Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Biomedicine

journal homepage:www.elsevier.com/locate/apjtb

Document heading doi:10.1016/S2221-1691(12)60371-2

Real time PCR quantification of WSSV infection in specific pathogen free (SPF) *Litopenaeus vannamei* (Boone, 1931) exposed to antiviral nucleotide

MA Badhul Haq[1*], K Kalai Priya[1], R Rajaram[2], R Vignesh[1], M Srinivasan[1]

[1] *Centre of Advanced Study in Marine Biology, Faculty of Marine Sciences, Annamalai University Parangipettai – 608 502, Tamilnadu, India*
[2] *Department of Marine Science, Bharathidasan University, Tiruchirappalli – 620 024, Tamil Nadu, India*

ARTICLE INFO

Article history:
Received 4 June 2012
Received in revised from 5 July 2012
Accepted 7 August 2012
Available online 28 August 2012

Keywords:
Aquaculture
Pathogen
Infection
Shrimps
Drugs
PCR
Genome

ABSTRACT

Objective: To investigate the level of WSSV transmission from the infected tiger prawn *Penaeus monodon* (*P. monodon*) to specific pathogen free *Litopenaeus vannamei* (*L. vannamei*) in laboratory captivity condition in relation to PCR detection, histopathological observation and viral genome sequence. **Methods:** Viral DNA was isolated from purified virions by treatment with proteinase K (0.2 mg/mL) and Sarkosyl (1%). The purity and concentration of the DNA were determined by agarose gel electrophoresis. Moribund and dead shrimp were removed and processed for indirect immunofluorescence (IIF) analysis. Histological observation of infected *L. vannamei* shrimps were revealed by the degenerated cells which were characterized by intranuclear inclusions in the tissues of WSSV infected mid-gut gland, lymphoid organ, gill lamellae and gut epithelium. Total DNA was extracted, from shrimp hemolymph and tissues, with a High Puree PCR template preparation kit. WSSV-DNA was detected using a commercial 2-step PCR detection kit. **Results:** The present study compares the real-time PCR results with SYBR Green I concentration ranging from 0.2 to 0.7×. The positive standard was used in the range of 10^2, 10^4 10^6, 10^8 and 10^{10} copies/ng of DNA in general. The PCR analysis showed the appearance of a prominent band from the PCR amplified product of WSSV-DNA at internal control band of 848 bp. Moderate and severe levels were observed as 650 bp and 910 bp (200 & 2 000 copies) in various transmission routes. The WSSV content in moribund shrimp of all the experimental species (*L. vannamei*) approximately ranged in nucleotide application by quantification method from 0.000 001 WSSV copies/μg of total DNA. In whole moribund infection animal, approximately 0.02 WSSV copies/μg of DNA was detected in nucleotide applied animal. **Conclusion:** These results indicate that wild brood stock and native culture shrimp *P. monodon* may be infected with WSSV and can get transferred into the SPF *L. vannamei* farming environment. Based on the studies, we made in captivity condition in different WSSV transmission route in dissimilar infection range with the use of nucleotide for antiviral drugs. There is an urgent need to address and develop antiviral drugs and molecular based viral genome technique for control measures to salt away the aquaculture environments.

1. Introduction

In many countries aquaculture is a major thrust area which plays vital role in improving community progress, food security, poverty mitigation, employment and other economic activities[1]. Global crustacean aquaculture is one of the most economically important sectors that are worth more than $10 billion per annum[2].

Diseases are one of the major constraints for the sustainable increase of shrimp production. Sustainable improvement of aquaculture relies on disease avoidance. With a strengthening of operations, the risk of disease occurrence and stretch of infectious diseases increases. Shrimp diseases can be divided into non infectious and infectious in origin[3]. Infectious diseases are caused by viruses, bacteria, fungi and parasites.

Biological factors such as microbial flora present in the pond play a role on the susceptibility of shrimp to pathogens. Proper management of the microbial flora is done by biosecurity measures, aeration, reduction or

*Corresponding author: Ministry of Science & Technology, Department of Science & Technology, New Delhi – 110 016.
Tel: 04144-243223
Fax: 04144-243555
E-mail: drmabhaqcas@gmail.com
Grant number: SR/FT/LS-050/2009
Funding Agency: SERC – Fast track – Young scientist

elimination of pathogens and their carriers, application of probiotics, sludge management, waste treatment, reduction of the amount of water exchange and treatment of the incoming water. These are all important issues in prevention of shrimp disease[4].

Viral infections are common diseases in crustaceans such as penaeid shrimp, which can be infected by more than twenty different viruses. In crustaceans, research on viruses has increased considerably since the first report on the occurrence of a virus in the crab Macropipus depurator. Crustacean viruses belong to or are related to various viral families like the Baculoviridae, Bunyaviridae, Herpesviridae, Picornaviridae, Parvoviridae, Reoviridae, Rhabdoviridae, Togaviridae, Iridoviridae or a new virus family, the Nimaviridae. Viral diseases of shrimp have caused negative effects on the economy in several countries in Asia, South America and America, where they have numerous shrimp culture industries.

The studies on the immunity of shrimp and other crustaceans have mainly focused on general aspects of immunity and as a consequence little is known about the antiviral responses in crustaceans.

Outbreak of disease is one of the major stumbling blocks in the development and sustainability of aquaculture. A number of approaches have been made in attempts to solve disease problem in aquaculture, one among these is vaccination[5]. The first major WSSV outbreak, reported in 1993, resulted in a 70% reduction in shrimp production in China[6] and this virus has remained a major concern for shrimp aquaculture throughout the world since. The presence of WSSV has been reported in wild and hatchery reared post larvae[7,8]. WSSV has become an epizootic disease and is not only a major threat to shrimp aquaculture, but also to marine ecology[9]. Epidemiological data has been reported for WSSV indicating that the presence of the virus does not necessarily result in white spot disease (WSD).

