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Research Article

PATHOGENESIS AND INFECTIVITY POTENTIAL OF WHITE SPOT SYNDROME VIRUS (WSSV) IN *LITOPENAEUS VANNAMEI*

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ABSTRACT

White spot syndrome virus (WSSV) infection is one of the most virulent pathogenic and divesting viruses of the cultured penaeid shrimps. In this attempt, we have analyzed the pathogenesis and infectivity potential of white spot syndrome virus (WSSV) in *Litopenaeus vannamei*. Transmission electron microscopic study revealed that, the presence of WSSV particles in the gill cells with pyknotic nuclei. Occasionally, two nucleocapsids within one envelope were also present amongst single enveloped nucleocapsids. A long rod-shaped structure that could reach 250 nm in length was present in the nuclei of some infected cells. Further, gill tissue samples collected from all the experimental shrimp were positive by one-step polymerase chain reaction (PCR), confirmed the severe WSSV infection on the shrimps. The experimental work fastest mortality was observed through the ingestion than water borne mode if infection. The water-borne mode caused the 100% cumulative mortality on 29th day, 21th day and17th day in tanks with 5, 10 and 15 *L. vannamei*, respectively. The ingestion mode caused the 100% mortality by the 22th day, 17th day and14th day in tanks with 5, 10 and 15 *L. vannamei* respectively.

Keywords: White Spot Syndrome Virus, *L. vannamei,* TEM, Infectivity test,

INTRODUCTION

White spot syndrome virus (WSSV), a large double-stranded DNA virus, infects a broad range of cultured and wild shrimps (Syed Musthaq *et al*., 2006). WSSV infection causes high mortality rates and severe economic losses in the shrimp aquaculture industry worldwide. Pre-screening of WSSV-free brood stock or larvae and regular surveillance of WSSV infection are important strategies to reduce the economic impacts of the disease on shrimp aquaculture. In India alone, the net loss has been estimated at several million dollars per year due to this pathogen (Anonymous, 1996). Formerly, numerous reports are available due to WSSV affect the cultivated shrimps in the eastern and western hemisphere, and it is the most virulent virus hitherto reported from the farmed shrimps (Flegel and Alday-Sanz, 1998; Van Hulten *et al*., 2000). The infected shrimp display clinical signs such as anorexia, lethargic, swollen branchiostegites due to fluid accumulation, white spots in the cuticle, separated/ loose cuticle from underlying epidermis, yellowish-white and enlarged hepatopancreas, hemolymph which fails to coagulate and reddish discoloration of the moribund shrimp (Lightner, 1996; Sahulhameed *et al.,* 1998; Wang *et al*., 1999). Since, there are no therapeutic treatments currently available for WSSV; the best management strategy is to prevent WSSV from entering a shrimp farming facility (Lotz, 1997) or use of optimal shrimp culture conditions (Browdy *et al.,* 1993).

The principle clinical sign of the affected shrimp will be white spots in the exoskeleton and epidermis (Sahulhameed *et al.,* 1998). This virus can cause 100% mortalities within 3-10 days of the onset of the above symptoms. The WSSV has a wide host range and it has been observed not only in shrimps but also in crabs and other arthropods such as copepods, insects and pest prawns. Viral diseases have become important limiting factors for shrimp production throughout the world (Flegel, 1997). The virus infects a broad host range, including wild and farmed shrimp (Flegel, 2001). WSSV survives only a few days extracellular and 30 day-period was long enough to exclude any possibility that subsequent infection of *P. monodon* might come from inoculated WSSV that were located extracellular (Maeda *et al.,* 1998). Effects of various treatments on white spot syndrome virus (WSSV) from *Penaeus japonicus* (Japan) and *P. monodon* (Thailand).Temperature is associated with mortality in Ecuadorian farms (Rodriguez *et al.,* 2003). Abrupt fluctuations in temperature and salinity due to heavy rains contribute to increased viral loads in shrimp in Mexico, resulting in 80% mortality (Peinado, 2006). Therefore, the present study was carried out Virological outcome of experimental infections with White Spot Syndrome Virus (WSSV) in *Litopenaeus vannamei*.

MATERIALS AND METHODS

Experimental animals:

The infected *Litopenaeus vannamei* were collected from the cultured pond at Vellapallam in south east India. Healthy *L. vannamei* (average individual weight 5g)were reared in individual culture tank (50 L)

and maintained under standard experimental conditions: temperature28± 2°C, salinity 29± 2 ppt, dissolved oxygen 4 ±2 ppm, pH 7.7 ±0.2, 12 ± 1 h (light/dark cycle)and fed with commercially available balanced pellet feed (Avanthi feed Limited, India). The shrimps were acclimatized for one week prior to the initiation of experiments.

