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WSSV Transmission Studies on Polychaete *Pereneris cultifera* to Pacific White Shrimp SPF *Litopenaeus vannamei* in Captivity

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ABSTRACT

The preparation viral inoculums were used in this study was isolated from infected polychaete P. cultrifera from the experimental tank. Viral DNA was isolated from purified virions by treatment with proteinase K followed by phenol and chloroform extraction and dialysis against TE. The inoculum (viral DNA) was used to challenge WSSV-negative animals under experimental conditions. Triplicate tanks were maintained to each species shrimp with oral route and intramuscular injection inoculation. 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. PCR analysis showed the appearance of a prominent band of PCR amplified product of WSSV-DNA at 848 bp at 24 hr up to day 7 of post infection in the pleopods although these band 296 bp (200 copies) continued from day 8 to 12. On days 13 and 14 the product band observed as 650 bp (2000 copies) and an equal amount of product band of 910 bp (20,000) during the days 15 to 18. The same product band was ranged between 296 to 910 bp during 19 to 25 days in the L. vannamei shrimps which exposed to WSSV by oral route. No band was observed in the control group. Histological observation of low, moderate and severely WSSV infected L. vannamei shrimps were revealed degenerated cells characterized by basophilic intranuclear inclusions in the tissues of WSSV infected mid-gut gland, lymphoid organ, gill lamellae, gut epithelium tissue of L. vannamei on the 9th day of post-inoculum. The survival days of L. vannamei extends up to 23 days for oral rooted and 24 days for intramuscularly injected shrimps of which mortality rate on the concerning days was 93.30% and 90.0 % respectively.

Key words: *PCR, Histopathology, WSSV, P. vannamei, P. cultrifera.*

INTRODUCTION

Litopenaeus vannamei is the most important shrimp species in terms of aquaculture production. The other important species are *Penaeu. monodon*, *P. chinensis*, *P. merguiensis*, *P. japonicus* and *Fenneropenaeus indicus*. The presence of *L. vannamei* is naturally along the pacific coast of Central and South America[1]. It was originally cultured in North, Central and South American countries.

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At the end of the 1970s, this species was introduced in Asia [2]. In 2005, 83% of global farm raised *L. vannamei* was produced in Asia. *L. vannamei* has several advantages compared to other cultured species. These include the availability of specific pathogen free (SPF) and specific pathogen resistant (SPR) strains, a higher growth rate, suitability to higher stocking density, tolerance to a wider range of temperature and salinity, a lower protein requirement in the diet, easier to breed and higher survival in larval rearing[2,3]. These aspects could explain the increasing preference to culture this species.

Pathogenicity describes the ability of a pathogen to cause disease; and virulence is the degree of pathogenicity within a group or species [4]. 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[11]. 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[11].

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[5-10]. WSSV infection could not be induced in the early larval stages of *P. monodon* (nauplii, zoea, and mysis) by immersion and oral challenge[8].

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 [12-14] WSSV virions can remain infective in the decaying belief that free virus cannot survive in nature waters more than 24 h [15] 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[11]. White spot syndrome virus (WSSV) is of rod shaped envelope containing double stranded DNA as genetic material which belongs to Nimaviridae family[16, 17]. 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 [18]. In India, almost all penaeid hatcheries use polychaete worms to promote maturation and spawning of wild caught brood-stock/ spawners of *P. monodon*. At present there is no remedy for the interference of WSSV with the wild occurrence and disease invasion[19]. 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[20]. Infectivity of WSSV to the Polychaete *Pereneris nuntia* and a possibility of WSSV transmission from the polychaete to the black tiger shrimp *P. monodon*, followed by Polychaete worms as a vector for WSSV were investigated by Laoaroon et al., 2005 [21] and Vijayan et al., 2005 [22]. Aim of the present investigation is to confirm the transmission of WSSV through the commercially important Polychaete viz., *Perenereis cultrifera* to the *Litopenaeus vannamei* in laboratory condition.

The Purpose of the present investigation was to confirm the transmission of WSSV through commercially important Polychaete *Perinereis cultrifera* to the *Litopenaeus vannamei* under laboratory condition.

MATERIALS AND METHODS

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.

Pacific white shrimp - *Litopenaeus vannamei*

90 day old specific pathogen-free (SPF) *L. vannamei* shrimps were collected from the commercial shrimp farm viz., SPDS aqua farm that located near railway station, Parangipettai. Earlier the brood stock shrimps were imported from the Ceniaqua (P) Ltd., Brazil; subsequently they were spawned and performed larval rearing in a commercial shrimp hatchery located at Marakkanam, East Coast of India. Average total length (TL) of the SPF shrimps were measured about 50–120 mm and were maintained in 25 Liters capacity tanks each was filtered, filled 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 10 – 20 g.

Feeding

The shrimp are 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.

Preparation of viral inoculum

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.

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 animals under experimental conditions. All challenged shrimps displayed signs of WSSV infection thus proving the presence of infectious WSSV white patch particles.

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).

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. vannamei*.

Clinical signs

The shrimp *L.vannamei* rarely displays white spots during WSSV infection as described by Nadala *et al*. 1998 [23] and Rodriguez *et al*. 2003 [24]. 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).

Time-course infectivity experiments

L. vannamei 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 polychaetes 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 as follows: 25 days for *L. vannamei*. Further, WSSV transmission trial repeated thrice.

Experimental Design

All the WSSV transmission design was followed by the procedure of Sahul Hameed *et al* [25] 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. Shrimp that died during isolation phase were fixed in Davidson's fixative following procedures outlined by Lightner D.V, 1996 [26]. Shrimps were kept in these isolation jars for 5 days, after which all surviving specimens were similarly fixed.

The filtrates containing white spot syndrome virus were injected intramuscularly into the second abdominal segment of the experimental shrimp (*L.vannamei*), 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, where in 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.

