Experimental challenge of white spot syndrome virus (WSSV) From Polychaete *Pereneris cultifera* To Peppermint shrimp *Lysmata wurdemanni* in captivity

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ABSTRACT

White Spot Syndrome Virus (WSSV), a large double-stranded DNA virus, is the major and most serious pathogen in the shrimp aquaculture industry. White spot syndrome virus (WSSV) collected from Polychaete - *Perinereis cultrifera* which was orally transmitted with white spot syndrome virus (WSSV) and conformed with PCR identification is transferred to peppermint shrimp *Lysmata wurdemanni* by intramuscularly injection and through oral rout.100% mortality of *L. wurdemanni* was resulted on 19th day in oral rooted and on 21st day for intramuscularly injected shrimp. The PCR analysis observed the appearance of a prominent band of PCR amplified product of WSSV-DNA at 848 bp at 24 hrs to 8 day of WSSV post-inoculum to the pleopods of *L. wurdemanni.* Whilst these band 650 bp, 296 bp were appeared from day 9 to 15. On day 14, 15,16 and 20 days of post inoculum the DNA bands were showed uniformity in the molecular weight of 650bp and it was encountered as 296, 650 and 910 bp an days 17, 18, 19, and 21 respectively in the *L. wurdemanni* exposed to WSSV by oral route and the WSSV appeared more virulent than in other penaeid species. There was no evidence of positive band observed in the unexposed *L. wurdemanni*. Mortality of 90.0 and 93.30 % were noticed in the *L. wurdemanni* shrimps treated oral intramuscular route of WSSV inoculum and was observed on 18 and 20 days. Nevertheless, a drastic change in the survival from 100 to 40.0 and 63.30 % was noticed. The day of experiment continued up to 21 days and on day 22 the experiment was terminated. 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 shrimp broodstock feed in hatchery sector.

Keywords: *Perinereris cultrifera,* , *Lysmata wurdemanni,* WSSV, PCR and hatchery

Introduction

White spot syndrome virus (WSSV) presently overshadows all other disease agents as the leading cause of production losses. It is considered to be one of the majority serious viral diseases of shrimp, and the causative organism has been identified as white spot syndrome virus (WSSV). It was first reported in Thailand as an accidental infection in laboratory-reared shrimp in early 1994, Wongteerasu- paya et al., 1995, but the first farm infections were not reported until late 1994 Wongteerasupaya et al., 1996. This virus has caused the loss of several million dollars in shrimp

culture industries in India and the loss continues even now, Anon., 1996. The WSSV has been isolated from *Penaeus monodon* and its morphology was studied Takahashi et al., 1994; Wongteerasupaya et al., 1995; Lo et al., 1996 and Sahul Hameed et al., 1998. WSSV has been found to be highly pathogenic to penaeid shrimp and has a wide host range that includes crabs, copepods and other arthropods.

Pathogenicity describes the ability of a pathogen to cause disease; and virulence is the degree of pathogenicity within a group or species, Rajesh et al., 2010. Virulence of a pathogen can be measured by the time of onset of disease (clinical signs), onset of mortality, time to reach cumulative mortality 100%, median lethal time (LT_{50}) and severity of infection in tissues. White Spot Syndrome Virus (WSSV) is a globally infective agent in shrimps causing high mortality and significant economic losses to the shrimp cultivation, Haq et al., 2012. Due to the worldwide economic and sociological significance of shrimp cultivation, and the development of high intensity in farming, progress of novel control measures against the WSSV infection become unavoidable, Haq et al., 2012. Under experimental conditions, intramuscular or oral inoculation of the virus, immersion in viral suspension, feeding of infected tissue or cohabitation with infected animals cause infection in shrimp at post larval stage onwards, Chou et al., 1998; Kanchanaphum et al., 1998; Prior et al., 2003; Yoganandhan et al., 2003; Leonardo et al., 2005 and Escobedo-Bonilla et al., 2006.

Horizontal transmission of WSSV from the affected shrimp farms to the neighbouring 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 and Scotti, 1986 and Mortensen 1993. WSSV virions can remain infective in the decaying belief that free virus cannot survive in nature waters more than 24 h Bondad-Reantaso et al., 2001, this virus could be transmitted to benthic crustaceans and predation.

The WSSV infected shrimp may it produces rapid development of white spots which ranges from 0.5–3.0 mm in diameter on the exoskeleton, appendages and inside the epidermis, Haq et al., 2012. White spot syndrome virus (WSSV) is of rod shaped envelope containing double stranded DNA as genetic material which belongs to Nimaviridae family, Musthaq et al., 2009; Haq et al., 2012 . 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 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 brood stock in hatcheries all over the world due to their high nutritive value, Bray and Lawrence, 1992. In India, almost all penaeid hatcheries use polychaete worms to promote maturation and spawning of wild caught brood-stock/ spawners of *L. wurdemannis*. At present there is no remedy for the interference of WSSV with the wild occurrence and disease invasion, Haq et al., 2012. 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.

The marine ornamental trade involves a wide variety of crustacean species, both wild and cultured. Ornamental shrimp are popular crustaceans in the aquarium trade and research has been done to establish culture conditions for many species, Lin and Zhang 2001; Simones et al., 2002 and Calado et al., 2003. The susceptibility of ornamental shrimp species to WSSV is currently unknown. The possibilities of potential transfer of WSSV from susceptible crustaceans to ornamental shrimp and vice versa are a real possibility both in culture systems and the wild. The aim of the present study was to determine the susceptibility of an ornamental shrimp species, *L. wurdemanni*, to WSSV, Laramore Susan 1997. Infectivity of WSSV to the Polychaete *P. nuntia* and a possibility of WSSV transmission from the polychaete to the *L. wurdemannis*, followed by Polychaete worms as a vector for WSSV were investigated by Laoaroon et al., 2005 and Vijayan et al., 2005. Aim of the present investigation is to confirm the transmission of WSSV through the commercially important Polychaete viz., *P. cultrifera* to the *L. wurdemanni* in laboratory condition. The Purpose of the present investigation was to confirm

the transmission of WSSV through commercially important Polychaete *P. cultrifera* to the *L. wurdemanni* under laboratory condition.

