WSSV TRANSMISSION EXPERIMENTS ON POLYCHAETE PERENERIS CULTIFERA TO FENNEROPENAEUS INDICUS IN CAPTIVITY

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Abstract
The present study was 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 Fenneropenaeus indicus by intramuscularly injection and through oral rout.100% mortality of Fenneropenaeus indicus was resulted on 13th day in oral rooted and on 15th day for intramuscularly injected shrimp. Mortality rate of 90.0 and 93.30 % were observed during 12 and 14 days of post infection of WSSV in Fenneropenaeus indicus 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 3 to 9. On days 8, 9, 10 and 14 the product band observed as 650 bp. The product band of 296 bp, 650 bp and 910 bp were noticed during 11, 12, 13 and 15 days of post-inoculum respectively. No evidence of DNA band observed in the control group. Hence it is conclude that the immunity of F.indicus is more against pathogens while comparing with other Phenaied shrimps. This result that F.indicus can survive more days than other shrimps. The histopathological analysis in hematopoietic tissue on the fifth day of post inoculated shrimp shows ranges on low level infection. 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 shrimp breeding purposes will immensely boot the industry.

***Keywords** : PCR, Diagnosis, WSSV, Fenneropenaeus indicus, Perinereis cultrifera and histopathology.*

I.INTRODUCTION

Penaeus indicus common name us Indian white prawn [1,2]. There also other important shrimp species are P. monodon, P. chinensis, P. merguiensis, P. japonicus and F. indicus for commercial purpose [3]. Penaeus indicus and Penaeus monodon are the most popular species of prawns cultured in India and among these two P. indicus is easily available and less expensive and can be easily maintained in laboratory [4]. The presence of L. vannamei is naturally along the pacific coast of Central and South America [3]. P. monodon, P. indicus,P. japonicus, P. chinensis, P. penicillatus, P. aztecus, Charybdis annulata, Grapsus albolineatus, Lithodes maja are found to be highly susceptible to WSSV [5-10] in which P. indicus and P. monodon are found to be highly pathogenic [9].In india WSSV infects P. indicus shrimps [9] and the outbreaks of WSSV affects as mass mortality [11].

Pathogenicity describes the ability of a pathogen to cause disease; and virulence is the degree of pathogenicity within a group or species [12]. 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 [13]. 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 [13]. 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 [14-19].

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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 [20-22] WSSV virions can remain infective in the decaying belief that free virus cannot survive in nature waters more than 24 h [23] 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 [13]. White spot syndrome virus (WSSV) is of rod shaped envelope containing double stranded DNA as genetic material which belongs to Nimaviridae family [24, 25]. 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 [26]. In India, almost all penaeid hatcheries use polychaete worms to promote maturation and spawning of wild caught brood-stock/ spawners of Fenneropenaeus indicus. At present there is no remedy for the interference of WSSV with the wild occurrence and disease invasion [27]. 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 [28]. Infectivity of WSSV to the Polychaete Pereneris nuntia and a possibility of WSSV transmission from the polychaete to the Fenneropenaeus indicus, followed by Polychaete worms as a vector for WSSV were investigated by Laoaroon et al., in 2005 [29] and Vijayan et al., in 2005 [30]. Aim of the present investigation is to confirm the transmission of WSSV through the commercially important Polychaete viz., Perenereis cultrifera to the Fenneropenaeus indicus in laboratory condition. The Purpose of the present investigation was to confirm the transmission of WSSV through commercially important Polychaete Perinereis cultrifera to the Fenneropenaeus indicus under laboratory condition.

II.MATERIALS AND METHODS:

A. 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 Litters 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.

B. Indian white shrimp - Fenneropenaeus indicus.

Wild F. indicus with 50–120 mm TL, were collected from Vellar estuary neighbouring Marine Virology Laboratory, CAS Marine Biology, Parangipettai, shrimps were kept up in 25 Litre engineered plastic tanks with filtered and aerated Vellar estuary water with the salinity20–25 ppt; temperature 28–32ºC; pH 8.0–8.2, and the ABW of shrimps ranged 7-15 g.