Histopathology

For histological consequence, polychaete organ tissues were collected from 12 (6 from each tank) moribund animals from the infected groups, and 6 (three from each tank) control shrimps, according to Vijayan *et al*., 1993 [27]. The polychaete organ along with the body setae were removed from the region between the mandibular and posterior dorsoventral muscle of the animal. Following organs were dissected and segregated such as gills, lymphoid organ, haematopoietic tissue, and stomach, mid and hind gut. Instigating the dissected organs was immediately fixed in Davidson's fixative for histology, and the other one was fixed in 95% ethanol for PCR. For histology, routine procedures were followed for preparation, sectioning, and

staining with haematoxylin and eosin[28]. The polychaete organs from the experimental and control shrimps were examined histologically for WSSV-specific manifestations following the routine diagnostic protocol of Lightner D.V, 1996 [26].

PCR analysis

Template DNA was prepared from polychaete *P.cultrifera* and shrimp 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 in the 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.

Mortality & Survival in the *L.vannamei*

Mortality of 93.30 and 90.0 % were noticed in the *L.vannamei* shrimps treated oral route and intramuscularly after post WSSV-inoculum and these mortality was observed on 23 and 24 days respectively. However, a drastic change in the survival from 100 to 56.70 and 46.70 % resulted in the *L.vannamei* shrimps. The total days of experiment continued up to 25 and on day 26 the experiment was terminated (Table 4).

Histopathology

Histological observation of low, moderate and severely WSSV infected *L.vannamei* shrimps were revealed degenerated cells characterized by basophilic intranuclear inclusions in the tissues of WSSV infected mid-gut gland, lymphoid organ, gill lamellae, gut epithelium. The gill epithelial cells were edematous and nuclei were hypertrophied with basophilic inclusions but no pathological changes or hypertrophied nuclei were observed in any of *L.vannamei* tissues post challenged to WSSV. In addition, the tubular epithelial cells of mid-gut gland were highly vacuolated as in crab *Paratelphusa hydrodomous*. Histological observation in the mid-gut gland revealed the presence of hypertrophied nuclei in the tissue of the shrimp on 9^{th} day of post-inoculum and these abnormality was encountered during low

sign of WSSV in *L.vannamei* (Figure 2) and the presence of necrosis, spheroid of lymphoid organ were showed evident (Figure 3). Gill lamella with large basophilic intramuscular inclusion bodies besides the occurrence of heavily infected cuticular epithelial cells with large basophilic inclusion were also found as an evident in *L.vannamei* (Figure 5) on day 15 of post-infection. Presence of large number of darkly stained, round intracytoplasmic inclusion, intramuscular inclusion in hematopoietic tissues and mid-gut gland respectively. The cellular degeneration of hepatopancreatic globules with the presence of WSSV inclusion bodies were also evidenced WSSV infection in different days of post-infection (Figure 9 & 10).

PCR analysis

The results of PCR analysis on different organs obtained from time-course experiments using experimentally WSSV-infected shrimps were presented in (Figure 12 to 20). The PCR analysis showed the appearance of a prominent band of PCR amplified product of WSSV-DNA at 848 bp at 24 hr up to day 7 of post infection in the pleopods although these band 296 bp continued from day 8 to 12. On days 13 and 14 the product band observed as 650 bp and a equal amount of product band of 910 bp during the days 15 to 18. The same product band was ranged between 296 to 910 bp during 19 to 25 days in the *L.vannamei* shrimps which exposed to WSSV by oral route. No band was observed in the control group.

Table1. Infectivity trial of WSSV from *P. cultrifera* to *L. vannamei* in experimental tanks

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

† Intramuscular; ***** No infected with WSSV / unexposed to WSSV

Table 2. Cumulative percent mortality of *L. vannamei* at different time intervals after inoculum (oral rate (o.r) and intra muscular (i.m) injection) with WSSV filtrate

Hours / days	Group 1 (*L. vannamei*)		Control for *L. vannamei*
	o.r	i.m	
24 hrs	Nil	Nil	Nil
36 hrs	Nil	Nil	Nil
48 hrs	Nil	Nil	Nil
3rd day	Nil	Nil	Nil
4th day	Nil	Nil	Nil
5th day	Nil	Nil	Nil
6th day	Nil	Nil	Nil
7th day	Nil	Nil	Nil
8th day	Nil	1	Nil
9th day	1	Nil	Nil
10th day	Nil	Nil	Nil
11th day	Nil	1	Nil
12th day	1	Nil	Nil
13th day	Nil	Nil	Nil
14th day	Nil	2	Nil
15th day	2	2	Nil
16th day	2	2	Nil
17th day	Nil	Nil	Nil
18th day	Nil	Nil	Nil
19th day	3	1	Nil
20th day	4	2	Nil
21st day	3	5	Nil
22nd day	7	5	Nil
23rd day	5	4	Nil
24th day	2	2	Nil
25th day	-	3	Nil

Table 3. PCR detection and cumulative percent mortality of *L. vannamei* at different time intervals after inoculum (oral route (o.r), intra muscular (i.m) injection) with WSSV filtrate