Materials and methods

(i)Polychaete - *Perinereis cultrifera*

P. cultrifera was collected from the intertidal region of Vellar estuary, (Lat.11^{0}49'E; Long. 79^0 46' N) southeast coast of India. Upon collection, they were washed with running tape water followed by distilled water. *P.cultrifera* was tested WSSV-negative by PCR was placed in a 25 Liters synthetic plastic tanks which was already filled with wet sand, during entire period of experiment the polychates were maintained 28-32°C at a density of 80 numbers in each tank with the ABW (absolute body weight) of 1.8 to 3.5 g. Triplicate tanks were maintained throughout the experimental period.

(ii) Peppermint shrimp - *Lysmata wurdemanni*

Wild *L. wurdemanni* with 40–70 mm TL, were collected from Gulf of mannar coastal waters, Tuticorin, shrimps were maintained in 25 Liter synthetic plastic tanks with filtered and aerated Vellar estuary water with the salinity 20–25 ppt; temperature 28–32ºC; pH 8.0–8.2, and the ABW of shrimps ranged 3-6 g.

(iii) Feeding

L. wurdemanni shrimp was treated with Avanthi pellet feed consisting 30% protein for about 20% of shrimp total body weight, four times a day (06:00, 11:00, 05:00 and 10:00 hrs). Earlier the shrimps were acclimated and starved for 48 hrs prior to initiation of viral transmission attempt. There were five shrimps in each species were picked out randomly for PCR analysis and healthy individuals were used for PCR examination. Further, a representative sample of these animals was subjected to nested PCR, using a WSSV-nested PCR kit (IQ2000 kit, India). Shrimps found to be healthy were used for the experiment.

(iv) Preparation of viral extract for oral challenge tests.

Pond reared, naturally infected *L. wurdemanni* with WSSV were used as the source of viral inoculum for primary laboratory infections. A stock inoculum prepared from primary infected shrimp was injected into more laboratory shrimp to provide WSSV infected tissue for oral challenge of polychaete *P.cultrifera*. In detail, white spot disease affected moribund *L. wurdemanni* were collected during an emergency harvest resulting from a white spot disease outbreak at two shrimp farms located in Nellore, Andhra Pradesh (India). The infected cephalothroacic tissues (gill, stomach, midgut, etc.) were homogenized in TN buffer (20 mM Tris-HCl and 400 mM NaCl, pH 7.4) at 0.1 g ml^{-1}. After centrifugation at 2000 × *g* for 10 minutes, the supernatant was filtered (0.22 µm) and injected (1:100 dilution in 0.9% NaCl) intramuscularly into the lateral area of the fourth abdominal segment of healthy shrimp of *L. wurdemanni*. Four days later, WSSV infection was verified by PCR, and abdominal muscle was collected and 10 g of this material and was homogenized in 100 ml phosphate buffer saline solution. This preparation was called viral feeding mixture (VFM) and stored at 4°C until used.

(v) Infectivity and vertical transmission procedure to Polychaetes

For WSSV infectivity studies, two replicate groups (80 individual each) of polychaete were fed VFM as described below. Before oral challenge, seawater was exchanged completely and the polychaetes were starved for 12 h. Polychaetes were then fed twice a day (06:00 and 10:00 hrs along with regular feeding schedule) with VFM at the rate of 1:1000 ml (v:v). During the + challenge, aeration was sufficient to give good oxygenation and keep the VFM in suspension. Seawater was replaced totally every 24 h by filtration through micromesh gauze, on which the polychaetes were gently but thoroughly rinsed with 3 × 100 ml sterilized seawater. The polychaetes were then transferred to a new tank and freshly prepared VFM was added for the next 24 h challenge cycle. On Days 3 to 9, seawater was exchanged and the polychaete*s* were fed with minced WSSV-PCR-negative shrimp meat instead of VFM to remove residual virus. Survival was determined on Day 30. After the last feeding, the challenged polychaetes were starved for 12 h before PCR analysis. The animals were also fed with WSSV-PCR-negative shrimp meat in another tank for control. Survival was determined daily until Day

30. Five polychaetes were picked out randomly from those polychaetes for PCR examination to exclude viral infection, because only healthy individuals were used.

(vi) Preparation of viral inoculums.

The virus used in this study was isolated from infected polychaete *P.cultrifera* from the experimental tank. WSSV infected tissues of polychaete along with the body setae were removed from the region between the mandibular and posterior dorsoventral muscle of the polychaetes were kept at -20ºC for experimental use. About 2 g tissues in total was homogenized in sterile marine phosphate-buffered saline (PBS) and centrifuged at 1600 g for 15 minutes at 4ºC. The supernatant fluid was then passed through a 450 nm pore size syringe filter. This virus containing supernatant fluid was diluted to 1 part filtrate to 10 parts PBS, and stored at -70ºC for infectivity studies.

(vii) Viral DNA purification

Viral DNA was isolated from purified virions by treatment with proteinase K (0.2 mg/ml) and Sarkosyl (1%) at 65°C for 2 h, followed by phenol and chloroform extraction and dialysis against TE. The purity and concentration of the DNA were determined by agarose gel electrophoresis. The inoculum (viral DNA) was used to challenge WSSV-negative *L. wurdemanni* under experimental conditions. All challenged shrimps displayed signs of WSSV infection thus proving the presence of infectious WSSV white patch particles.

(viii) Intramuscular inoculation protocol

Three experiments were performed using the intra muscular route. In each experiment, 3 groups of 10 shrimp (MBW = 9.40 ± 4.92 g, n = 120) were inoculated with 10, 30 or 90 ID50. In addition, 3 groups of 10 shrimp were mock-inoculated with 50 µl PBS and used as controls. Shrimp were injected between the 3rd and 4th segments of the pleon. Before and after injection, this surface was wiped with 70% ethanol. These experiments were run until all the infected shrimp died. Control shrimp were sacrificed at 360 h post inoculation (hpi)

(ix) Oral inoculation procedure

Triplicate tanks were maintained to each species shrimp with oral route and intramuscular injection inoculation. In each experiment, 3 groups of 10 shrimp (MBW = 9.72 ± 2.24 g, n = 120) were inoculated with 1 of 3 doses (10, 30 and 90 SID50). Three groups of 10 shrimp were mock-inoculated with 50 µl PBS and used as controls. Oral inoculation was performed as follows: shrimp were placed in a tray ventral side up, a flexible and slender pipette tip (790004 Biozym) was introduced into the oral cavity, and the inoculum was delivered into the lumen of the foregut. These experiments were run until all the infected shrimp died. Control shrimp were sacrificed at 600 and 360 hpi with *L. wurdemannis*.