It has been shown that if the risk is minimized then the disease can be avoided or reduced. Thus, successful shrimp crops can be harvested when WSSV and other shrimp viruses are present at low viral prevalence[10], when stress is reduced or when the virus is detected early enough, and outbreaks can be prevented despite the presence of WSSV. The portals of entry of virus have not yet been clearly defined[11]. There are a lot of studies for preventing and controlling shrimp WSSV infection, such as improvement of environmental conditions, induction of non-specific antiviral response with antivirus drugs or immunostimulants[12]. Vaccines kindle the immune system to help fight off diseases and the application of these methods to control infectious diseases is growing in significance[13].

WSSV is pathogenic to at least 78 species, mainly to decapod crustaceans including marine and freshwater shrimp, crab, crayfish and lobsters[9,14]. The first outbreak due to WSSV was reported in shrimp farms in Taiwan in 1992[15] followed by other shrimp farming countries of South East Asia, Middle East, North, Central and South America[9,14]. White spot syndrome virus (WSSV), the pathogen of shrimp white spot disease, is a rod-shaped enveloped dsDNA virus belonging to Nimaviridae family[16].

WSSV occurs worldwide and causes high mortality and considerable economic damage to the shrimp farming industry. Considering the global environmental, the economic and sociological importance of shrimp farming, and the constraints of high intensity cultivation, development of novel control measures against the outbreak of WSSV become inevitable.

The recent progress in molecular biology techniques has made it possible to obtain information on the factors, mechanisms and strategies used by this virus to infect and replicate in susceptible host cells. It has been reported that sick and dying shrimp lead to rapid progression of WSD and there is increasing evidence that the ingestion of sick or dying shrimp is the major mode of transmission[17]. Yet, further research is still required to fully understand the basic nature of WSSV, its exact life cycle and mode of infection

In penaeid shrimp, C-type lectins have been shown to function as antimicrobial and antiviral proteins[18,19] proteins of pattern recognition[20], proteins of pathogen clearance[21], and they have been shown to participate in the innate immune response[22]. It has also been documented that in the hemolymph and in other tissues of penaeid shrimp, the expression of C-type lectins is regulated at the transcriptional and translational levels after bacterial and viral infection[23,24].

Recently, loop-mediated isothermal amplification (LAMP) protocols were developed for WSSV detection[25-27]. Shrimp, like other invertebrates, does not produce antibodies and relies on the innate immune system to recognize and react to pathogens or environmental antigens. However, protective immune responses in the shrimp induced by DNA vaccines against intramuscularly infected white spot syndrome virus have been reported in recent papers[28].

An ethanolic extract bis(2-methylheptyl) phthalate from *Pongamia pinnata* (*P. pinnata*) leaves was incorporated in a diet and tested for antiviral activity against WSSV infection in *Penaeus monodon* (*P. monodon*). *P. monodon* were fed for 4 days before and 15 days after WSSV challenge (200 and 300 μg/g body weight of shrimp/d). Increased survival (40% to 80%) was found with the diet containing the highest concentration of the extract[24].

Several antiviral molecules have been isolated and characterized recently from decapods. Characterization and identification of these molecules might provide a promising strategy for protection and treatment of these viral diseases. Similarly the existence of antiviral properties of several peptides has been reported in penaeid shrimps. PmAV protein, a c-type lectin from the black tiger shrimp, *P. monodon*, strongly inhibits virus-induced cytopathic effects in a fish cell culture.

Recent studies showed evidences of an antiviral immunity in shrimp including the existence of toll like receptors (TLR), RNA interference, antiviral substances

in tissues and immune genes. TLR in mammals function as activators of immune cells, intracellular signaling against infection, recognition of LPS, peptidoglycan, lipoprotein and are involved in antiviral immunity. TLR have been discovered in *Litopenaeus vannamei* (*L. vannamei*)[29] and *P. monodon*[30]. Choi[31] carried out a study for the molecular level identification of recombinant protein vaccine efficacy, by oral feeding against white spot syndrome virus infection, with the comparison of viral mRNA transcriptional levels in shrimp cells.

Viral structural proteins, especially the envelope proteins, are important because of not only virion morphogenesis but also that they are the first molecules to interact with the host cell[12].

L. vannamei is the most important shrimp species in terms of aquaculture production. The other important species are *P. monodon*, *P. chinensis, P. merguiensis, P. japonicus* and *F. indicus*. *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. These aspects could explain the increasing preference to culture this species.

The objective of the present investigation was to determine the level of WSSV transmission from the infected tiger prawn *P. monodon* to SPF *L. vannamei* in laboratory captivity condition, in connection to PCR detection, Histopathological observation and viral genome sequence. Out come from this study would immensely support few ideas to the aquaculture and molecular industry to sprint smoothly. This study was to update recent knowledge of innate immunity against viral infections in shrimp *L. vannamei*. This information will expand our knowledge and may contribute to developing effective prophylactic or therapeutic measures.

A serious of recent studies has shown that viruses have the ability to manipulate the life histories and understanding of the hosts in remarkable ways, challenging our understanding of the almost invisible world of viruses[3,4].

2. Materials and methods

2.1. Preparation of viral inoculums

White spot disease affected moribund *P. monodon* were collected during an emergency harvest resulting from a WSSV outbreak at two shrimp farms located in Nellore, Andhra Pradesh (India). Two grams of infected cephalothroacic tissues (gill, stomach, midgut) were homogenized in sterile marine phosphate-buffered saline (PBS) and centrifuged at 1 600 g for 15 min at 4 ℃. 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 −70 ℃ for infectivity studies.