Experimental design:

The infectivity experiment was conducted on *L. vannamei,* with three different routes of infection (ingestion and waterborne). The shrimps were divided into three groups each comprising of 15 shrimps (All experiments are triplicates) as details given bellow:

Group I: The shrimp (WSSV Negative) were scrapped from mechanized boat hull and kept along with the control shrimps throughout the experiment.

Group II: The shrimps were fed *ad–libitum* with WSSV infected shrimp flesh. From second day onwards the shrimps were fed with dry commercial pellet feed and the control animals were fed with beef liver on the day of experiments and followed by pellet feed.

Group III: Infection trial was performed using the filtrate of WSSV infected shrimp. The infected shrimp meat was removed from the shell and homogenized in brackish water at 4°C at a ratio of 1:9. After being centrifuged at 8515× g for 5 min., the supernatant fluid was filtered through a 0.45 µm membrane and diluted 500 times with brackish water in order to use as water borne inoculation.

Transmission electron microscopy (TEM):

The WSSV positive shrimp tissues were fixed in 3 % glutaraldehyde (pH 7.2), washed in sodium cocodylate buffer (pH 7.2), post fixed in 1% osmium tetroxide and further washed in buffer, dehydrated through an ascending series of graded alcohol 50% to 100% cleared in propylene oxide. The tissue was further infiltrated by propylene oxide and embedded in epoxy resin. The embedded mound was kept in an incubator at 60°C for 48 h for cooling down. One micron semi thin sections were cut using an ultra–microtome (Leica Ultra–cut UCT) with a glass knife and stained with 1% toludine blue. Subsequently, the ultra–thin sections were taken on copper grid and stained with Uranyl acetate and Reynold's solution. The sections were examined using a TEM (Phillips model 201–C, Phillips Electron Instruments, Mahwah, NJ). After TEM analysis, virus infected shrimp tissues were further analyzed PCR analysis.

Molecular characterization:

The moribund and dead shrimps were tested for WSSV using same method (PCR) of testing WSSV in the moribund shrimps. Total DNA was extracted from the gill tissues from control and experimental groups of shrimps by Trizol reagent (Invitrogen, Carlsbad, CA) and purified by QiagenDNeasy Mini Kit (Qiagen, Venlo, The Netherlands). Purified total DNA was amplified, forward (5'- AATGGTCCCGTCCTCATCTCA-3') and reverse (5'-GCTGCCTTGCCGGAAATT-3') primers specific, which were successively analyzed semi-quantitatively using the GoTaq PCR master mix and quantitativelyusing the PCR master mix (Applied Biosystems, FosterCity, CA). Amplicons were analyzed subsequently on a 12% polyacrylamide gel in TAE

buffer (40mMTris, 20 mM acetic acid and 1mM EDTA) and visualized by ethidium bromide staining.

RESULTS

The present study demonstrated that the density of shrimps lead to severe or fast mortality of shrimps exposed to WSSV inoculation**.** The normal and WSSV infected carapace from the WSSV infected *L.*vannamei (Fig. 1 a, b and c). The white spots were seen in the experimentally infected *L. vannamei*. The white spots ranged between 1.5–2.8 mm in diameter embedded in the carapace (Fig 2a and b).The viral particles viewed under TEM in the experimentally infected *L.vannamei* ranged between 170 and 250 µm (Fig. 3a). The white spot syndrome virus (WSSV) lesions in the cuticular epithelium of the gill of an experimentally infected *L.vannamei* (Fig. 3b).The epitheloid cells had a hypertrophied nucleus with intra nuclear inclusions bodies. Nuclei contain Rod to ovoid-shaped virions particles. Some virions with a terminal projection observed in gill filaments of *L.vannamei* during the epizootic outbreaks (Fig. 3c and d).The sensitive methods of PCR were utilized with commercial diagnostic kits to detect white shrimp virus disease, and diagnostic results showed that WSSV were detected in the diseased shrimp (Fig. 4 and 5).

The experimental work we have selected two different methods (i) Water borne and (ii) ingestion. Among all modes of infection, the fastest mortality was observed through the ingestion and water - borne modes. The water - borne mode caused the 100% cumulative mortality by the 29th day, 21th day and17th day in tanks with 5, 10 and 15 *L. vannamei*, respectively (Fig. 6). The ingestion mode caused the 100% mortality by the 22th day, 17th day and14th day in tanks with 5, 10 and 15 *L. vannamei*, respectively (Fig. 7). However, water borne infection mode of infection showed almost similar result on the cumulative mortality of *L. vannamei*. Density of the *L.vannamei* in the tank influenced the cumulative mortality. With increasing the *L.vannamei* density, the mortality also increased in all mode of ingestion. In tanks with 18 *L. vannamei*, the complete mortality was reported by 1stday, 5thday, 12thday and 17th day in the ingestion, water borne mode, respectively. In tanks with 5 shrimps per tank all were died by the 14th day and 21stday in ingestion and water borne mode, respectively (Fig. 8).