(x) Clinical signs

The shrimp *L. wurdemanni* rarely displays white spots during WSSV infection as described by Nadala *et al*. in 1998 [31] and Rodriguez *et al*. in 2003 [32]. Empty guts and reduced response to mechanical stimulation observed as a first clinical sign to appear in WSSV-diseased shrimp, and are good indicators of infection and mortality. These clinical signs were used to monitor the onset of disease in shrimp inoculated by intra muscular (i.m) or oral routes (o.r).

(xi) Time-course infectivity experiments

L. wurdemanni was infected by i.m and o.r of WSSV strain. The animals (30 per tank) were maintained in a 25 liters plastic tank at room temperature (28–32ºC) with the salinity ranging between 20 and 25 ppt. In the experimental tank A, shrimps were treated with WSSV infected polychate worms through oral route at 10% of total body weight. In experimental tank B, shrimps were injected intramuscularly between the second and fourth abdominal segment with 50µl of viral extract from infected shrimp using 1ml insulin syringes. Control shrimps were injected with hemolymph from WSSV uninfected shrimp. Tissues and hemolymph were collected from experimental shrimps for PCR analysis. Shrimps were sacrificed at 24h, 48h, 3, 6, 12, 18 and 25 days interval, and stored at -20 ºC for further investigation. The total transmission evaluation performed in the wet laboratory is about 15 days for *L. wurdemanni*. Further, WSSV transmission trial repeated thrice.

(xii) Experimental Design

All the WSSV transmission design was followed by the procedure of Sahul Hameed et al., 2002, excluding the test with *Artemia* with virus phytoplankton adhesion route and immersion challenge. To ensure viral transmission, the exposed susceptible shrimp were isolated after the 24 h exposure period into 1 litre jars (Table.1). The time of death of isolated shrimp was recorded. The filtrates containing white spot syndrome virus were injected intramuscularly into the second abdominal segment of the experimental shrimp (*L. wurdemanni*), each shrimp received 50 µl inoculum. Initially there were thirty-two shrimps exposed to WSSV. The control groups comprising a tank with eight shrimps each were kept isolated from the experimental sets, wherein eight shrimps in one of the tanks were injected with extraction DNA of healthy polychaete, and the other two tanks with eight shrimps each were intramuscularly viral exposed. Maintenance and feeding in the control sets (unexposed to WSSV) were similar to that of experimental sets.

(xiii) PCR analysis

Template DNA was prepared from polychaete *P.cultrifera* and *L. wurdemanni* sample according to the instructions given in the test kit. Briefly, 20 mg samples was added to an eppendorff tube containing 100 µl lysis buffer and homogenized using a sterilized tooth pick. After centrifugation at 2000 × *g* for 2 min, 5 µl silica was added to the supernatant followed by gentle agitation at 4°C for 10 min. The mixture was centrifuged at 2000 × *g* for 15 s, the supernatant was discarded and the pellet was washed with 200 µl 70% ethanol and suspended in 10 µl distilled deionised water followed by incubation at 55°C for 5 min. After centrifugation at 4000 × *g* for 5 min, the supernatant was used as a template for PCR analysis.

WSSV-DNA was detected using a commercial 2-step PCR detection kit. The PCR was performed using the method of 2-step WSSV diagnostic nested PCR, described by IQ 2000 Farming Intelligene Tech. Corp, Taipei, Taiwan using first PCR primer for the preliminary amplification and the nested PCR primer for the second nested amplification. The first PCR profile were carried out in 7.5 µl reaction master mixture containing 2 µl of template DNA (approximately 100 ng) and 0.5 µl of IQzyme DNA Polymerase and nested PCR were carried out in 14 µl of reaction mixture containing 1 µl IQzyme DNA Polymerase and make up 25 µl final volume. Amplification was performed in a thermocycler (PCR Express) using the following protocol: 1 cycle at 94ºC for 2 min, then 94°C for 20 sec; 62°C for 20 sec; 72°C for 20 sec, repeated 15 cycles, then add 72°C for 30 sec 20°C for 30 sec at the end of the final cycle. The second PCR profile was carried out in 94°C for 20 sec, 62°C for 20 sec; 72°C for 30 sec, repeat 30 cycles, then added 72°C for 30 sec 20°C for 30 sec at the end of the concluding cycle, followed by a final extension for 5 min at 72°C. Electrophoresis was executed by loaded 12 µl of the amplified product and 5 µl DNA molecular markers onto 1.5% agarose gel with 1× TBE (Trizma, boric acid, EDTA) buffer. The gel was stained using ethidium bromide solution (1 µg ml^{-1}) for 30 min, and the bands were visualized by UV transillumination and GelDoc system. The WSSV negative and positive results were interpreted with help of UV exposure GelDoc System.

Results

The physiochemical characteristics of the experimental tanks were determined, temperature, PH and DO ranged from 29-30.5˚c, 8.7-9.0, 30-35ppt and 4.4-6.5 mg/l, respectively. The clinical signs observed in experimentally infected shrimps that showed lethargy and lack as appetite. The uropods, telsons, pereiopods and pleopods became reddish in colour. The white spots were observed in the cephelothoraic region most of the dying shrimps. The behavior pattern included reduced swimming activity, deorientation during swimming and swimming on one side.

