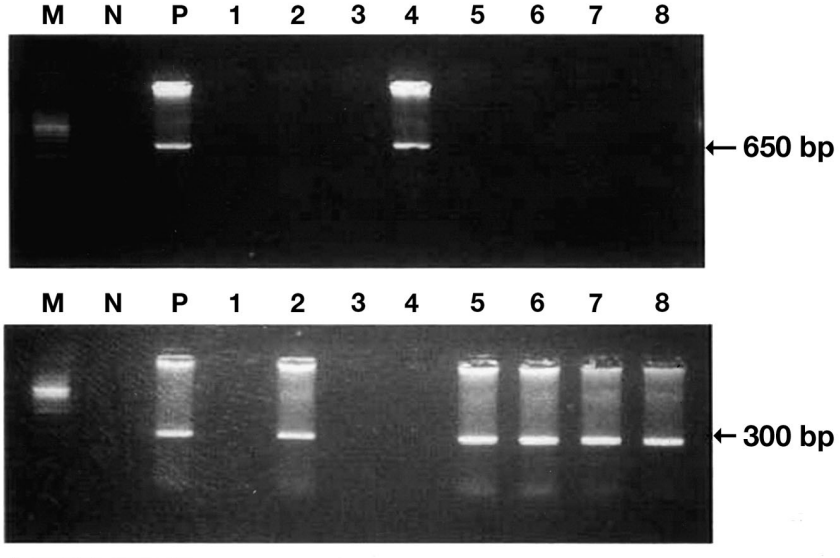

Fig. 3. *Marphysa gravelyi.* Polymerase chain reaction (PCR) analysis for the presence of WSSV in polychaete worms. Top panel: 1st step PCR; bottom-panel: 2nd step PCR. Lanes: M, DNA marker; N, negative control; P, positive control; 1 to 8, sampling sites. Note: sample from Stn 4, (tested 1-step PCR positive) not tested for 2-step PCR reaction

Table 1. PCR test results of WSSV in polychaete worms *Marphysa gravelyi* and tiger shrimp *Penaeus monodon* broodstock

Groups	1-step PCR positive	2-step PCR positive	2-step PCR negative	Prevalence (%)
Polychaetes exposed to WSSV (n = 30)	1/30	18/30	11/30	63
Polychaetes unexposed control (n = 20)	0/20	0/20	0/20	0
P. monodon broodstock exposed to WSSV (n = 6)	0/6	5/6	1/6	83
P. monodon broodstock control (n = 6)	0/6	0/6	6/6	0

other biochemical qualities of the worms which help in the gonadal maturation of the shrimp, and needs to be studied further. Another alternative is the production of polychaetes in controlled virus-free conditions. Polychaete farming technology has been developed and is practiced in Europe (Olive 1999). Although shrimp hatcheries in India and other Asian countries depend almost entirely on natural polychaete stocks, contamination of wild polychaete populations with lethal pathogens such as WSSV demonstrates the need to produce pathogen free polychaete worms through aquaculture.

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