Time post injection required for detection	PCR detection for WSSV		Histopathology detection for WSSV		Mortality & survival range of experimental tank A – Oral route (%)		Mortality & survival range of experimental tank B – intramuscular injection (%)		PCR detection, mortality & survival range of control Tank C (%)		
	Tank A	Tank B	Tank A	Tank B	Mortality	Survival	Mortality	Survival	PCR	Mortality	Survival
24 hrs	-ve	-ve	NA	NA	0	100	0	100	-ve	0	100
36 hrs	-ve	-ve	NA	NA	0	100	0	100	-ve	0	100
48 hrs	-ve	-ve	-ve	-ve	0	100	0	100	-ve	0	100
3rd day	-ve	-ve	NA	NA	0	100	0	100	-ve	0	100
4th day	-ve	-ve	NA	NA	0	100	0	100	-ve	0	100
5th day	-ve	-ve	-ve	-ve	0	100	0	100	-ve	0	100
6th day	-ve	-ve	NA	NA	0	100	0	100	-ve	0	100
7th day	+ve	-ve	NA	NA	0	100	0	100	-ve	0	100
8th day	-ve	+ve	NA	NA	0	100	3.3	96.7	-ve	0	100
9th day	+ve	+ve	-ve	-ve	3.3	96.7	0	96.7	-ve	0	100
10th day	+ve	+ve	NA	NA	0	96.7	0	96.7	-ve	0	100
11th day	+ve	+ve	NA	NA	0	96.7	6.7	93.3	-ve	0	100
12th day	+ve	+ve	NA	NA	6.7	93.3	0	93.3	-ve	0	100
13th day	+ve	+ve	+ve	-ve	0	93.3	0	93.3	-ve	0	100
14th day	+ve	+ve	NA	NA	0	93.3	13.3	86.7	-ve	0	100
15th day	+ve	+ve	NA	NA	13.3	86.7	20.0	80.0	-ve	0	100
16th day	+ve	+ve	NA	NA	20.0	80.0	26.7	73.3	-ve	0	100
17th day	+ve	+ve	+ve	+ve	0	80.0	0	73.3	-ve	0	100
18th day	+ve	+ve	NA	NA	0	80.0	0	73.3	-ve	0	100
19th day	+ve	+ve	NA	NA	30.0	70.0	30.0	70.0	-ve	0	100
20th day	+ve	+ve	NA	NA	43.3	56.7	36.7	63.3	-ve	0	100
21st day	+ve	+ve	+ve	+ve	53.3	46.7	53.3	46.7	-ve	0	100
22nd day	+ve	+ve	NA	NA	76.7	23.3	70.0	30.0	-ve	0	100
23rd day	+ve	+ve	NA	NA	93.3	6.7	83.3	16.7	-ve	0	100
24th day	+ve	+ve	NA	NA	100	0	90.0	10.0	-ve	0	100
25th day	+ve	+ve	+ve	+ve	-	-	100	0	-ve	0	100

Sampling Method: PCR analysis - The experimental shrimp pleopods was sampled every 12 h in first 2 days, every 24 h in after third day onwards. Histopathology detection - The experimental shrimp were sacrificed every 48 h in first 4 days, every 3 day in after seventh day onwards. NA: Not Analysied

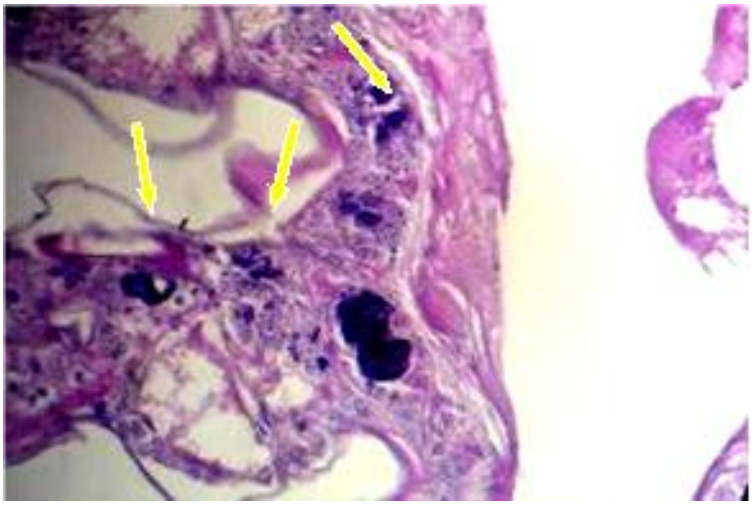

Fig. 1: Micrograph of cuticular epithelium of a healthy (control) hepatopancreas of *L. vannamei*. Arrow indicates the normal subcuticular epithelial cells with normal nuclei. (H & E) 1000 X

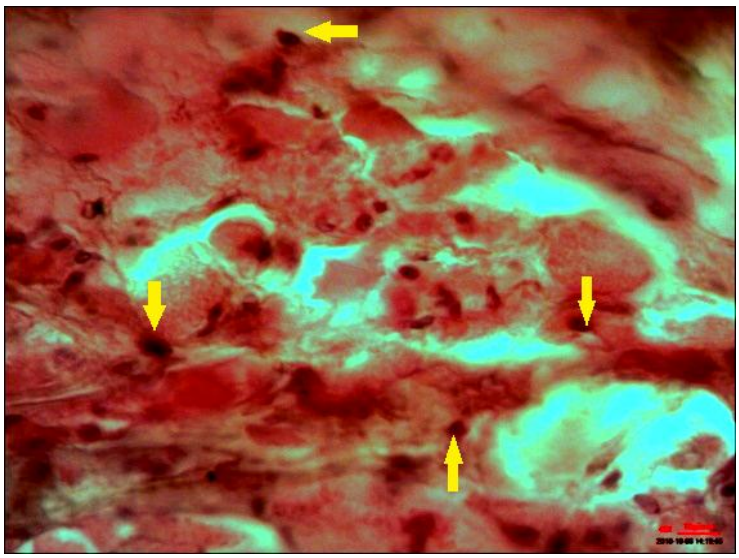

Fig. 2: Micrograph of cuticular epithelium in the hematopoietic tissue of *L. vannamei* 9 day of post-inoculum (low level infection range), arrow shows hypertrophied nuclei. (H &E) 1000 X