1. Gross Pathology

Grossly visible white spots were usually rounded and consisted of a peripheral whitish-brown ring enclosing a brownish central area demarcated by small cavities assembled in bead-like rows. Numerous scattered melanised spots and cavities were found in the central area. White spots first appeared on the carapace and on the fifth-sixth abdominal segments, and later on the shell of the whole body. Sizes of the spots varied from barely visible dots to spots of 3 mm in diameter. The initial microscopic spots mainly appeared as separate tiny dots but they were sometimes also arranged in

bead-like order. The spots appeared yellowish-brown and opaque under the microscope rather than white as seen by the naked eye. They were mainly embedded in the cuticle but some portions extended to its inner surface. Large, whitish patches visible to the naked eye also occurred when the spots enlarged and coalesced, resulting in an overall whitish discoloration of the shell.

2. Mortality & Survival in the *L. wurdemanni*

Mortality of 90.0 and 93.30 % were noticed in the *L. wurdemanni* shrimps treated oral route and intramuscularly after post WSSV-inoculum and these mortality was observed on 19 and 21 days respectively (Figure 1). However, a drastic change in the survival from 100 to 40.0 and 63.30 % resulted in the *L. wurdemanni* shrimps. The total days of experiment continued up to 21 and on day 22 the experiment was terminated (Figure 2).

Table 1. Infectivity trial of WSSV from *P.cultrifera* to *L. wurdemanni* in experimental tanks

Species	*L. wurdemanni*		
Mode of transmission	Oral route	i.m	Control
Quantity treated	5 % of total body weight	50 µl / shrimps	Control

3. PCR analysis

The results of PCR analysis on different organs obtained from time-course experiments using experimentally WSSV-infected shrimps were presented in (Fig 1-2). The PCR analysis observed the appearance of a prominent band of PCR amplified product of WSSV-DNA at 848 bp at 24 hrs to day 9 of post-inoculums in the pleopods of *L. wurdemanni* whilst, these band of 650 bp, 296 bp continued from day 10 to 12. On days 13th to 17th the product band observed as 650 bp and which was recorded as 296 bp, 650 bp and 910 bp 16th to 21st days of post-inoculum respectively to *L. wurdemanni* exposed to WSSV by oral route. There was no evidence of DNA band in the control group of *L. wurdemanni* in both the group tanks.

Discussion

The susceptibility of marine ornamental peppermint shrimp, *L. wurdemanni* to white spot syndrome virus (WSSV) was tested by oral route and intramuscular injection. The results revealed that these *L. wurdemanni* were as highly susceptible as marine shrimp when the WSSV was administered intramuscularly. The practice of feeding unscreened *P.cultrifera* increase the risk of pathogen transmission, especially the worm are collected from shrimp farming areas where WSSV is prevalent. Logically, when the *P.cultrifera* had WSSV filtrate through oral route in their body and the virus remained infectious, *L. wurdemanni* that feed on this infected polychaete should have been infected before 3rd day of post-inoculum. The find that the shrimp were not infected suggested that the WSSV in the polychaete became non-infectious at a certain period in the polychaete bodies. The presences of WSSV are viral DNA was confirmed by nested PCR. This find raises the question when the bested PCR results were falls – positive and if the polychaete had not been infected by WSSV from the beginning and the argument is less likely since the chance of forming the pattern of bands from the non-specific amplify should be very low, especially the three band pattern of the severe grading. In addition, the DNA sequence of the PCR product also confirmed the specificity of reduction. Alternately that it is possible the *P.cultrifera* was infected by WSSV, but the virus could not replicate in the polychaete tissues and and became non-virulent in the host. This consequence was also confirmed by an absence of histological features of WSSV infection in the WSSV- infected polychaete. Therefore, it can be concluded that *P.cultrifera* is acting as a reservoir and carriers fir WSSV under among oral route and immersion infectious, in particular oral route showed more effective than intramuscular route. Further for practical purposes, the use of *P.cultrifera* in shrimp hatcheries should be safe regarding WSSV infection is some precaution are followed. Probably the only procedure needed is to make certain that the polychaete do not contain infections WSSV particles in their gut lumens, as wild polychaetes may

feed on WSSV-infected shrimp carcasses. Wild polychaete should be kept in captivity for about one week before use, to excrete WSSV from the gut lumen.

The injection challenge sought to rectify viral load differences that may have occurred between per os experiments by injecting viral filtrate based on body weight. The typical pattern seen in studies comparing oral and injection challenges is that similar mortality is seen between methods, Lightner et al., 1998; Maeda et al., 2000; Sahul Hameed et al.,2001. A greater percent mortality is seen with injection challenge, Lotz ,1997 and, Supamattaya et al., 1998. Shrimp used were acquired at the same time and from the same source as in the second per os experiment. Previous experiments with *L. vannamei* had shown that a [10.sup.-6] dilution injected at 20[micro] l/g of body weight caused 100% mortality. Other WSSV injection challenge experiments have used similar dilution factors, Prior et al., 2003. Based on the per os experiments, in the present study, intramuscular injection and oral routes were used to test the pathogenicity of WSSV isolated from infected shrimp and these routes of inclusion resulted in rapid mortality. The PCR result showed that intramuscular inoculation of different sample preparations except abdominal caused death of the entire experimental animal within 10 to 21 days post infection (p.i) and strongly implied the presence of infectious virus in all these tissues and organs that treated with WSSV-inoculum. Similarly, Laramore Susan 1997 were reported that, adult *L. wurdemanni* appear somewhat susceptible to the virus, whereas the juveniles appear refractory to the virus (40% versus 0% mortality), however as the adults and juveniles were collected from different geographical areas, genetic variation cannot be discounted. Although there are no reports to date of a natural WSSV infection in Lysmata or other ornamental shrimp species, the finding that adult Lysmata are susceptible to WSSV has implications for the ornamental industry.

The PCR findings revealed that the shrimps treated with WSSV post-inoculated 32 days old *P.cultrifera* tissues strongly suggest the possibility of WSSV transmission from polychaete to *L. wurdemanni*. Logically, when the polychates had WSSV in their body and if the virus remained infectious, *L. wurdemanni* that on *P.cultrifera* should have been infected. The findings that the shrimps were not infected suggested that the virus in the polychaete became non-infectious after a certain period in the polychaete bodies. The presence of virus of nucleic acid of WSSV was confirmed by nested PCR. Although there are no reports to date of a natural WSSV infection in Lysmata or other ornamental shrimp species, the finding that adult Lysmata are susceptible to WSSV has implications for the ornamental industry.