2.2. Virus purification

Viral DNA was isolated from purified virions by treatment with proteinase K (0.2 mg/mL) and sarkosyl (1%) at 65 ℃ 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.

2.3. Experimental animals-shrimp – L. vannamei

About 30 samples of SPF *L. vannamei*, adult shrimp and larvae were collected from prime division of Tamil Nadu aquaculture environment for the study. The shrimps used in culture were imported from Brazil aquaculture grounds through CP Aqua India Pvt, Ltd and other leading aquaculture industry, Parangipettai. Shrimp *L. vannamei* were maintained in four 50 L plastic tanks at room temperature (28-30 ℃) with salinity between 20 and 25 ppt. Filtered and sterilized estuary water was used for all the experiments.

The species, *L. vannamei* were fed with formulated shrimp feed (30% protein) at 10% body weight, once a day. They were kept in these tanks for 7 d for acclimatization prior to the experiments. About five shrimps were picked out randomly from those shrimps for PCR examination to exclude viral infection, because only healthy individuals were used. Further, a representative sample of these animals was subjected to nested PCR, using a WSSV-nested PCR kit (IQ2000 kit, India).

2.4. Preparation of viral tissue and extract for oral challenge and intramuscular injection tests

Pond-reared *P. monodon* shrimp naturally infected 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 intramuscular and oral route challenge of *L. vannamei*.

2.5. Intramuscular inoculation procedure

Three experiments were performed using the i.m. route. In each experiment, 3 groups of 10 shrimp (MBW = (9.40 ± 4.92) g, n = 120) were inoculated with 10, 30 or 90 SID_{50}. In addition, 3 groups of 10 shrimp were mock-inoculated with 50 μL PBS or healthy heamolymphand 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 120 h post inoculation (hpi).

2.6. Oral inoculation procedure

Three experiments were performed using the oral route. 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 SID_{50}). 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 (no. 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 120 hpi.

2.7. Preparation of antiviral drugs

The commercial shrimp feed (100 g), procured at SPDS shrimp farm of Agaram village in Tamil Nadu. Added 1 g of antidrug nucleotide in distilled water and blended into the feed. After drying the feed was mixed with 1 g of boiled sugar for binder in total amount of pellet feed, for 1 h air dry, and then the coated drugs were given two experimental tank shrimps. Normal commercial feed was used as control.

2.8. Effect of the nucleotide on the survival rate of WSSV infected shrimp L. vannamei

Healthy shrimps weighing 5-8 g and 12-15 g were used in this study. Test on both groups were performed separately in duplicate with 10 shrimps in each treatment. Nucleotide coated was fed by mixing it with shrimp feed 10% body weight of shrimp. Nucleotide was fed for 4 d before challenging the shrimp with WSSV.

The shrimp were challenged by incubation in the virus solution for 2.5 h. This virus concentration caused the control shrimp to die in 3-5 d. The nucleotide was fed continuously for 15 d. The same pellet without nucleotide was fed to the control groups, which were also challenged with WSSV. The shrimp were monitored for 10 d after infection and all mortality were recorded.

2.9. Evaluation of WSSV infection

Inoculated shrimp were monitored every 12 h throughout the experiment. Moribund and dead shrimp were removed and processed for indirect immunofluorescence (IIF) analysis. Control shrimp were also analyzed by IIF.

Clinical signs: *L. vannamei* rarely display white spots during WSSV infection[32]. Empty guts and reduced response to mechanical stimulation are the first clinical signs 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 i.m. or oral routes.

2.10. Time-course infectivity experiments

Shrimp (*L. vannamei*) were infected by intramuscular injection and oral route of WSSV strain. Experimental tank A: animals were given WSSV infected meat through oral route at 10% body weight basis. The second experimental tank B: animals were injected intramuscularly in the second and fourth abdominal segment with filtrate 50 μL viral inoculum per animal, prepared from WSSV infected shrimp using 1 mL insulin syringes. Control animals were injected with hemolymph from WSSV uninfected shrimp. Tissue samples and hemolymph were collected from experimental animals for PCR.

Three per sample for histological analysis were sacrificed at 24 h, 48 h, 3 d, 6 d, 12 d, 18 d, 25 d interval, and stored at -20 ℃ for further study. The total experimental study period were maintained for 22 d in *P. vannamei* in different time period respectively.

2.11. DNA extraction

Total DNA was extracted, from shrimp hemolymph and tissues, with a high pure PCR template preparation kit.

2.12. 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 IQ2000 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 94 ℃ for 2 min, then 94 ℃ for 20 s; 62 ℃ for 20 s; 72 ℃ for 20 s, repeated 15 cycles, then add 72 ℃ for 30 s; 20 ℃ for 30 s at the end of the final cycle. The second PCR profile was carried out in 94 ℃ for 20 s; 62 ℃ for 20 s; 72 ℃ for 30 s, repeat 30 cycles, then added 72 ℃ for 30 s; 20 ℃ for 30 s at the end of the concluding cycle, followed by a final extension for 5 min at 72 ℃.

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) for 30 min, and the bands were visualised by UV transillumination and GelDoc system. Accomplished WSSV negative and positive results were interpreted with the help of performed gel, under UV exposure GelDoc System.

2.13. Histopathology

For histological consequence, *L. vannamei* organ tissues were collected from WSSV infected experimental tanks (three from each tanks). The species, *L. vannamei*

Table 1.
PCR result of samples collected from different transmission route of WSSV in *L. vannamei*.