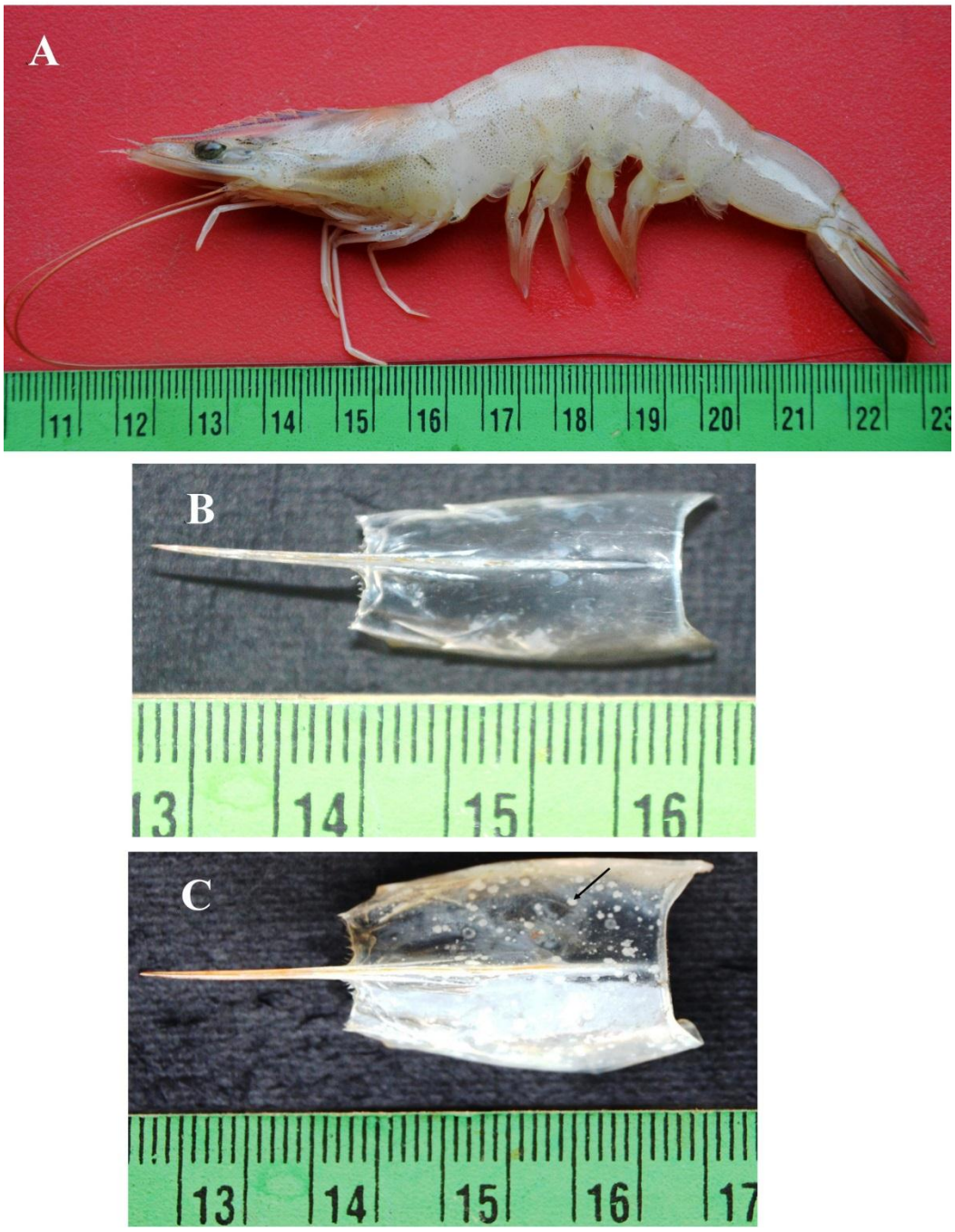

Figure **1:** Normal *Litopenaeus vannamei*(a); *Litopenaeus vannamei* normal carapace (b); *Litopenaeus vannamei* carapace infected with WSSV (c).