The results from the study strongly suggest developing specific pathogen resistant brooders would immensely useful in rearing of shrimp for commercial purposes. Even though, shrimp hatcheries in India and other Asian countries depend almost entirely all natural polychaete stocks, continuation of while polychaete population with lethal pathogen such as WSSV demonstrate the need to produces pathogen free polychaete worm especially *P.cultrifera* through aquaculture. However, by implementing quarantine prior bring *P.cultrifera* commercial marine ornamental shrimp breeding purposes will immensely boot the industry.

Conclusion

Mortality rate of 90.0 and 93.30 % were observed during 19 and 21 days of post infection of WSSV in *L. wurdemanni* treated by oral route and intramuscularly. Whilst the appearance of a prominent band of PCR amplified product of WSSV-DNA at 650 bp and 296 bp continued from day 9 to 15. On days 14, 15, 16 and 21 the product band observed as 650 bp. The product band of 296 bp, 650 bp and 910 bp were noticed during 16th, 19th and 21st days of post-inoculum respectively. No evidence of DNA band observed in the control group. Hence it is conclude that the immunity of *L. wurdemanni* is more against pathogens while comparing with other penaied shrimps. The results were revealed that, *L. wurdemanni* can survive more days than other crustacean organisms.

Acknowledgement

The authors are thankful to the supports form University Grants Commission, Govt of India research project No: F. No: 41- 4/2012 (SR) for providing funds and authority of Annamalai University for providing facility for this work.

AGE gel photographs

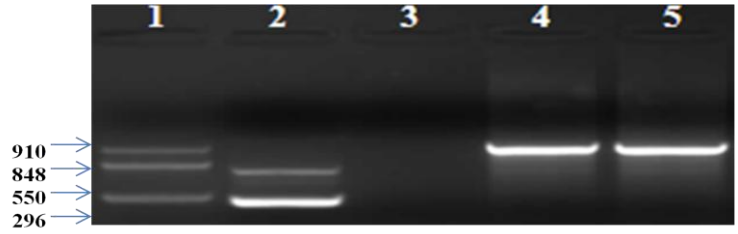
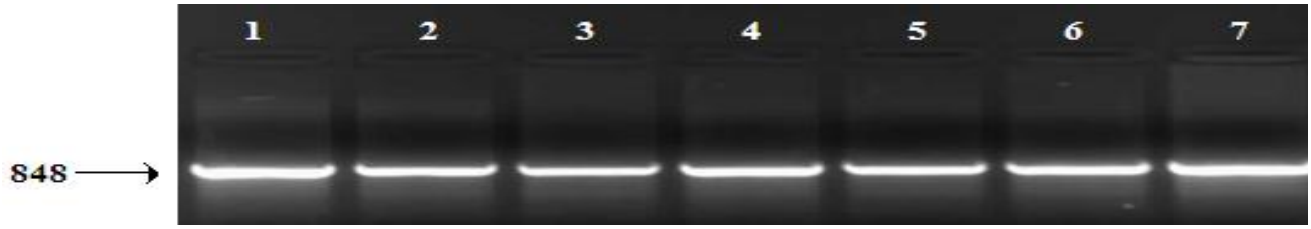
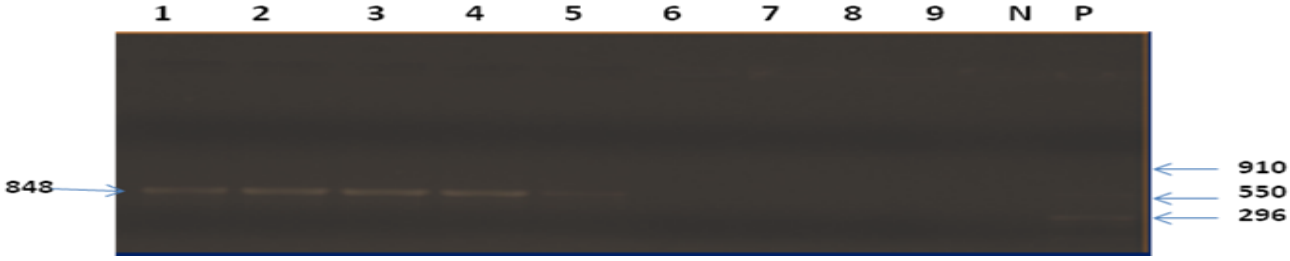


Figure 1. Detection of WSSV in Polychaete P.cultrifera tissue in post viral inoculum by two-step PCR result: Lane 1, Marker; Lane 2, positive control; Lane 3 negative control ; Lane 3, Day 34 WSSV moderate level +ve sample (296bp and 650 - 200 and 20 copies) ; Lane 4, Day 34 WSSV moderate level +ve sample (296bp and 650 – 200 and 20 copies)



A

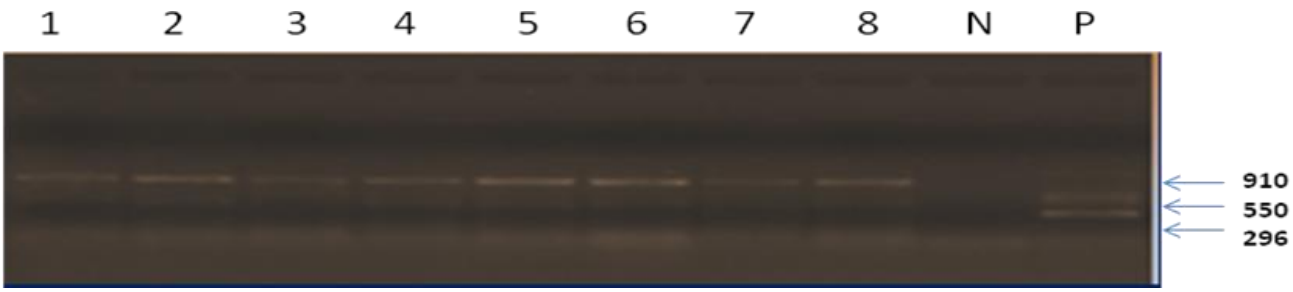
Lane 1	24 hrs WSSV Negative -ve sample (848bp) – Tank-A-sample
Lane 2	36 hrs WSSV Negative -ve sample (848bp) – Tank-A-sample
Lane 3	48 hrs WSSV Negative -ve sample (848bp) – Tank-A-sample
Lane 4	Day 3 WSSV Negative -ve sample (848bp) - Tank-A sample
Lane 5	Day 5 WSSV Negative -ve sample (848bp) - Tank-A sample
Lane 6	Day 7 WSSV Negative -ve sample (848bp) - Tank-A sample
Lane 7	Day 9 WSSV Negative -ve sample (848bp) - Tank-A sample