Days	Infection range/Viral copies/Base pair	Type of WSSV transmission route	Histology analysis result	Nested PCR result
1–6	Negative internal control band/848 bp	i.m & oral	Negative	Negative
7–12	Negative internal control band/848 bp	i.m & oral	Negative	Negative
13–14	Low/20/296 bp	i.m	Negative	Positive
15–16	Moderate/200/650, 296 bp	i.m	Negative	Positive
17–18	Severe/2000/910, 650, 296 bp	i.m & oral	Positive	Positive
19–20	Severe/2000/910, 650, 296 bp	i.m & oral	Positive	Positive
21–22	Severe/2000/910, 650, 296 bp	i.m & oral	Positive	Positive

were dissected and segregated the following organs *viz.*, 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 rest was fixed in 95% ethanol for PCR. For histology, routine procedures were followed for preparation, sectioning, and staining with haematoxylin and eosin.

The polychaete organs from the experimental and control shrimps were examined histological for WSSV-specific manifestations following the routine diagnostic protocol of Lightner[14].

3. Results

3.1. Gross pathology of WSSV

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.

3.2. Nested PCR detection of WSSV

The results of PCR analysis on different organs obtained from different transmission route of WSSV in dissimilar level of infection range. The PCR analysis showed the appearance of a prominent band from the PCR amplified product of WSSV-DNA at internal control band 848 bp at non-infected in control tank. Although low infection positive band (20 copies) were showed at 296 bp continued from initial stage of the infection in oral route. On moderate and severe level were observed as 650 bp and 910 bp (200 & 2 000 copies) in both transmission routes. The PCR product band was ranged between 296 to 910 bp was found during entire period of experimental studies of SPF *L. vannamei* shrimps which exposed to WSSV through native shrimp of *P. monodon*.

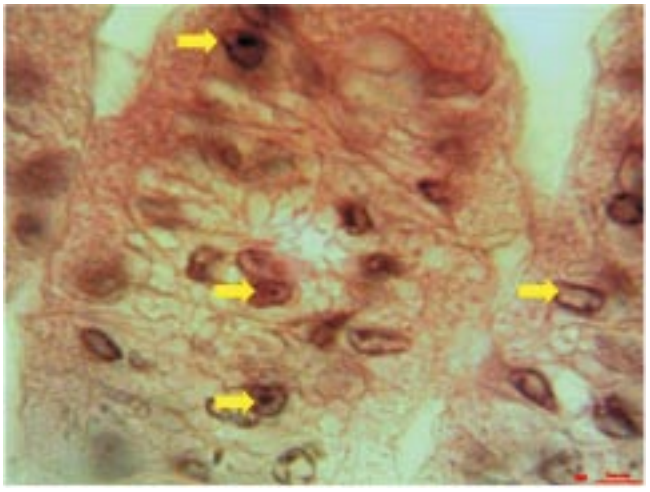


Figure 1. Intranuclear inclusion bodies characteristic of WSSV infection (arrows) in the gill Lamella cells of *L. vannamei* showing of WSSV – 21 d of post inoculum in B group (H & E 100×).

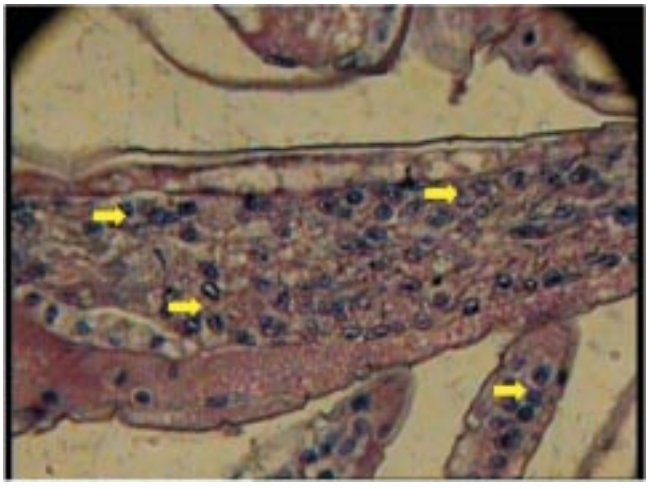


Figure 2. Intranuclear inclusion bodies characteristic of WSSV infection (arrows) in the hind gut region cells of *L. vannamei* (intramuscular injection route) showing signs of WSSV – 22 d of post inoculum in A group (H & E 100×)

3.3. PCR result of samples collected from different transmission route of WSSV in L. vannamei

The 50 μL of the WSSV inoculum was a dilution of 10-7. The average percent mortality between the four highest treatments with antiviral nucleotide (0.01 mg/mL) in associated with different transmission routes (Table 1). The results suggest that the minimal concentration of extract that inhibited WSSV *in vitro* was 0.01 mg/mL.

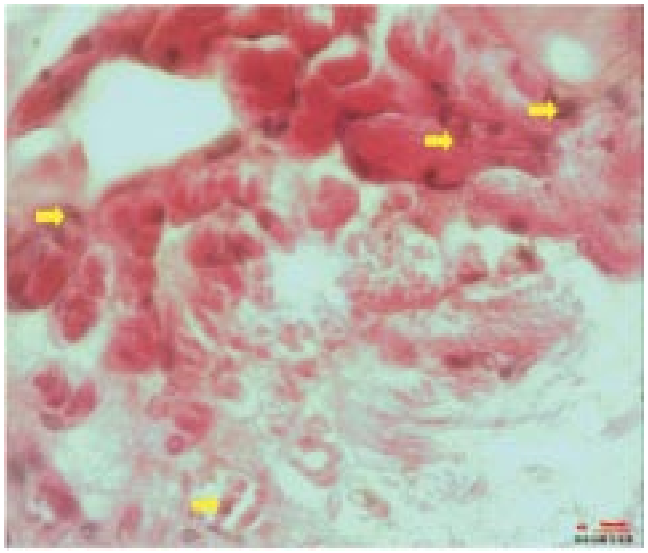


Figure 3. Hematopoetic tissue with basophilic intranuclear inclusion bodies (arrows) characteristic of white spot syndrome virus (WSSV) (oral route) in *L. vannamei* in post inoculum of 18 d in B group (H & E 100×).