C. Feeding.

Fenneropenaeus indicus 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.

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D.Preparation of viral extract for oral challenge tests.

Pond reared, naturally infected Fenneropenaeus indicus 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 Fenneropenaeus indicus 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 Fenneropenaeus indicus. 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.

F. Infectivity and vertical transmission method to Polychaetes.
For WSSV infectivity studies, two replicate groups (80 individual each) of polychaetewere 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 polychaeteswere 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 polychaetes 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.

G. 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 dorso-ventral 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.

H. Viral DNApurification.
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 F. indicus under experimental conditions. All challenged shrimps displayed signs of WSSV infection thus proving the presence of infectious WSSV white patch particles.

I. 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

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experiments were run until all the infected shrimp died. Control shrimp were sacrificed at 360 h post inoculation (hpi)

J. 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 F. indicus.

K. Clinical signs.

The shrimp F.indicus 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).

L. Time-course infectivity experiments.

F.indicus 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 F.indicus. Further, WSSV transmission trial repeated thrice.

M. Experimental Design.

All the WSSV transmission design was followed by the procedure of Sahul Hameed et al. in 2002 [33] 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, in 1996 [36]. 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 (Fenneropenaeus indicus), 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.

N. 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 [34]. The polychaete organ along with the body setae were removed from the region between the mandibular and posterior dorso-ventral muscle of the animal. Following organs of shrimps 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,

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sectioning, and staining with haematoxylin and eosin [35]. 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, in 1996 [36].

O. PCR analysis.

Template DNA was prepared from polychaete P.cultrifera and F. indicus 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.

III.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.

A. 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.

B.Mortality & Survival in the F.indicus.

Mortality of 90.0 and 93.30 % were noticed in the F.indicus shrimps treated oral route and intramuscularly after post WSSV-inoculum and these mortality was observed on 12 and 14 days respectively (Table 3). However, a drastic change in the survival from 100 to 40.0 and 63.30 % resulted in the F.indicus shrimps. The total days of experiment continued up to 15 and on day 16 the experiment was terminated (Table 5).

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C. Histopathology

Histological observation of low, moderate and severely WSSV infected F.indicus 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 histopathologcial evidence of post WSSV infected F.indicus tissues namely gut and mid-gut gland were compared with normal histological observations **(**Fig 6**)**. After five days of post-inoculum with low level infection range visulizes hypertrophied nuclei (Fig 2) in cuticular epithelium of the hematopoietic tissue. The intranuclear inclusion bodies characteristic of WSSV infection in the hematopoietic tissue cells after ninth day expresses large numbers of darkly stained, round intracytoplasmic inclusion bodies as shown in Fig 5. On the twelveth day of post-transmission the cuticular epithelium of the gill lamellae with large basophilic intranuclear inclusion bodies are degenerated and heavily infected become large basophilic inclusions as shown in Fig 3. The subcuticular gut epitheliums are affected with hypertrophied nuclei containing basophilic intranuclear inclusion bodies after 12 days of transmission (Fig 4). On the fifteenth day of post inoculum F.indicus showing cellular degeneration of hepatopancreatic globules with presence of WSSV inclusion bodies (Fig 7).

D.PCR analysis

The results of PCR analysis on different organs obtained from time-course experiments using experimentally WSSV-infected shrimps were presented in (Fig 9-13). 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 48 hrs of post-inoculum in the pleopods of F.indicus whilst, these band of 650 bp, 296 bp continued from day 3 to 6. On days 8, 9,10 and 14 the product band observed as 650 bp and which was recorded as 296 bp, 650 bp and 910 bp 11, 12, 13 and 15 days of post-inoculum respectively to F.indicus exposed to WSSV by oral route. There was no evidence of DNA band in the control group of F. indicus.

IV.DISCUSSION

The baculoviruses divided into three sub groups: the nuclear polyledrosis virus (A), the granulosis virus (B) and the non-occluded virus (C) [37]. 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 [37] 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, F.indicus 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 / 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 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. 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

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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. Similarly the distribution of WSSV in the different organs and tissues 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 that treated with WSSV-inoculum.