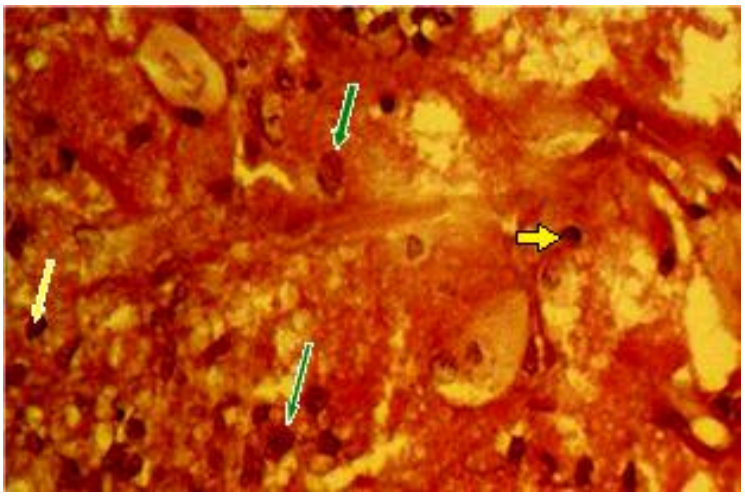

Fig. 3: Micrograph of lymphoid organ necrosis (LON) & lymphoid organ spheroid (LOS) in *L. vannamei*. Green arrow shows LON and yellow arrows show LOS-12d of post inoculum 1000 X

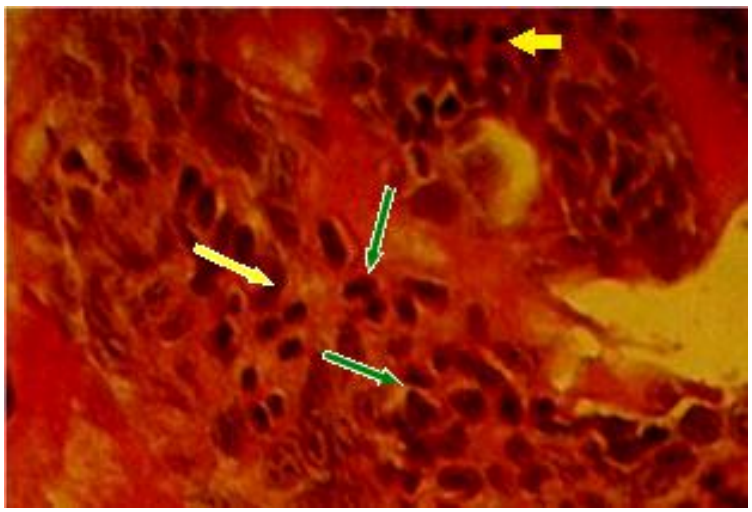

Fig. 4: LON & LOS in *L. vannamei*. Green arrow shows LON and yellow arrows shows LOS – 7d of post inoculum 400 X

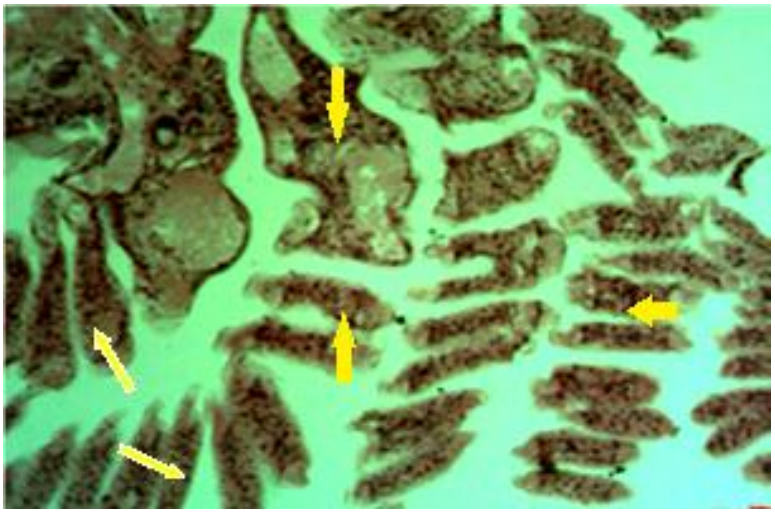

Fig. 5: Micrograph of cuticular epithelium of the gill lamellae with large basophilic intranuclear inclusion bodies characteristic of *L. vannamei*, 15d of post-transmission. Arrow shows degenerated and heavily infected cuticular epithelial cells showing large basophilic inclusions characteristic of WSSV infection. (H &E) 400 X

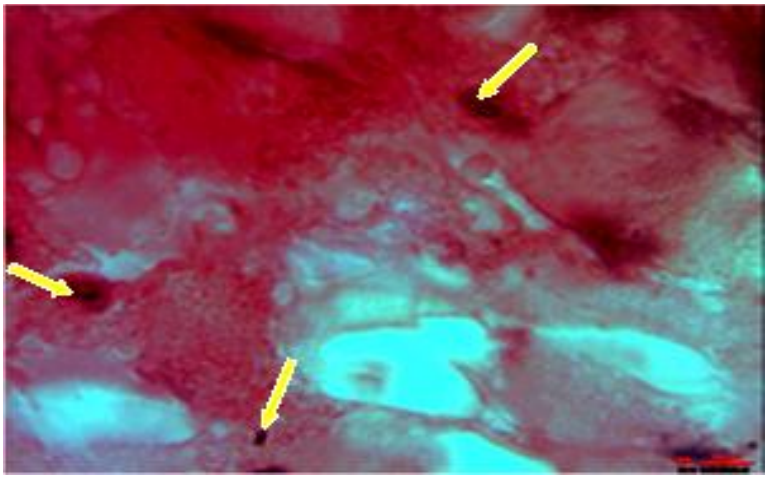

Fig. 6: Micrograph of subcuticular gut epithelium with hypertrophied nuclei containing basophilic intranuclear inclusion bodies characteristic of WSSV infection (arrow) in *L.vannamei* showing gross signs of WSSV on 15 d. (H&E) 1000 X