B

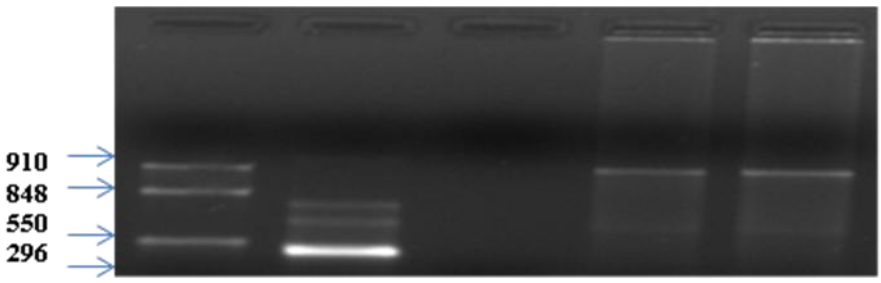
Lane 1	24 hrs WSSV Negative -ve sample (848bp) – Tank-B-sample
Lane 2	36 hrs WSSV Negative -ve sample (848bp) – Tank-B-sample
Lane 3	48 hrs WSSV Negative -ve sample (848bp) – Tank-B-sample
Lane 4	Day 3 WSSV Negative -ve sample (848bp) - Tank-B sample
Lane 5	Day 5 WSSV Negative -ve sample (848bp) - Tank-B sample
Lane 6	Day 7 WSSV Negative -ve sample (848bp) - Tank-B sample
Lane 7	Day 9 WSSV Negative -ve sample (848bp) - Tank-B sample
Lane 8&9	Negative control - dH2O
Lane 10	Negative control - Yeast tRNA
Lane 11	Positive control – 910, 550 and 296 bp

Figure 2 - (A & B). PCR product of *L. wurdemanni* shrimp samples in control tank A and B (24 hrs- 9 d).



A

Lane 1	Day 10 WSSV mod. level +ve sample (550, 296bp) –Tank-A sample
Lane 2	Day 13 WSSV mod. level +ve sample (550, 296bp) –Tank-A sample
Lane 3	Day 16 WSSV mod. level +ve sample (910, 550, 296bp) –Tank-A sample
Lane 4	Day 19 WSSV mod. level +ve sample (910, 550, 296bp) –Tank-A sample
Lane 5	Day 10 WSSV mod. level +ve sample (550, 296bp) –Tank-B sample
Lane 6	Day 13 WSSV mod. level +ve sample (550, 296bp) –Tank-B sample
Lane 7	Day 16 WSSV mod. level +ve sample (910, 550, 296bp) –Tank-B sample
Lane 8	Day 19 WSSV mod. level +ve sample (910, 550, 296bp) –Tank-B sample
Lane 9	Negative control - Yeast tRNA
Lane 10	Day 21 WSSV mod. level +ve sample (550, 296bp) –Tank-B sample



B

Lane 1	Molecular wt marker (848,650,333bps)
Lane 2	Positive control – 910, 550 and 296 bp (2000, 200 and 20 copies)
Lane 3	Negative control - Yeast tRNA
Lane 4	Day 21 WSSV mod. level +ve sample (910, 550, 296bp) –Tank-B sample
Lane 5	Positive control – 550 and 296 bp (200 and 20copies)

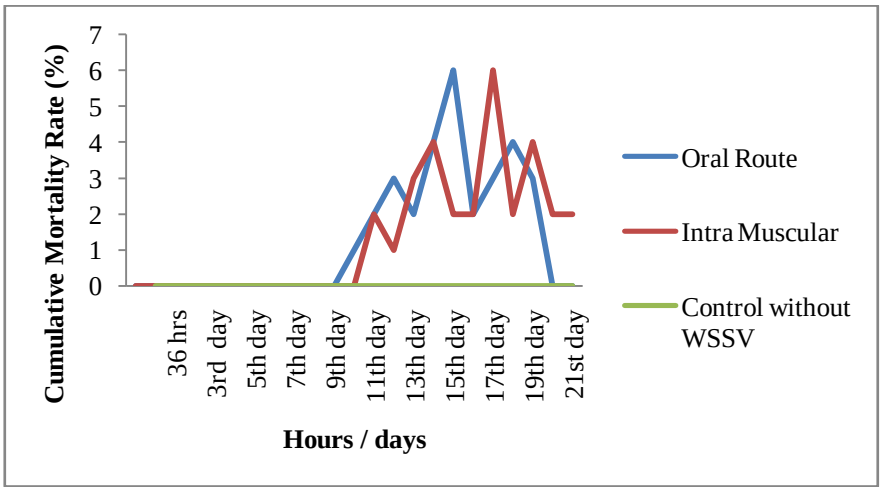
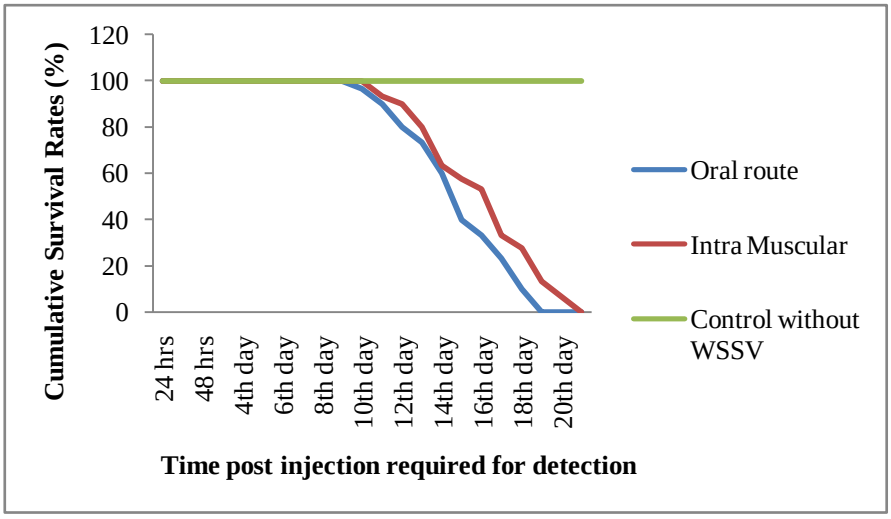