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 F.indicus. Logically, when the polychates had WSSV in their body and if the virus remained infectious, F.indicus 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 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 shrimp breeding purposes will immensely boot the industry.

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Table 1.Infectivity trial of WSSV from P.cultrifera to F.indicus in experimental tanks

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

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

Hours / days	F.indicus infected with WSSV		Control with out WSSV
	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	1	Nil	Nil
5th day	2	2	Nil
6th day	3	1	Nil
7th day	2	3	Nil
8th day	4	4	Nil
9th day	6	2	Nil
10th day	2	2	Nil
11th day	3	6	Nil
12th day	4	2	Nil
13th day	3	4	Nil
14th day	-	2	Nil
15th day	-	2	Nil

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Table 3. PCR detection and cumulative percent mortality of F.indicus 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 - F.indicus	IHC detection for WSSV - F.indicus	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	3.3 96.7	0 100	-ve 0 100
5th day	+ve +ve	-ve -ve	10.0 90.0	6.7 93.3	-ve 0 100
6th day	+ve +ve	NA NA	20.0 80.0	10.0 90.0	-ve 0 100
7th day	+ve +ve	NA NA	26.7 73.3	20.0 80.0	-ve 0 100
8th day	+ve +ve	NA NA	40.0 60.0	36.7 63.3	-ve 0 100
9th day	+ve +ve	+ve +ve	60.0 40.0	43.3 57.7	-ve 0 100
10th day	+ve +ve	NA NA	66.7 33.3	46.7 53.3	-ve 0 100
11th day	+ve +ve	NA NA	76.7 23.3	66.7 33.3	-ve 0 100
12th day	+ve +ve	NA NA	90.0 10.0	73.3 27.7	-ve 0 100
13th day	+ve +ve	+ve +ve	100 0	86.7 13.3	-ve 0 100
14th day	+ve +ve	NA NA	- -	93.3 6.7	-ve 0 100
15th day	+ve +ve	NA NA	- -	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 Analysis

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Histopathology slides

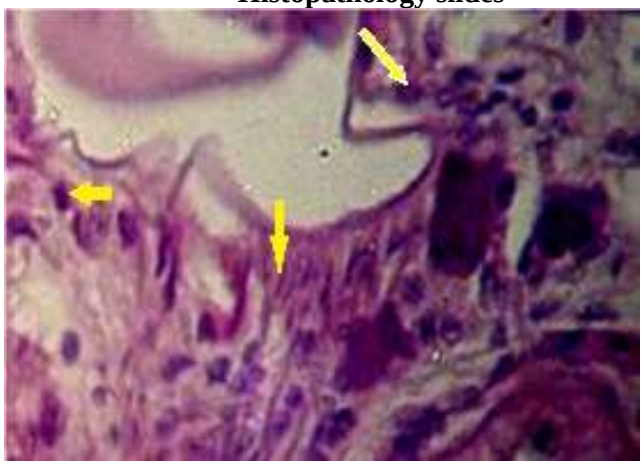

Figure 1. Micrograph of cuticular epithelium of a healthy (control) hepatopancreas F. indicus. Arrow indicates the normal subcuticular epithelial cells with normal nuclei. (H & E) 1000 X

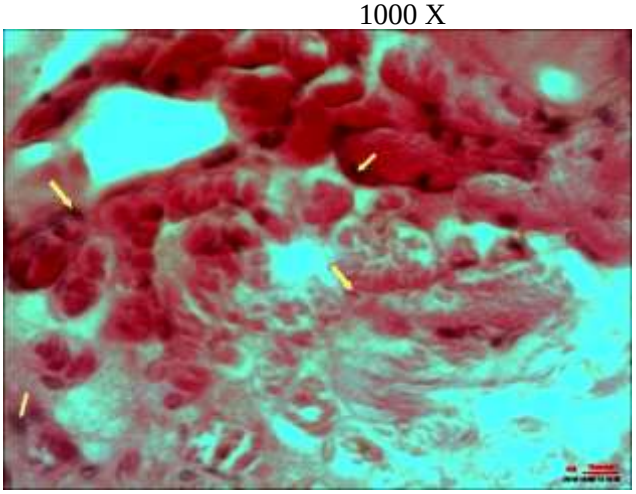

Figure 2. Micrograph of cuticular epithelium of the hematopoietic tissue of F.indicus, 5 d of post-inoculum (low level infection range), arrow shows hypertrophied nuclei (H&E) 1000 X