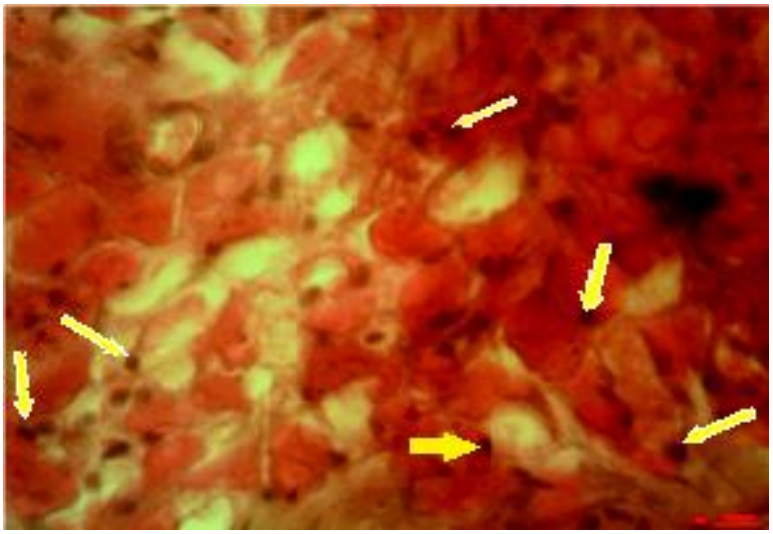

Fig. 7: Micrograph of intranuclear inclusion bodies characteristic of WSSV infection (arrow) in the hematopoietic tissue cells of *L.vannamei* showing gross signs of WSSV on 15 d. Note the presence of large numbers of darkly stained, round intracytoplasmic inclusion bodies (arrowheads) (H&E) 1000 X

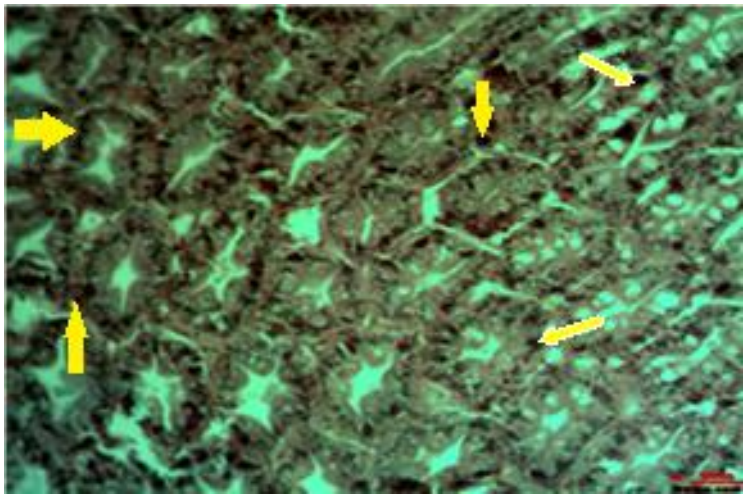

Fig. 8: Micrograph of intramuscular inclusion bodies characteristic of WSSV infection (arrow) in the mid-gut gland tissue cells of *L. vannamei* showing severity (H& E) 400 X

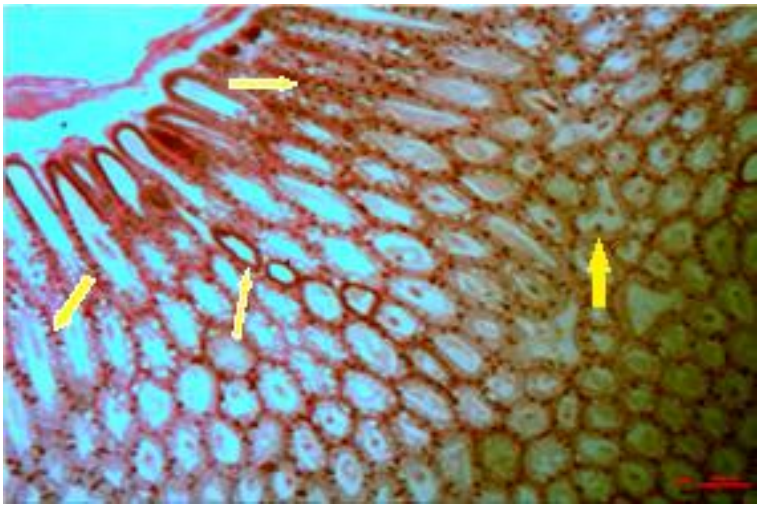

Fig. 9: Micrograph of mid-gut gland on 18d of post transmission in *L.vannamei* showing cellular degeneration of hepatopancreatic globules (arrow) with presence of WSSV inclusion bodies 400 X

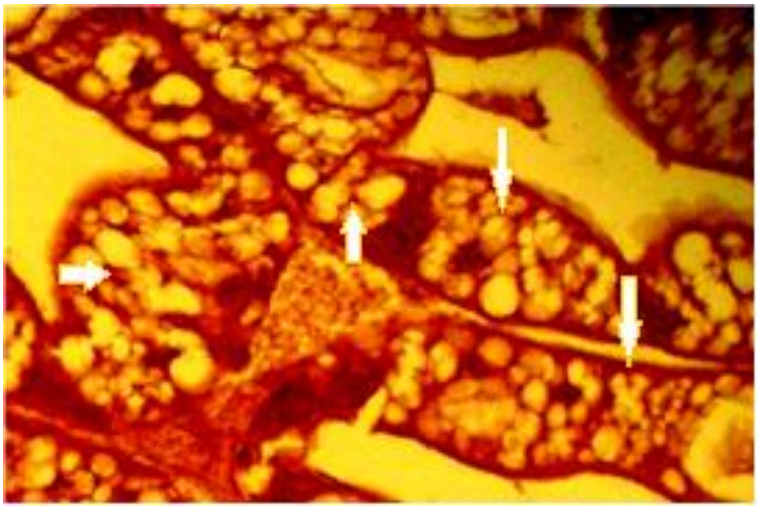

Fig. 10: Micrograph of normal mid-gut gland of healthy *L.vannamei* on 24day showing cellular generation of hepatopancreatic globules (arrow) with absence of WSSV inclusion bodies 1000 X