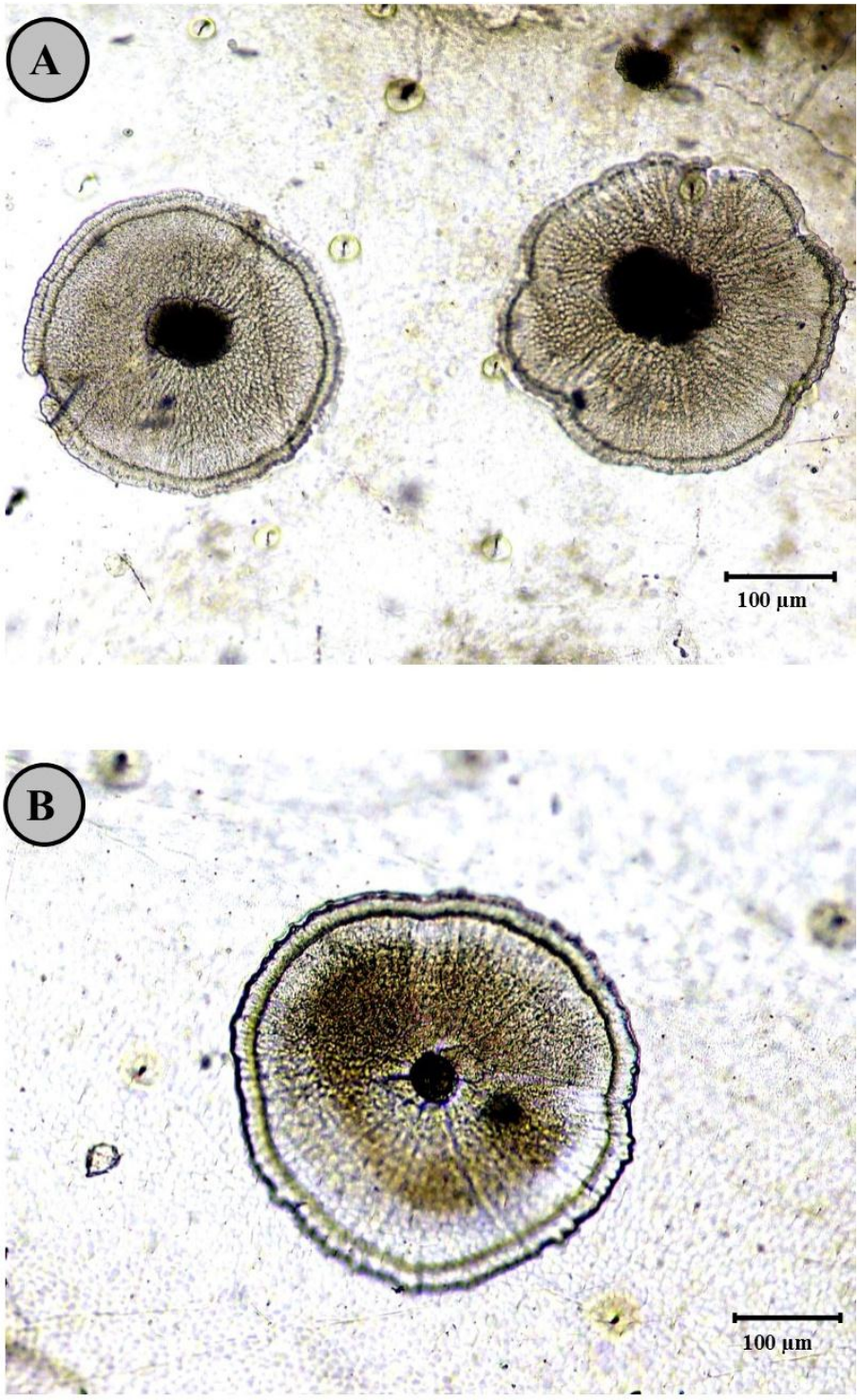

Figure 2: BWS as opaque brownish lichen-like lesions with a crenated margin (a and b).