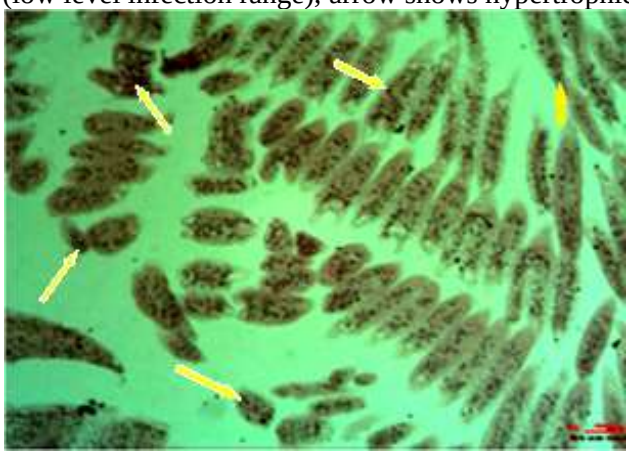

Figure 3. Micrograph of cuticular epithelium of the gill lamellae with large basophilic intranuclear inclusion bodies characteristic of F.indicus, 12 d of post-transmission. Arrow showsdegenerated and heavily infected cuticular epithelial cells showing large basophilic inclusions characteristic of WSSV infection (H& E) 400 X

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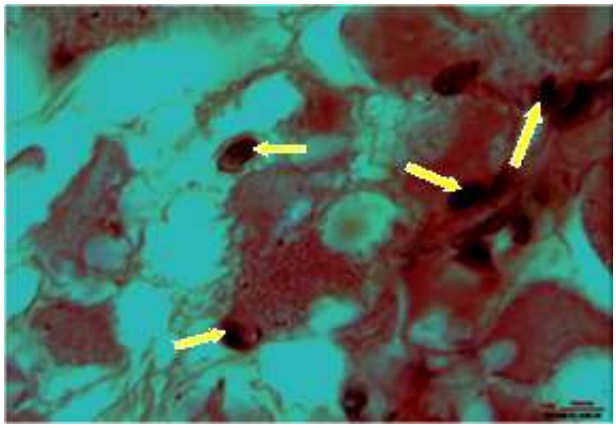

Figure 4. Micrograph of subcuticular gut epithelium with hypertrophied nuclei containing basophilic intranuclear inclusion bodies characteristic of WSSV infection (arrow) in F.indicus showing gross signs of WSSV on 12 d. (H&E) 1000 X

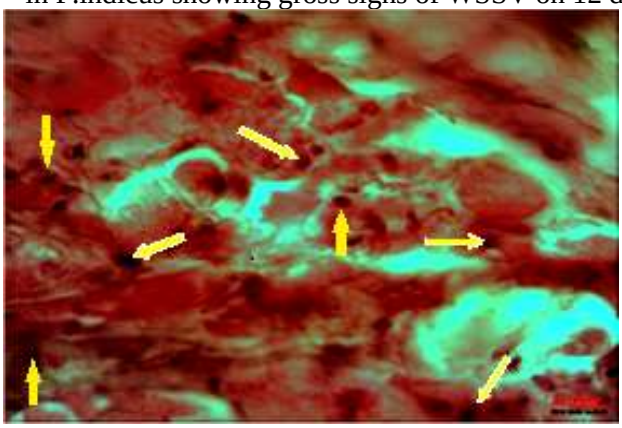

Figure 5. Micrograph of intranuclear inclusion bodies characteristic of WSSV infection (arrow) in the hematopoietic tissue cells of F.indicus showing gross signs of WSSV on 9 d. Note the presence of large numbers of darkly stained, round intracytoplasmic inclusion bodies (arrowheads) (H&E) 1000 X