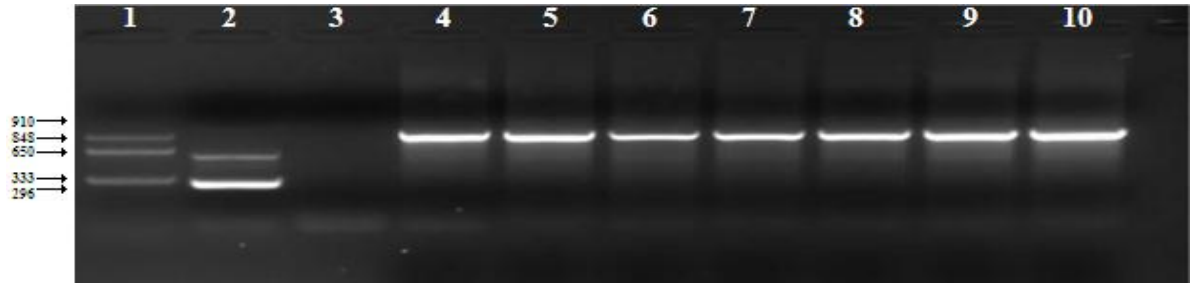

Fig. 12: PCR analysis in *L.vannamei* –Post viral inoculum (24 hrs- 7 d)

Lane 1 Molecular wt marker (848,650,333bp)
Lane 2 Positive control (910, 630 and 296bp)
Lane 3 Negative control (yeast tRNA)
Lane 4 & 5 24 hrs to 3 d WSSV Negative -ve sample (8486bp) – Tank-A-sample
Lane 6 Day 4 to 6 WSSV Negative -ve sample (8486bp) - Tank-A-composite sample
Lane 7 24 hrs to 3 d WSSV Negative -ve sample (8486bp) - Tank-B composite Sample
Lane 8 Day 4 to 5 WSSV Negative -ve sample (8486bp) - Tank-B composite sample
Lane 9 Day 6 WSSV Negative -ve sample (8486bp) - Tank-B Sample
Lane 10 Day 7 WSSV Negative –ve sample (8486bp) - Tank-B sample

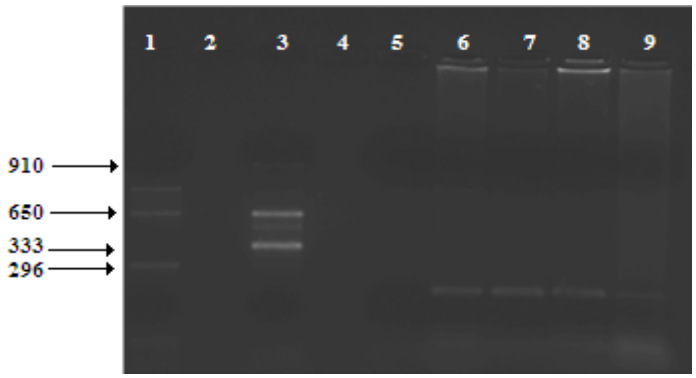

Fig. 13: PCR analysis in *L.vannamei* - Post viral *inoculums* (7- 20 d)

Lane 1&2 DNA molecular weight Marker Negative control (yeast tRNA)
Lane 3 Positive control (910, 630 and 296bp)
Lane 4&5 Negative control (dH2O, culture water and Master mix)
Lane 6&7 Day 7 to 8 WSSV lower level +ve sample (296bp) –Tank-A-sample
Lane 8&9 Day 9 to 10 WSSV lower level +ve sample (296bp) –Tank-A-sample

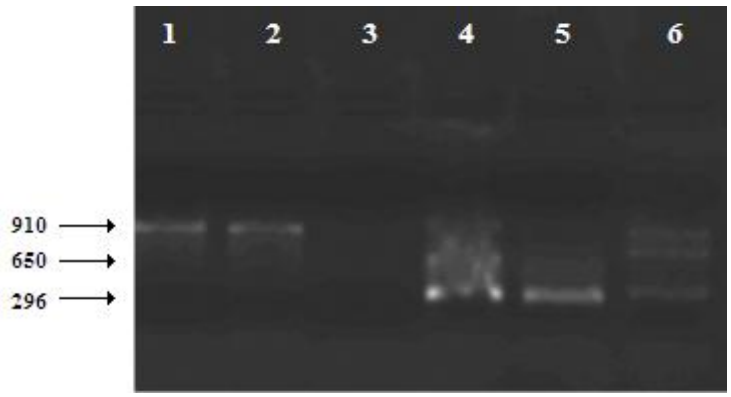

Fig. 14: PCR analysis in *L.vannamei* - Post viral inoculum (7- 20 d)

Lane 1&2 Day 11 to 12 WSSV low level +ve sample (296bp) –Tank-A- composite sample
Lane 3 Negative control (yeast tRNA)
Lane 4 Day 13 to 14 WSSV moderate level +ve sample (650bp) –Tank-A-composite sample
Lane 5 Day 15 to 16 WSSV sever level +ve sample (910bp) –Tank-A- composite sample
Lane 6 Day 17 to 18 WSSV sever level +ve sample (910bp)-Tank-A- composite sample

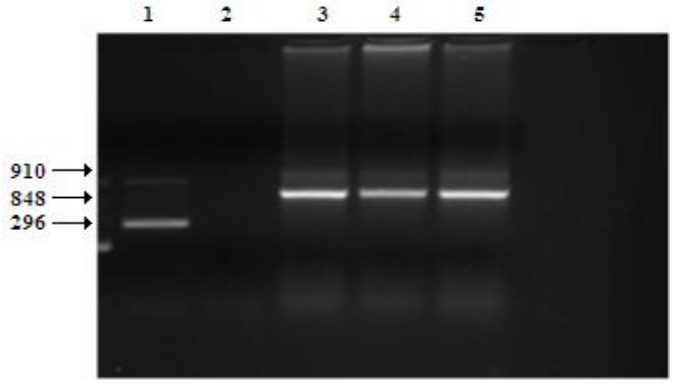

Fig. 15: PCR analysis in *L.vannamei* - *Post viral inoculums* (7- 20 d)