3.4. Antiviral activity of nucleotide against WSSV in L. vannamei in oral and intramuscular injection routes

The maximum survivals (100%) were recorded in control group and minimum was recorded in positive control group. The nucleotide applied groups (oral and i.m route) were presented lower mortality range (60% & 70%) than compared to other first two groups.

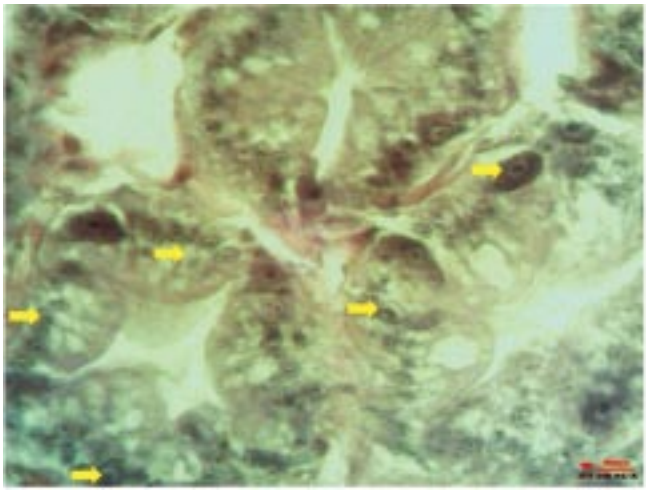
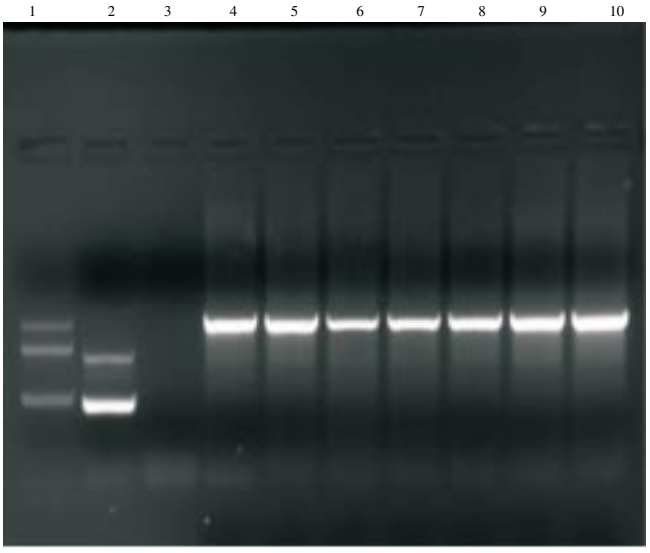
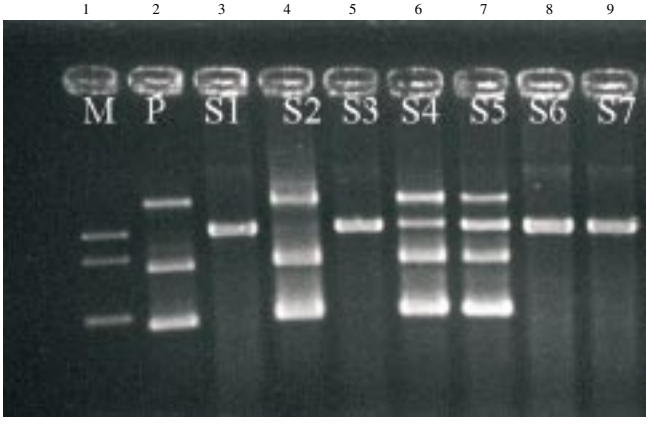


Figure 4. Intranuclear incusion bodies charecteristic of WSSV infection (arrow) in the hepatopancrease tissue of *L. vannamei* showing signs of WSSV (H & E 40×).



WSSV control. Lane 1: Molecular wt marker (848, 650 and 333 bp); Lane 2: Positive control (910, 650 and 296 bp); Lane 3: Negative control (yeast tRNA); Lane 4: Day 1 & 2 WSSV-ve sample (848 bp); Lane 5: Day 3 & 4 WSSV-ve sample (848 bp) (composite samples); Lane 6, 7: Day 5 & 6, 7 & 8 WSSV-ve sample (848 bp) (composite samples); Lane 8, 9: Day 9 & 10, 11 & 12 WSSV-ve sample (848 bp) (composite samples); Lane 10: Day 13 & 14 WSSV-ve sample (848 bp) (composite samples).



Group A & B. Lane 1: Molecular wt marker (848, 650 and 333 bp); Lane 2: Positive control (910, 650 and 296 bp); Lane 3: Day 1to12 WSSV-ve sample (848 bp) a, group; Lane 4: Day18 -20 WSSV severe +ve sample - 910, 650, 296 bp-a, group (composite samples); Lane 8: Day13-17 WSSV low +ve sample-296 bp - a, group (composite samples); Lane 5: Day 1 to 13 WSSV-ve (848 bp) b, group (composite samples); Lane 6: Day19-22 WSSV severe +ve sample-910, 650, 296 bp - b, group (composite samples); Lane 9: Day14-18 WSSV low +ve sample-296 bp - b, group (composite samples)

Figure 5. Photograph of agarose gel electrophoresis of PCR product of the gill and hepatopancreas samples oral, intramuscular route (A&B) and control obtained from WSSV affected and non-affected *L. vannamei*.