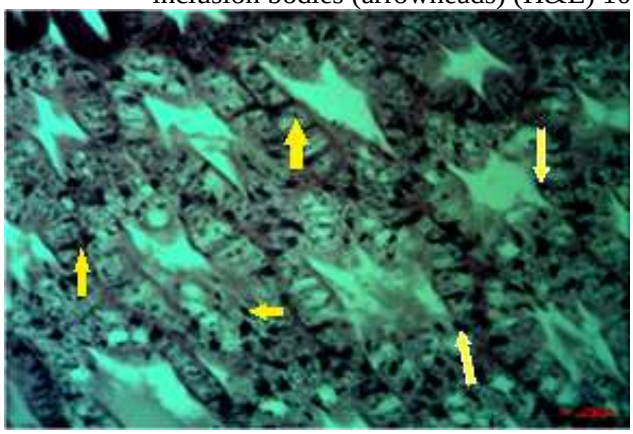

Figure 6. Micrograph of intramuscular inclusion bodies characteristic of WSSV infection (arrow) in the mid-gut gland tubule cells of F. indicus showing severity (H& E) 400 X

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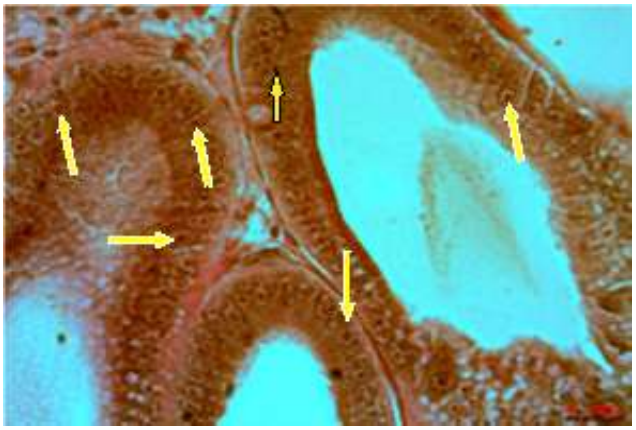

Figure 7. Micrograph of mid-gut gland of 15 d. post inoculum F.indicus showing cellular degeneration of hepatopancreatic globules (arrow) with presence of WSSV inclusion bodies 1000 X

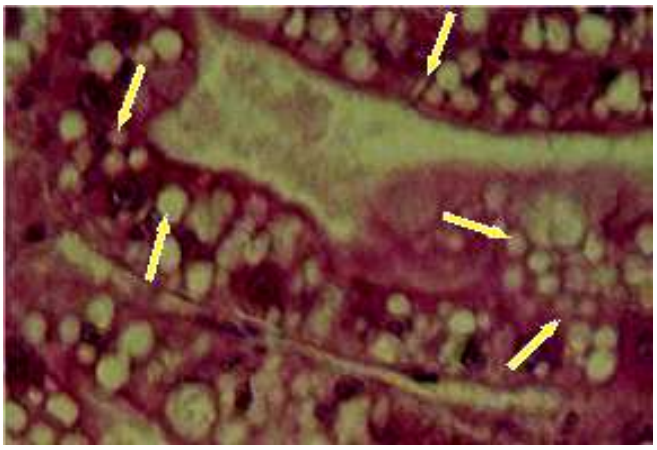

Figure 8. Micrograph of normal mid-gut gland of F.indicus on 15day showing cellular generation of hepatopancreatic globules (arrow) with absence of WSSV inclusion bodies 1000 X