Fig. 3. Cumulative mortality Rates (%) of *L. wurdemanni* at different time intervals after inoculum (oral route (o.r) and intra muscular (i.m) injection) with WSSV filtrate.

Fig. 4. Cumulative Survival Rates (%) of *L. wurdemanni* at different time intervals after inoculum (oral route (o.r) and intra muscular (i.m) injection) with WSSV filtrate.



References

[1] Anon., 1996, Report of Marine Products Export Development Agency, In: The Hindu, June 4, p. 17.
[2] Bondad-Reantaso M.G, Mcgladdery S.E, East J and Subasinghe R.P., 2001, Asia diagnostic guide to aquatic animal diseases, FAO. Fish. Tech. Pap. No. 402/2, pp 237.
[3] Bray W.A and Lawrence A.L., 1992, Reproduction of Penaeus species in captivity. Elsevier, Amsterdam, pp 93-170.
[4] Calado, R., L. Narciso, S. Morais, A. L. Rhyne & J. Lin. 2003. A rearing system for the culture of ornamental decapod crustacean larvae. Aquaculture 218, pp 329-339.
[5] Canzonier W.J., 1971, Accumulation and elimination of coliphage S-13 by the hard clam Mercenaria mercenaria. Appl. Microbial, 21, pp 1024-1031.
[6] Chou H.Y, Huang C.Y, Lo C.F and Kou G.H., 1998, Studies on transmission of white spot syndrome associated baculovirus (WSBV) in *Penaeus monodon* and *Penaeus japonicus* via brone contact and oral ingestion, Aquaculture, 164, pp 263-276.
[7] Escobedo-Bonilla C.M, Audoorn L, Wille M, Alday-Sanz V, Sorgeloos P, Pensaert M.B and Nauwynck H.J., 2006, Standardized white spot syndrome virus (WSSV) inoculation procedures for intramuscular or oral routes, Dis. Aqua. Org, 68, pp 181-188.
[8] Haq M.A.B, Banu M.N,Vignesh R, Shalini R and Meetei K.H.B., 2012, Identification and sequence based detection of WSSV infecting SPF *Litopeneaus vannamei* (Boone, 1931) in culture environment of Tamil Nadu coastal waters, Asian Pacific Journal of Tropical Biomedicine, 1, pp 1-11.
[9] Haq M.A.B, Kavitha N, Vignesh R and Shalini R., 2012. Identification and sequence based detection of WSSV infecting SPF L*itopeneaus vannamei* (boone, 1931) in captivity, International Journal of Pharma and Bio Sciences; 3, pp 547 – 559.
[10] Haq M.A.B, Priya K.K, Rajaram R, Vignesh R and Srinivasan M., 2012, Real time PCR quantification of WSSV infection in specific pathogen free (SPF) *Litopenaeus vannamei* (Boone, 1931) exposed to antiviral nucleotide, Asian Pacific Journal of Tropical Biomedicine, S1, pp 121 -129.