Lane 1	Positive control (910, 630 and 296bp)
Lane 2	Negative control (yeast tRNA)
Lane 3	Day 7 to 8 WSSV moderate level +ve sample (650bp) –Tank-B-sample
Lane 4	Day 9 to 10 WSSV moderate level +ve sample (650bp) –Tank-B-sample
Lane 5	Day 11 to 12 WSSV moderate level +ve sample (650bp) –Tank-B- composite sample

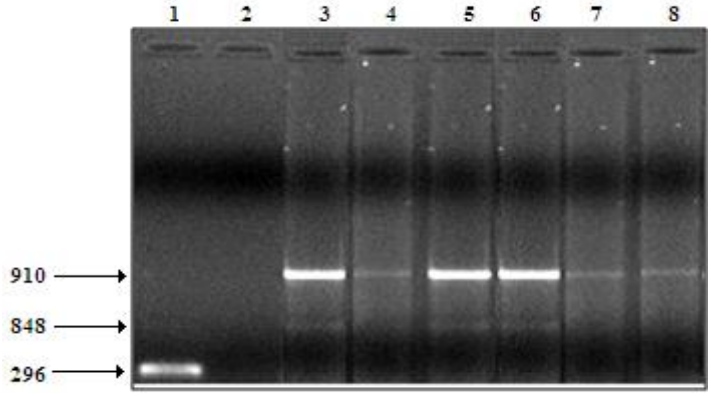

Fig. 16: PCR analysis in *L.vannamei* - *Post viral inoculums* (7- 20 d)

Lane 1&2	Positive control (296bp) lower level Negative control (yeast tRNA)
Lane 3&4	Day 13 to 14 WSSV moderate level +ve sample (650bp) –Tank-B-sample
Lane 5&6	Day 15 to 16 WSSV moderate level +ve sample (650bp) –Tank-B-sample
Lane 7	Day 17 to 18 WSSV moderate level +ve sample (650bp) –Tank-B- composite sample
Lane 8	Day 19 to 20 WSSV sever level +ve sample (910bp)-Tank-B- composite sample.

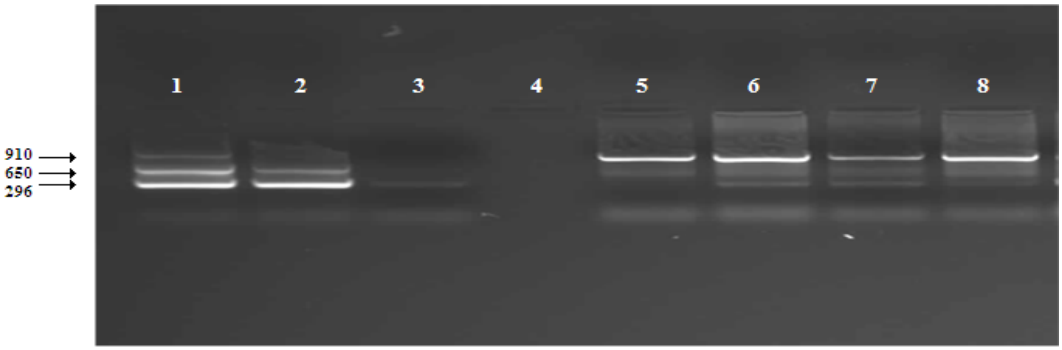

Fig. 17: PCR analysis in *L.vannamei* - *Post viral inoculum* (19– 25 d)

Lane 1	Day 19 WSSV lower level +ve sample (910,650,296bp) –Tank-A-sample
Lane 2	Day 20 WSSV moderate level +ve sample (650,296bp) –Tank-A-sample
Lane 3	Day 21 WSSV moderate level +ve sample (296bp) –Tank-A-sample
Lane 4	Negative control (yeast tRNA)
Lane 5	Day 22 WSSV moderate level +ve sample (650,296bp) –Tank-A-sample
Lane 6	Day 23 WSSV sever level +ve sample (910,650,296bp) –Tank-A-sample
Lane 7	Day 24 WSSV sever level +ve sample (910,650,296bp) –Tank-A-sample
Lane 8	Day 25 WSSV sever level +ve sample (910,650,296bp) –Tank-A-sample

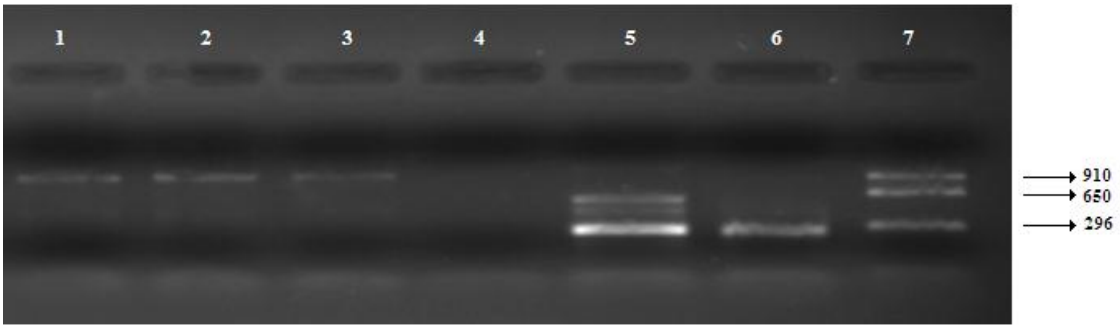

Fig. 18: PCR analysis in *L.vannamei* - *Post viral inoculum* (19– 25 d)