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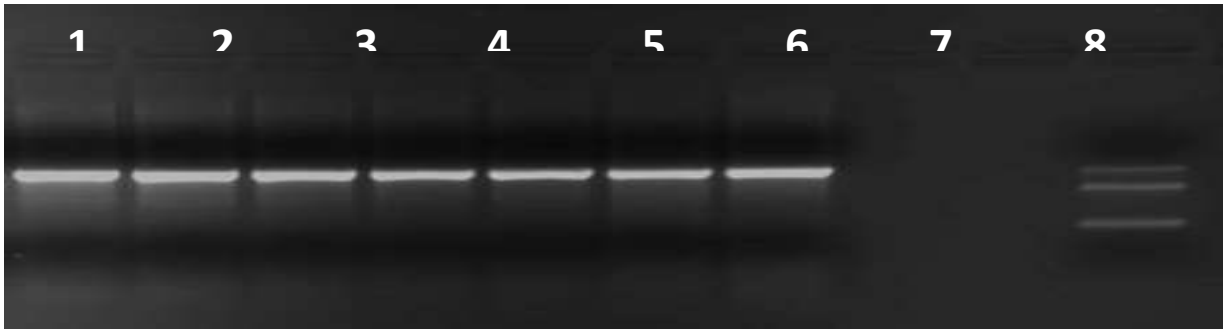
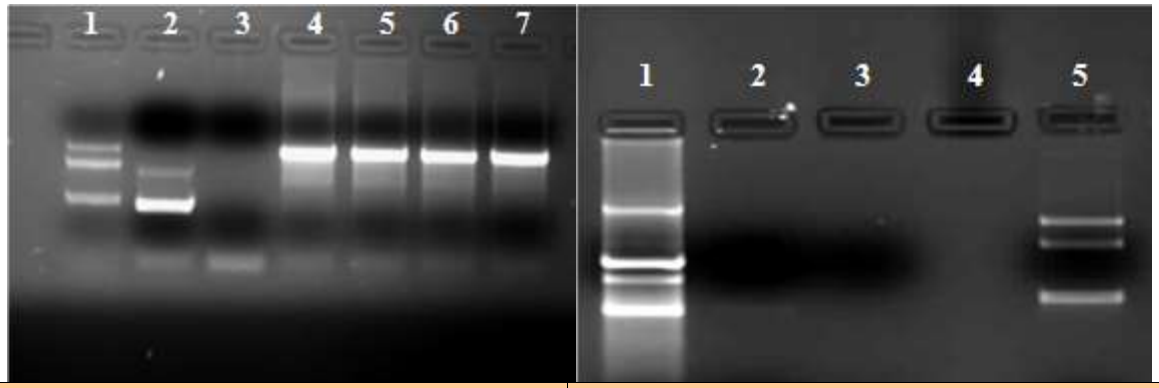
AGE GELS



a. F.indicus Post viral inoculum			
Lane 1	24 hrs WSSV Negative -ve sample (848bp) – Tank-A-sample	Lane 6	48 hrs WSSV Negative -ve sample (848bp) – Tank-B-sample
Lane 2	36 hrs WSSV Negative -ve sample (848bp) – Tank-A-sample	Lane 7	Day 3 WSSV Negative -ve sample (848bp) - Tank-B sample
Lane 3	48 hrs WSSV Negative -ve sample (848bp) – Tank-A-sample	Lane 8	Negative control (yeast tRNA)
Lane 4	24 hrs WSSV Negative -ve sample (848bp) - Tank-B composite sample	Lane 9	Negative control (ddH_2O)
Lane 5	36 hrs WSSV Negative -ve sample (848bp) - Tank-B composite samples	Lane10	Positive control (910, 630 and 296bp)

Figure 9 PCR analysis in F.indicus (24 hrs- 3 d).



b.F.indicus Post viral inoculum		c. F.indicus Post viral inoculum	
Lane 1&2	DNA molecular weight marker Positive control (910, 630 and 296bp)	Lane 1	Day 6-7 WSSV sev. level +ve sample (910,650, 296bp) –Tank-B-sample
Lane 3	Negative control (yeast tRNA)	Lane 2	Day 4 WSSV low. level +ve sample (296bp) –Tank-B-sample
Lane 4&5	Day 3 to 4 WSSV mod. level +ve sample (650, 296bp) –Tank-A-sample	Lane 3	Day 5 WSSV low. level +ve sample (296bp) –Tank-B-sample
Lane 6&7	Day 5 to 6 WSSV mod. level +ve sample (650, 296bp) –Tank-A-sample	Lane 4	Negative control (yeast tRNA)