[11] Hay B and Scotti P., 1986, Evidence for intracellular absorption of virus by the Pacific oyster, Crassostrea gigas, N.Z .J. Mar. Fresh. Res, 20, pp 655-659.
[12] Kanchanaphum P, Wongteerasupaya C, Sitidilokratana N, Boonsaeng V, Panyim S, Tassanakajon A, Withyachumnarnkul B and Flegel TW., 1998, Experimental transmission of white spot syndrome virus (WSSV) from crabs to shrimp *Penaeus monodon,* Dis. Aqua. Org, 34, pp 1–7.
[13] Laoaroon S, Boonnat A, Poltana P, Kanchanaphum P, Gangnonngiw W., 2005, Infectivity of white spot syndrome virus (WSSV) to the polychaete *Perenesis nunita* and a possibility of WSSV transmission from the polychaete to the black tiger shrimp *Penaeus monodon,* Diseases in Asian Aquaculture, 5, pp 353-361.
[14] Laramore. Susan 1997, Susceptibility of the peppermint shrimp *Lysmata wurdemanni* to the white spot syndrome virus, J Shellfish Res 26 (2), pp 623-627.
[15] Leonardo D.V.A, Bonnichon V, Roch P, Parrinello N and Bonami J.R., 2005, Comparative WSSV infection routes in the shrimp genera Marsupenaeus and Palaemon, J. Fish. Dis, 28, pp 565–569.
[16] Lightner, D. V., K. W. Hasson, B. L., White and Redman, R.M., 1998, Experimental infection of Western hemisphere penaeid shrimp with Asian White Spot Syndrome Virus and Asian Yellow Head Virus, J. Aquat. Anita. Health 10, pp 271-281.
[17] Lin, J. and D. Zhang, 2001. Effect of broodstock diet on reproductive performance of the peppermint shrimp, *Lysmata wurdemanni,* J. Shellfish Res. 20, pp 361-363.
[18] Lo, C.F., Leu, J.H., Ho, C.H., Chen, C.H., Peng, S.E., Chen, Y.T., Yeh, P.Y., Huang, C.J., Wang, C.H., Kou, G.H., 1996. Detection of baculovirus associated with white spot syndrome (WSSV) in penaeid shrimps using polymerase chain reaction, Dis. Aquat. Organ. 25, pp 133–141.
[19] Lotz, J. M., 1997, Effect of host size on virulence of Taura Virus to the marine shrimp *Penaeus vannamei* (Crustacea: Penaeidae), Dis. Aquat. Organ, 30, pp 45-51.
[20] Maeda, M., T. Itami E. Mizuki R. Tanaka, Y. Yoshizu, K. Doi, C., Yasunaga-Aoki, Y., Takahashi and Kawarabata, 2000, Red swamp crawfish (*Procambarus clarkii*): an alternative experimental host in the study of white spot syndrome virus. Acta Virol. 44, pp 371 374.
[21] Mortensen SH., 1993, Passage of infectious pancreatic necrosis virus (IPNV) throught invertebrates in an aquatic food chain, Dis. Aquat. Org, 16, pp 41-45.
[22] Musthaq S.S, Madhan S, Sahul Hameed A.S and Kwang J., 2009, Localization of VP28 on the baculovirus envelope and its immunogenicity against white spot syndrome virus in *Penaeus monodon,* Virology, 391, pp 315-324.
[23] Nadala E.C.B and Loh P.C., 1998, A comparative study of three different isolates of white spot virus, Dis. Aquat. Org, 33, pp 231–234.
[24] Nunes A.J.P, Gesteria T.C.V and Goddard S., 1997, Food consumption and assimilation by the Southern brown shrimp *Penaeus subtilis* under semi-intensive culture in N.E Brazil. Aquaculture, 144, pp 371-386.
[25] Prior S, Browdy C.L, Shepard E.F, Laramore R and Parnell P.G., 2003, Controlled bioassay systems for determination of lethal infective doses of tissue homogenates containing Taura syndrome or white spot syndrome virus, Dis. Aqua. Org, 54, pp 89-96.
[26] Rajesh S, Anusha S, Rajeshwari R, Ramya R, Kavitha R and Narayanan R B., 2010, Molceular cloning, sequence analysis and tissue expression of penaeidin from white spot syndrome virus infected *Penaeus monodon* shrimps of Indian coastal region, Indian J. Biotechnol, 10, pp 57-263.
[27] Sahul Hameed A.S, Murthi B.L.M, Rasheed M, Sathish S, Yoganandhan K, Murugan V and Jayaraman K., 2002, An investigation of Artemia as a possible vector for white spot syndrome virus (WSSV) transmission to *Penaeus indicus*. Aquaculture, 204, pp 1-11.
[28] Sahul Hameed, A. S., K. Yoganandhan, S. Sathish, M. Rasheed, V. Murugan and Jayaraman, K., 2001, White spot syndrome virus (WSSV) in two species of freshwater crabs (*Paratelphusa hydrodomous* and *P. pulvinata*). Aquaculture 201, pp 179-186.
[29] Sahul Hameed, A.S., Anilkumar, M., Stephen Raj, M.L., Jayaraman, Kunthala, 1998. Studies on the pathogenicity of systemic ectodermal and mesodermal baculovirus and its detection in shrimp by immunological methods, Aquaculture 160, pp 31–45.

[30] Shapiro-Ilan D.I, Fuxa J.R, Lacey L.A, Onstad D.W and Kaya H.K., 2005, Definitions of pathogenicity and virulence in invertebrate pathology, J. Invert. Path, 88, pp 1–7.
[31] Simones, F., Ribeiro F and Jones, D.A., 2002, Feeding early larval stages of fire shrimp, *Lysmata debelius* (Caridea Hippolytidae), Aquaculture International 10, pp 349-360.
[32] Supamattaya, K., R. Hoffmann, W, Boonyaratpalin, S and Kanchanaphum, P., 1998, Experimental transmission of white spot syndrome virus (WSSV) from black tiger shrimp *Penaeus monodon* to the sand crab *Portunus pelagieus*, mud crab *Scylla serrata* and krill Acetes sp, Dis. Aquat. Organ. 32, pp 79-85.
[33] Takahashi, Y., Itami, T., Maeda, M., Fujii, R., Tomonaga, S., Supamattaya, K., Boonyaratpalin, S., 1994, Electron microscopic evidence of bacilliform virus infection in kuruma shrimp *Penaeus japonicas,* Fish Pathol. 292, pp 121–125.
[34] Vijayan K.K, Mohamed S and Diwan A.D., 1993, On the structure and moult controlling function of the Y-organ in the prawn *Penaeus indicus,* J. World Aqua Society, 24, pp 516-521.
[35] Vijayan K.K, Raj SV, Balasubramanian C.P, Alavandi S.V, Sekhar T.V and Santiago T.C., 2005, Polychaete worms- a vector for white spot syndrome virus (WSSV), Dis. Aquat. Org, 63, pp 107-111.
[36] Wongteerasupaya C, Vickers JE, Sriurairatana S, Nash C.L, Akarajamorn A, Boonsaeng V, Panyirn S, Tassanakajon A, Withyachumnarnkul B, Flegel T.W., 1995, A non-occluded, systelnlc baculovirus that occurs in cells of ectodernlal and mesodermal origin and caused hlgh mortality in the black tiger prawn *Penaeus monodon,* Dis Aquat Org 21, pp 69-77.
[37] Wongteerasupaya C., Wongwisansri S., Boonseang V., Panyim S., Pratanpipat P., Nash GL, Withayachumnarnkul B, Flegel T.W., 1996, DNA fragment of *Penaeus monodon* baculovirus PmNOBII gives positive in situ hybridization with white-spot viral infections in six penaeid shrimp species Aquaculture 143, pp 23-32.
[38] Wongteerasupaya, C., Vickers, J.E., Sriurairatana, S., Nash, G.L., Akarajamorn, A., Boonsaeng, V., Panyim, S., Tassanakajon, A., Withyachumnarnkul, B., Flegel, T.W., 1995, A non-occluded systemic baculovirus that occurs in cells of ectodermal and mesodermal origin and causes high mortality in the black tiger prawn *Penaeus monodo*n, Dis. Aquat. Org. 21, pp 77–96.
[39] Yoganandhan K, Narayanan R.B and Sahul Hameed A.S., 2003, Larvae and early post-larvae of Peaneus monodon (Fabricus) experimentally infected with white spot syndrome virus (WSSV) show no significant mortality, J. Fish. Dis, 26, pp 385-391.