3.5. Histopathological observation of WSSV

Histological observation of severely WSSV infected *L.vannamei* shrimps were revealed degenerated cells characterized by intranuclear inclusions in the tissues of WSSV infected mid-gut gland, lymphoid organ, gill lamellae, gut epithelium. Hematopoetic tissues with basophilic intranuclear inclusion bodies characteristic were observed in moderate infection of

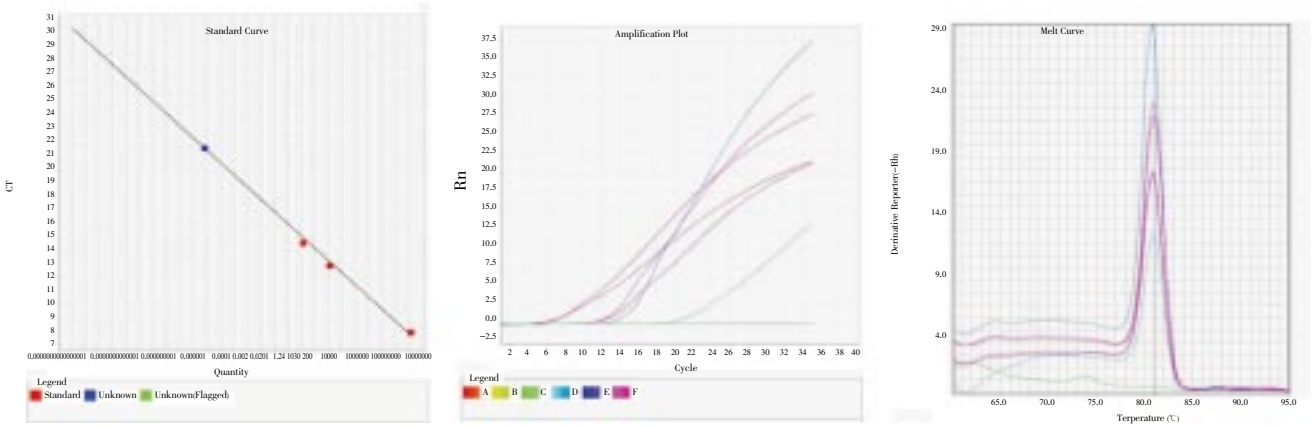


Figure 6. Standard curve, amplification plot and melt curve of sample 1.

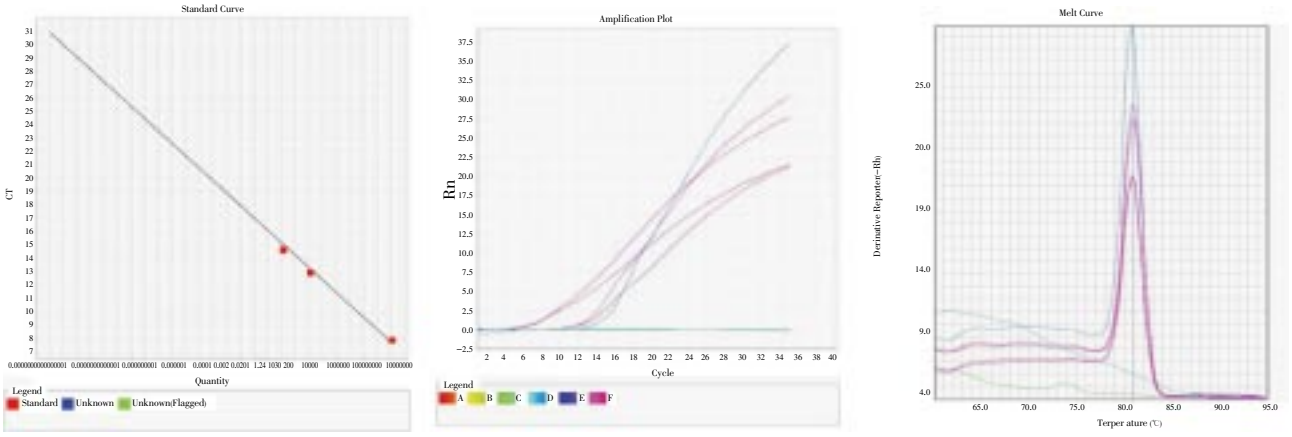


Figure 7. Standard curve, amplification plot and melt curve of sample 2.

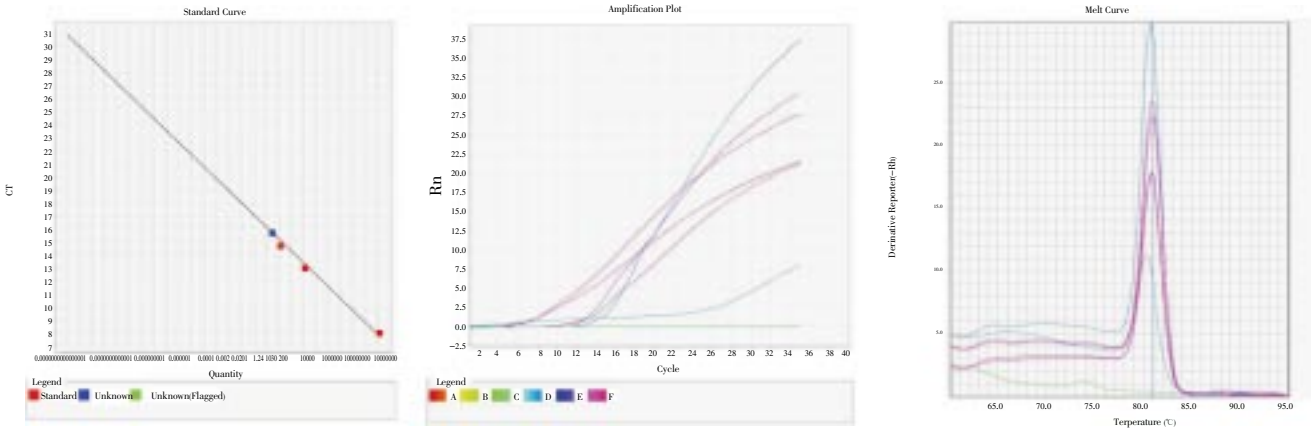


Figure 8. Standard curve, amplification plot and melt curve of sample 3.