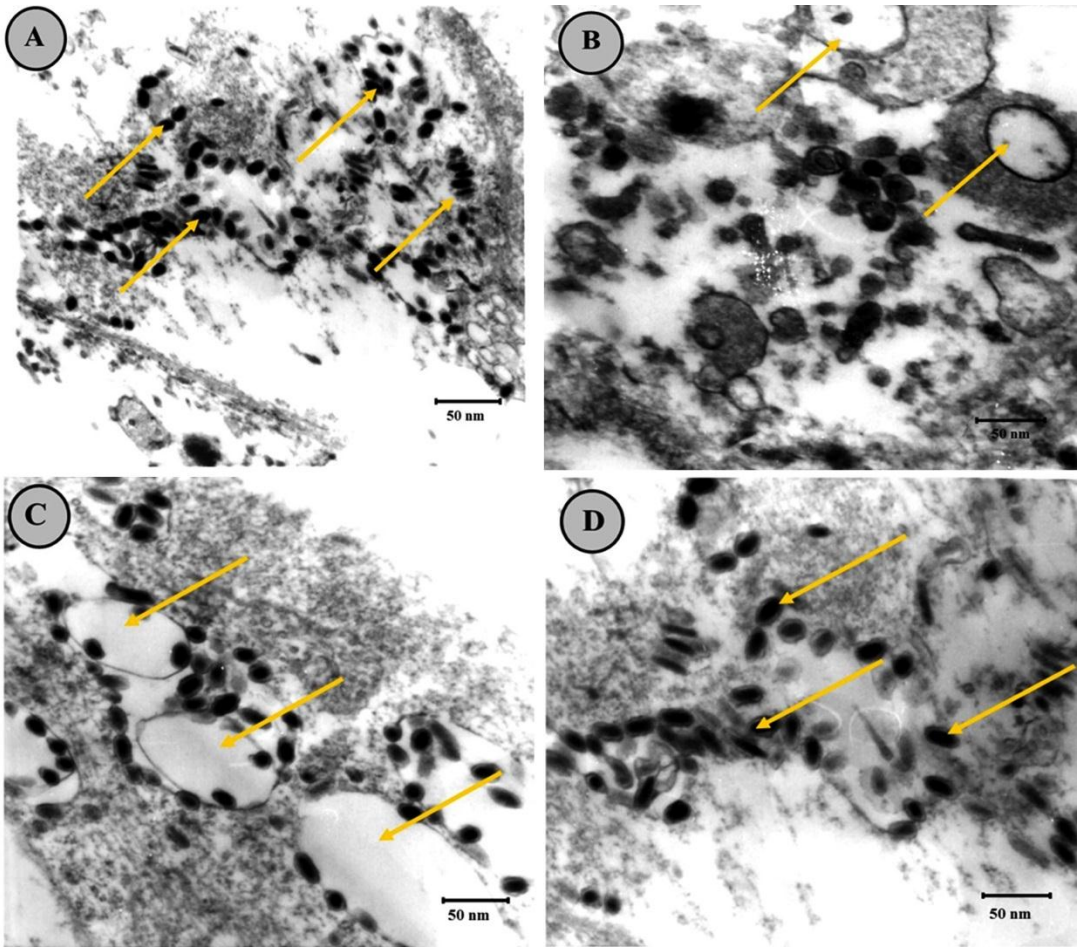

Figure 3: Hypertrophied nuclei with intra nuclear WSSV inclusions (arrows) (a); Rod- to ovoid-shaped intranuclearvirions (b); WSSV viral suspension from *L.vannamei* hepatopancreas (c); Oval-shaped and bacilliform particles (large arrow) and outer envelope (lower arrow) (d).

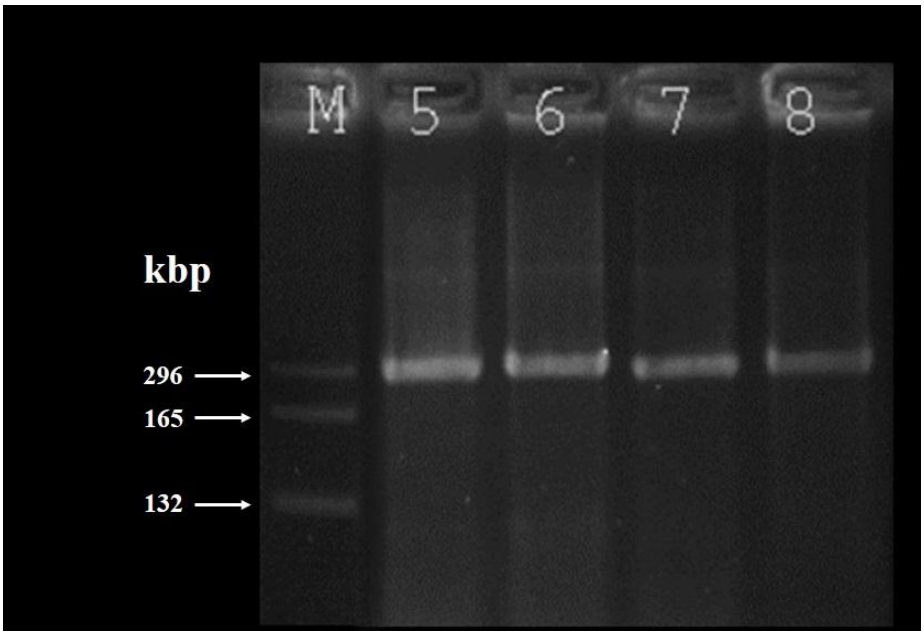

Figure 4: M – Marker, 5,6,7,8 – Amplification of DNA.