Lane 1 Day 21 WSSV moderate level +ve sample (650,296bp) –Tank-B-sample
Lane 2 Day 22 WSSV moderate level +ve sample (650,296bp) –Tank-B-sample
Lane 3 Day 23 WSSV moderate level +ve sample (650, 296bp) –Tank-B-sample
Lane 4 Negative control (yeast tRNA)
Lane 5 Positive control (910, 630 and 296bp)
Lane 6 Day 24 WSSV lower level +ve sample (296bp) –Tank-B-sample
Lane 7 Day 25 WSSV Sever level +ve sample (910,650,296bp) –Tank-B-sample

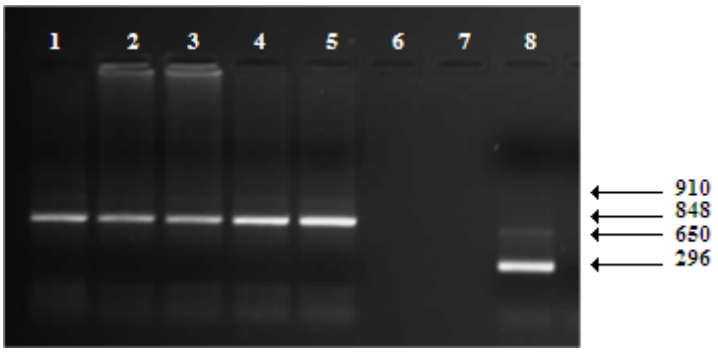

Fig. 19: PCR analysis in the healthy *L.vannamei*– *Control tank-A*.

Lane 1 24 hrs-5d WSSV Negative -ve sample (848bp) – Tank-A-composite sample
Lane 2 6 – 10 d WSSV Negative –ve sample (848bp) – Tank-A-composite sample
Lane 3 11-15d WSSV Negative -ve sample (848bp) – Tank-A-composite sample
Lane 4 5 16-25d WSSV Negative -ve sample (848bp) - Tank-A composite samples
Lane 6,7 Negative control (yeast tRNA) Negative control (dH2O, Master mix)
Lane 8 Positive control (910, 630 and 296bp)

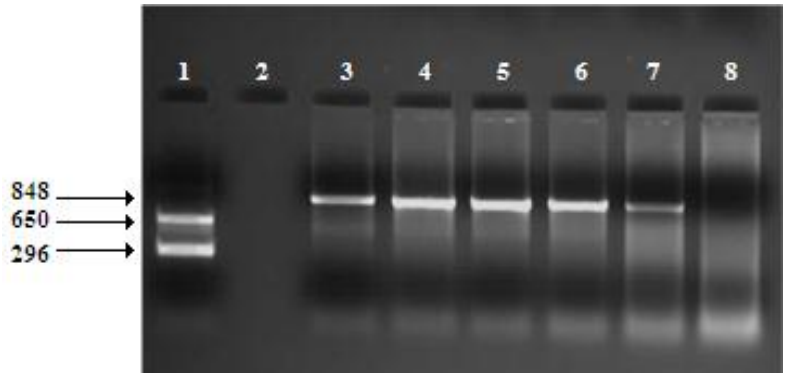

Fig. 20: PCR analysis in the healthy *L.vannamei* - *Control tank-B*

Lane 1,2 Positive control (910, 630 and 296bp) Negative control (yeast tRNA)
Lane 3,4 24 hrs-6d WSSV Negative -ve sample (848bp) – Tank-B-composite sample
Lane 5,6 7 – 12 d WSSV Negative –ve sample (848bp) – Tank-B-composite sample
Lane 7 13-18d WSSV Negative -ve sample (848bp) – Tank-B-composite sample
Lane 8 19-25d WSSV Negativ
Lane 8 19-25d WSSV Negative -ve sample (848bp) – Tank-B-composite sample

DISCUSSION

The baculoviruses divided into three sub groups: the nuclear polyledrosis virus (A), the granulosis virus (B) and the non-occluded virus (C) [29]. Based on the morphology, size, site of assembly, cellular pathology and nucleic acid content, the present virus belongs to the former group C of the family Baculoviridae. Two type C baculovirus have been reported in Penaeid shrimp [29] and these two viruses differ from the virus described in the present study in size and site of assembly. 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.vannamei* that feed on this infected polychaete should have been infected before 7th 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 / or was attenuntated 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 in the shrimps. 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. However, the best management is to establish polychaete culture in a WSSV-free environment and use WSSV-free polychaete to feed broodstock. This can be possible by implementing domestication program to the polychaetes.

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 in *L.vannamei.* The distribution of WSSV in the different organs and tissues of *L.vannamei* was diagnosed using histopathological tools. The results from both histopathology and PCR showed that intramuscular inoculation of different sample preparations except abdominal caused death of the entire experimental animal within 3 to 5 days post infection (p.i) and strongly implied the presence of infectious virus in all these tissues and organs of *L.vannamei* that treated with WSSV-inoculum. In the case of *L.vannamei* the survival extended up to day 9 of p.i and heavy mortality observed between days 18 and 19 of p.i in the shrimps treated with oral route and intramuscularly. The similar trend was observed between days 8 and 9 of p.i. This indicates that the *L.vannamei* can with stand to more days against WSSV than the ultimate candidate species in the Indian water.

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.vannamei*. It was also found that the proportion of WSSV infected *P.cultrifera* survival in the experimental tanks declined from 99% to zero survival within 4

days of severe phase WSSV infection. It shows that the severity of WSSV in *P.cultrifera* did not cause an increase in the proportion of mortality till day 31 of attempt. Severity of WSSV in the polychaete was also higher during later phase of infection. Present findings closely resemble the infectivity effort carried out in *Perinereis nuntia* to *Penaeus* monodon[30]. Logically, when the polychaete had WSSV in their body and if the virus remained infectious, *L.vannamei* 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.

The results from the study strongly suggest developing specific pathogen resistant brooders would immensely useful in rearing the shrimps 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 shrimp breeding purposes will immensely boot the industry.

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