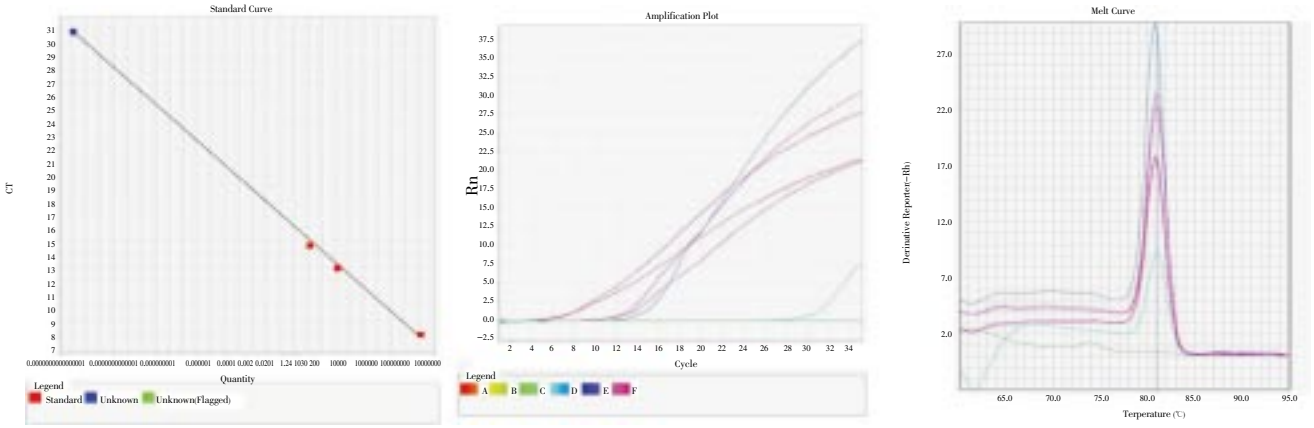


Figure 9. Standard curve, amplification plot and melt curve of sample 4.

WSSV in *L. vannamei*. 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 in WSSV uninfected region. Intranuclear inclusion bodies characteristic of high level of WSSV infection presented in the gill region of *L. vannamei*. However, the tubular epithelial cells of mid-gut gland were highly vacuolated as in low level of infection shrimps (Figure 1-4).

3.6. Real-time PCR analysis of WSSV gene quantification from the L. vannamei

In the present study, a highly sensitive and specific SYBR Green real-time PCR was developed to quantify white spot syndrome virus (WSSV) infections in captivity farm-reared sub-adult of Pacific white shrimp, *L. vannamei*. Quantitative real time PCR was applied in this study to quantify the white spot syndrome virus (WSSV) in infection range of different immune and cephalothorax region of shrimp tissue.

A total of three type infection range in different individuals organ 3 experimental tanks were maintained in Marine Virology Laboratory, Faculty of Marine Sciences. The WSSV infections in 2 experimental animals were showed application of antiviral drug nucleotide in wide range, from 0 to 20 copies/ng of DNA in general. The control tank was divided into 2 groups; one was WSSV positive control through viral inoculum transmission. Another one was WSSV non-infected tank. The WSSV positive control sample were showed mass mortality in 22nd day of the experimental.

The survival was noticed 0% of control 1 (WSSV positive) and 100% were in control 2 (WSSV negative). The nucleotide applied animals were noticed 40% and 30% of mortality in day 22nd of experimental period. The positive standard were used in the range of 10^2, 10^4 10^6,10^8 and 10^{10} copies/ng of DNA in general.

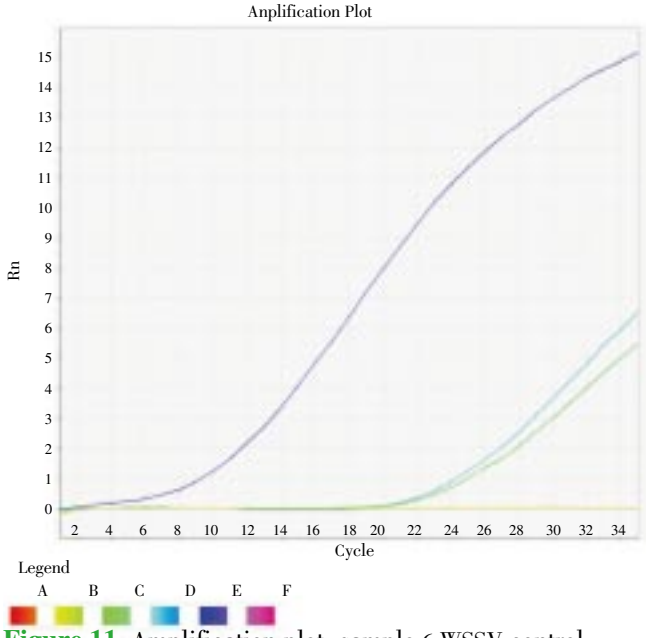


Figure 11. Amplification plot-sample 6 WSSV control.

The WSSV content in moribund shrimp of all the experimental tank species to be tested (*L. vannamei*) approximate ranges are showing in nucleotide application in quantification method from 0.000 001 WSSV copies/μg of total DNA. In whole moribund infection animal, approximately 0.02 WSSV copies/μg of DNA was detected in nucleotide applied animal (Figure 5).The positive WSSV (control) animals were recorded 10^2 WSSV copies/μg, which was equivalent to severe or more of infection range. The comparison of WSSV content with nucleotide antiviral drug application in between different tissues showed that lymphoid organ, hematopoitic and gill lamellae and hepatopancreas tissues contain several times less virus (0.000 000 000 000 000 1 copies/μg of total DNA) than other part of regions. With inocula of known virus content, bioassays by oral route and intramuscular were showed in different logs of WSSV copies (0, 0.01, 0.2, and 20 WSSV copies/μg) was necessary to establish disease in the challenge shrimp.