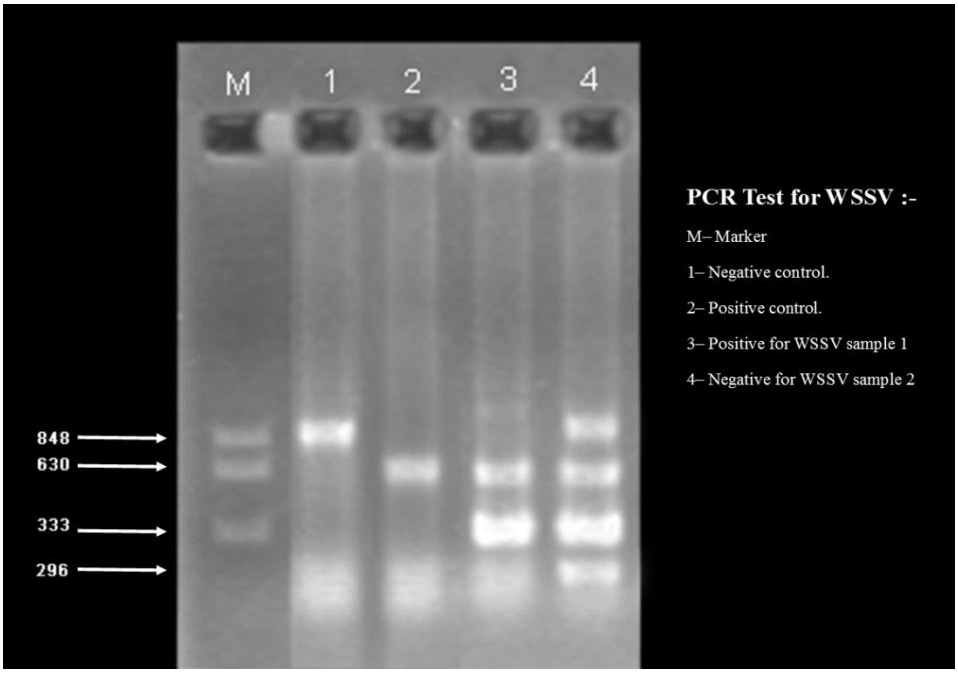

Figure 5: PCR amplification of a fragment of the WSSV gene. A 1% agarosegel electrophoresis stained with ethidium bromide, revealing the PCR amplification product of 848 bp corresponding to a fragment of the WSSV gene. M: marker (100 pb DNA ladder); (1) negative control (PCR reaction without DNA); (2) Positive control (PCR reaction without DNA) (3 & 4) amplification of the WSSV sample from infected shrimp genomic DNA.