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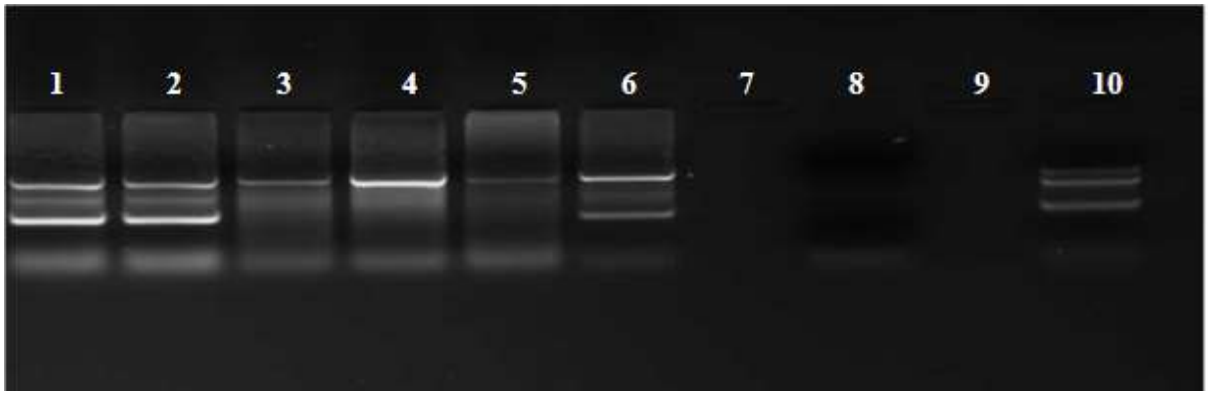
b.F.indicus Post viral inoculum		c. F.indicus Post viral inoculum	
Lane 8&9	Day 7 to 9 WSSV mod. level +ve sample (650, 296bp) –Tank-A-sample	Lane 5	Day 6 WSSV sev. level +ve sample (296, 650,910bp)-Tank-A-sample.

Figure 10.PCR analysis in F.indicus (3-7 d).



d. F.indicus Post viral inoculum			
Lane 1	Day 12 WSSV sev. level +ve sample (296, 650, 910bp)-Tank-A-sample	Lane 6	Day 13 WSSV sev. level +ve sample (296, 650, 910 bp) –Tank-A-sample
Lane 2	Day 11 WSSV sev. level +ve sample (296, 650, 910bp)-Tank-A-sample	Lane 7	Negative control (yeast tRNA)
Lane 3	Day 10 WSSV mod. level +ve sample (650bp) –Tank-A-sample	Lane 8	Day 14 WSSV low. level +ve sample (296bp) –Tank-A-sample
Lane 4	Day 9 WSSV mod. level +ve sample (650bp) –Tank-A-sample	Lane 9	Negative control (dH2O, culture water and Master mix)
Lane 5	Day 8 WSSV mod. level +ve sample (296, 650bp)-Tank-A-sample	Lane 10	Day 15 WSSV sev. level +ve sample (296, 650, 910 bp) –Tank-A-sample

Figure 11.PCR analysis in F.indicus (8- 15 d)

a.F.indicus Post viral inoculum			
Lane 1	Day 8 WSSV mod. level +ve sample (296, 650bp)-Tank-B-sample	Lane 6	Molecular wt marker (848,650,333bp)
Lane 2	Day 9 WSSV mod. level +ve sample (650bp) –Tank-B-sample	Lane 7	Negative control (yeast tRNA)
Lane 3	Day 10 WSSV mod. level +ve sample (650bp) –Tank-B-sample	Lane 8	Day 12 WSSV mod. level +ve sample (650bp) –Tank-B-sample
Lane 4	Day 11 WSSV mod. level +ve sample (650bp) –Tank-B-sample	Lane 9	Day 13 WSSV mod. level +ve sample (650bp) –Tank-B-,sample

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Lane 5	Positive control (910, 630 and 296bp)	Lane 10,11	Day 14 to 15 WSSV mod. level +ve sample (650bp)-Tank-B-comp.samp.