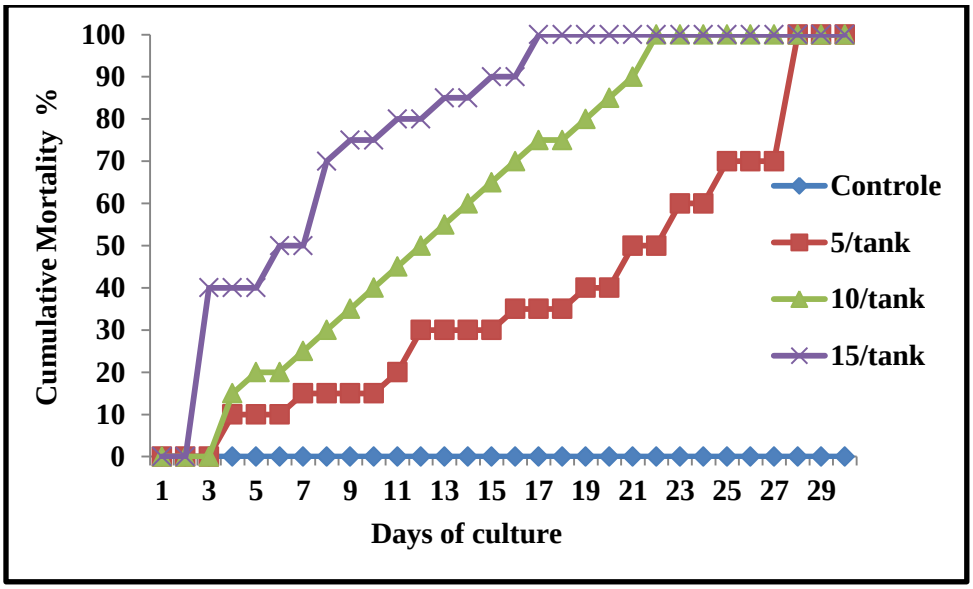

Figure 6: Cumulative mortality in water borne

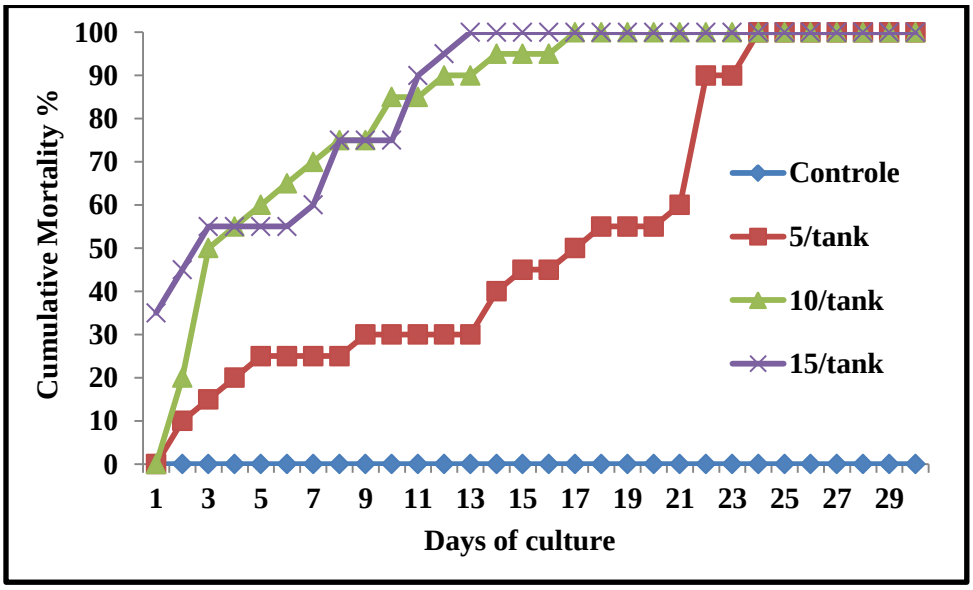

Figure 7: Cumulative mortality in ingestion

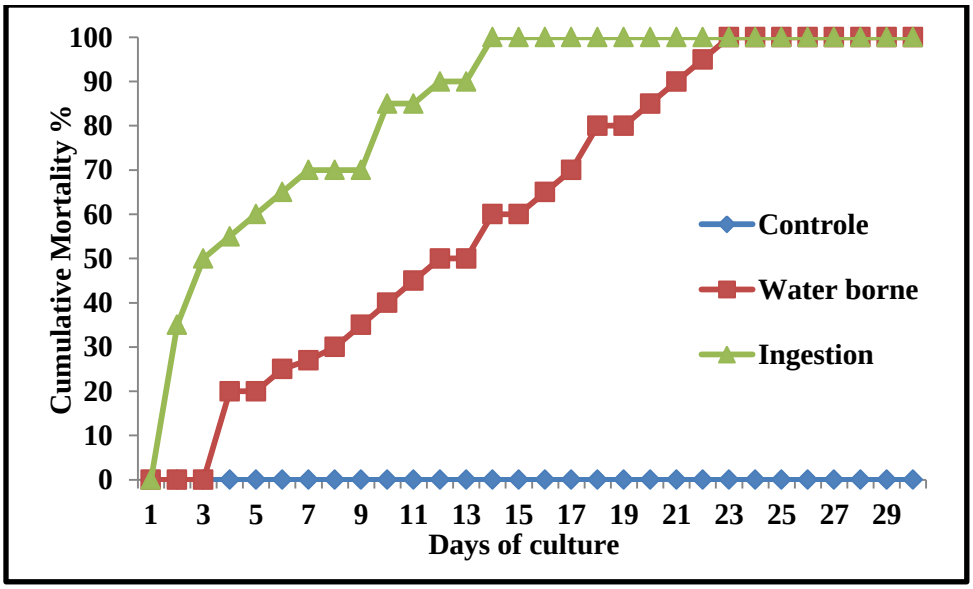

Figure 8: Cumulative mortality in compatibility report

DISCUSSION

The shrimps tolerate a wide variation in salinities, temperature, pH and dissolved oxygen. Therefore, they are available in almost all coastal and marine ecosystems including the brackish water ecosystem. In the present study, the WSSV infection has been confirmed in the spontaneously infected shrimp collected from a shrimp pond. Our TEM observations of viral structure and morphogenesis agree with the descriptions of other authors on penaeid shrimp infected by WSSV. The structure we refer to as LRS has been reported in *P.vannamei* (Durand, 1997) and *P. monodon* (Wang *et al*., 1999) infected with WSSV. We observed up to eight longitudinal electron dense bands and four lighter bands in one LRS, as reported by Wang *et al.* (1999). Durand *et al.* (1997) considered this structure as an assembly of nucleocapsid precursors, while Wang *et al.* (1999) proposed that the LRS may compromise WSSV nucleosomes that are associated with early viral replication.

The occasional presence of two nucleocapsids within one envelope amongst singly enveloped nucleocapsids as observed here has been reported in *Fenneropenaeu schinensis* infected by WSSV (Zhan *et al.,* 1998) infected by a rod-shaped nuclear virus, although it is considered as a rare occurrence in crabs (Johnson, 1988). This occurrence was also uncommon in penaeids infected by WSSV, as it has not been reported in other ultrastructural investigations in *P. vannamei* (Durand *et al*. 1997;Tapay, *et al*., 1997; Rajendran *et al*., 1999; Wang *et al*. 1999a) or other penaeid shrimps (Inouye *et al.*1994; Takahashi *et al*.,

1994;Wongteerasupaya *et al*.,1995;Wang *et al*.1999a and b).