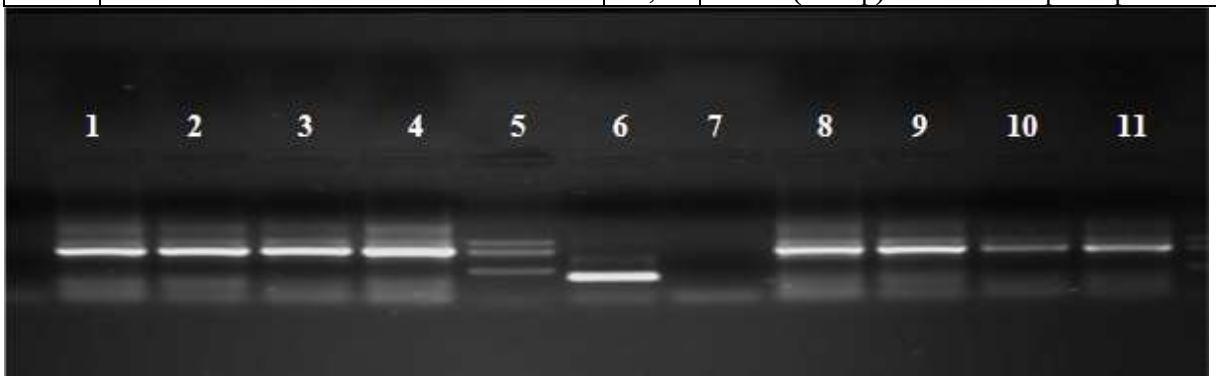
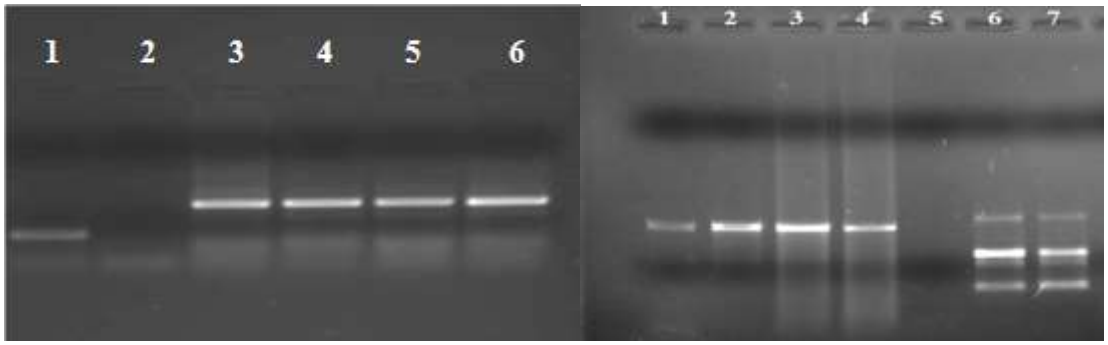


Figure 12.PCR analysis in F.indicus (8- 15 d)



b. F.indicus – Control tank-A		c. F.indicus– Control tank-B	
Lane 1,2	Positive control (910, 630 and 296bp) Negative control (yeast tRNA)	Lane 1,2	24 hrs-7d WSSV Negative -ve sample (848bp) – Tank-B-composite sample
Lane 3	24 hrs-3d WSSV Negative -ve sample (848bp) – Tank-A-composite sample	Lane 3	8 –12 d WSSV Negative –ve sample (848bp) – Tank-B-composite sample
Lane 4	4-6d WSSV Negative -ve sample (848bp) – Tank-A-composite sample	Lane 4	13 –15 d WSSV Negative –ve sample (848bp) – Tank-B-composite sample
Lane 5	7-12d WSSV Negative -ve sample (848bp) – Tank-A-composite sample	Lane 5	Negative control (yeast tRNA)
Lane 6	13-15d WSSV Negative -ve sample (848bp) – Tank-A-composite sample	Lane 6,7	Positive controls (910, 630 and 296bp)
Lane 8	Positive control (910, 630 and 296bp)		

Figure 13PCR analysis in the healthy F. Indicus(24h- 15 d) -(Control Tank – C)

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