The PCR performed in this study clearly revealed a rapid increase in viral load in systemic circulation of WSSV-challenged animals within 48 hpi, and they eventually died few hours later with profound expression of all clinical symptoms typical for white spot syndrome (Sanchez-Paz, 2010). In the present study, detected viral particles in the nucleus of the gill cells were rod shaped and the size ranged between 150 and 350 µm in lengths and 20 to 60 µm in width. The PCR methods for detecting penaeid shrimp viruses were developed as genome sequence information became available for each of the viruses of concern (Teng *et al.,* 2006). The PCR were developed and have added even more potential sensitivity to the detection capabilities of shrimp viruses. The molecular technology for diagnosing all of the important penaeid shrimp viruses has been developed and made commercially available as diagnostic kits. In the present study, the sensitive methods of PCR were utilized with commercial diagnostic kits (Farming IntelliGene) to detect white shrimp virus disease, and diagnostic results showed that WSSV were detected in the diseased shrimp.

The present findings agree with previous work where mortality was reduced in WSSV infected *L.vannamei* maintained at 32°C (Vidal *et al*., 2001; Granja *et al*., 2003 and 2006).The 95.25% mortality has been observed on the17th day through the oral ingestion route, but in the case of water-borne infection, it was 95% on the 23rd day. Earlier, Chou *et al.* (1995) recorded 100% infection in 14 days for the water-borne infection route, but only in 7 days for the oral ingestion route. At constant 33°C (24 h 33°C), absence of clinical signs and reduced mortalities (0- 10%) of WSSV infected shrimp is in accordance with other studies (Vidal *et al*., 2001, Rahman *et al.,*2006a).Other studies done in vivo with WSSV-infected shrimp M. *japonicus* or crayfish *P*. *leniusculus* showed that maintaining these species at water temperature below 16°Cwas also effective in reducing mortality (Jiravanichpaisal *et al*., 2004).

This study also gives experimental evidences that the density of *L. vannamei* also influenced the cumulative mortality through different mode of infections. The cumulative mortality was increasing with increasing of *L*. *vannamei* density. There is no previous study comparing the effects of the *L*. *vannamei* density on its cumulative mortality through different modes of viral infection. However, through the infectivity test, the *L.vannamei* were experimented with different mode of infection. The results revealed that the cumulative mortality was fastest (all animal died by 5 days) through the ingestion mode at the highest stocking density (20 shrimps/tank). Almost similar and fastest mortality was also observed by (Di Leonardo *et al.,* 2005) on *Palemon* sp. and *Marsupenaeus japonicus*. He reported that the 100% mortality of M. *japonicus* was occurred on 4th day through the injection mode of infection. With respect to the ingestion mode caused the 100%mortality by the 22th day, 17th day and 14th day in tanks with 5, 10 and 15 *L. vannamei*, respectively. Since the study was indicated the temperature is 34.6 °C, However, the benefit from exposure to 33 °C of WSSV infected shrimp in the field has its limits because of the short time span during which the temperature has to be increased (between 12 and 24 hpi), negative effects of 33 °C on shrimp (Ponce Palafox *et al*., 1997;

Le Moullac and Haffner, 2000; Cheng *et al*., 2005; Zhang *et al*., 2006). Previous study revealed acceleration in the mortality rate of *P.vannamei* shrimp when dually infected with WSSV and *V.campbellii* (Phuoc *et al*., 2008). These findings agree with (Gopalakrishnan, 2012), who reported the 100% mortality of the shrimp by the 17th day of the experiment through the ingestion of the WSSV infected crab (S. *tranquebarica*) flesh. (Chou *et al.,* 1995) reported the 100% mortality by the 7th day through the ingestion mode of infection. In the present study, the water - borne mode caused the 100% cumulative mortality by the 29th day, 21th day and 17th day in tanks with 5, 10 and 15 *L. vannamei*, respectively. These agree with (Chou *et al.,* 1995), who reported that the 100% mortality by the 14 days in the shrimps experimented through the water–borne mode of infection. In the present study, the shrimp mortality was delayed through the water borne mode of infection compared to the other modes of infection. The reason might be the time needed to transfer viral particles from the shrimp tissue via water medium to the *L.vannamei*. Generally, more time is needed to transfer viral particles from the shrimp to the *L. vannamei* in the case of water–borne than the ingestion mode of infection (ingestion and water–borne infection) because the water–borne results comparatively slow viral contamination. However, the water–borne mode of infection is one of the important routes since the *L. vannamei* spread the infection through this mode only.

CONCLUSION

This study also gives experimental evidences that the density of *L.vannamei* also influenced the cumulative mortality through different mode of infections. The cumulative mortality was increasing with increasing of *L. vannamei* density. There is no previous study comparing the effects of the *L. vannamei* density on its cumulative mortality through different modes of viral infection. According to the density is a crucial factor that affects all states of physiology including disease susceptibility, feeding, behavior, growth of animal.

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