The positive control sample (without nucleotide application) was observed in high level of WSSV copies. In example, five logs of WSSV copies inject into shrimp muscle produce a 50 μL of 2 d onwards. This real time polymerase chain reaction (PCR) technique is sensitive (five copies), specific (negative with DNA or positive from shrimp baculoviruses and parvoviruses), dynamic (seven logs) and easy to perform (96 tests in <4 h).

3.7. RT-PCR result interpretation

The SYBR Green real-time PCR were determined 5 samples in different criterion manner. The following sample descriptions were analyzed in different immune organs in different time course and nucleotide application.

The amplification temperature was performed whole cycles *viz.*, 94 ℃, 60 ℃ and 72 ℃ respectively. Total cycling parameters were completed 35 in different amplification steps. The amplification plot was compared with fluorescence intensity in different value. The WSSV PCR product sample with positive standard was amplified uniformly in beneath identical standard copies.

The melting curve was observed in cycling parameters *i.e*, denaturation and annealing and elongation or extension in particular temperature and particular derivative reporter (-Rn) in wide range of 5.0 - 25.0. Real-time PCR results with SYBR Green I concentration ranging from 0.2 to 0.7X. The positive standard were used in the range of 10^2, 10^4 10^6, 10^8 and 10^{10} copies/ng of DNA in general. The sample 1 was recorded in approximately 0.02 WSSV copies/μg of DNA was detected in nucleotide applied *L. vannamei* (Figure 6) The sample 2 was recorded in approximately 0.000 01 WSSV copies/μg of DNA was detected in nucleotide applied *L. vannamei* (Figure 7). Sample 3 (Figure 8) was not detected and in sample 4 and 5 it

was recorded approximately 20 WSSV copies/μg of DNA without nucleotide applied positive control of *L. vannamei* sample (Figure 9 & 10). Figure 11 represents the amplification Plot-sample 6 (WSSV +ve control).

The sample 1 was recorded in very low level of WSSV infection approximately 0.000 000 000 000 000 1 WSSV copies μg^{-1} of DNA was detected in before nucleotide applied *L. vannamei* sample.

4. Discussion

The molecular mechanisms involved in WSSV infection are still unknown and only a few studies have been carried out on the activity of its structural proteins. A study was carried out to evaluate polymerase chain reaction (PCR), histopathology and quantitative PCR for the detection of white spot syndrome virus (WSSV) in *L. vannamei* and its molecular characterization. White spot syndrome virus (WSSV) is a contagious viral disease of penaeid prawns and is caused by white spot syndrome virus (WSSV), an enveloped, rod-shaped virus containing a double-stranded DNA genome.

WSSV is classified as a member of the family Whispoviridae. The virus infects a wide range of crustaceans but clinical disease is limited to the penaeid prawns including *P. monodon*, *P. japonicus*, *P. chinensis*, *F. indicus*, *P. merguiensis* and *L. setiferus*.

For the present analysis the WSSV gene were collected from SPF *L. vannamei* through in captivity and experimental aspects in laboratory condition with particular application of antiviral drug nucleotide The nested PCR product band was ranged between 296 to 910 bp was found during entire period from the experimental studies of SPF *L. vannamei* shrimps. The viral copies were noticed from 20 - 2 000 copies. The house keeping gene was observed in 848 bp in control treatment tank. Histopathological surveillance was performed from WSSV carrier shrimp SPF *L. vannamei* at cephalothorax region. Intranuclear inclusion bodies characteristic of high level of WSSV infection presented in the gill region of *L. vannamei*. However, the tubular epithelial cells of mid-gut gland were highly vacuolated as in low level of infection shrimps. The Real-time PCR study examined the viral copy number as determined by real-time RT-PCR, in different tissue samples from *P. vannamei* exposed to WSSV.

Two routes of exposure, injection and oral route, was investigated. Six different body parts from each shrimp were assessed for viral copy numbers. Ten shrimp were analyzed per treatment. In addition, ten specific pathogen free (SPF) *P. vannamei* were analyzed and served as a negative control. Another set of animals were maintained for WSSV positive control. Real-time PCR results with SYBR Green I concentration ranging from 0.2 to 0.7×.

The positive standard were used in the range of 10^2, 10^4 10^6, 10^8 and 10^{10} copies/ng of DNA in general. The tissue samples examined included: whole tail muscle, tail muscle (shell removed), gills, pleopods, head (cephalothorax with the hepatopancreas included) and tail fan. The results from these experiments showed a significant level of difference between the SPF and the injection treatments. As was expected, there was also a significant difference between the negative control and the nucleotide treatment groups.

There was no significant difference between the viral copy numbers contained in different body parts from the injection experiment. But WSSV infected with nucleotide treatment *L. vannamie* was presented major significant difference between the WSSV injected without application of Nucloetide. The mean viral copy number per nanogram of total DNA (cn/ng tDNA) extracted in the injection study ranged from 0-20 in the whole experiments. In the oral and i.m experiments, the cn/ng of extracted tDNA ranged from 0-0.02 in the immune organ in the head. When these values are converted to mean viral copy number per gram (cn/g) of tissue, the values increased in range.

The WSSV content in moribund shrimp of all the experimental tank species to be tested (*L. vannamei*) approximate ranges are showing in nucleotide application in quantification method from 0.000 001, 0.001, 0.02 and 20.0 WSSV copies/μg of total DNA. In whole moribund infection animal, approximately 0.02 WSSV copies/μg of DNA was detected in nucleotide applied animal. The positive WSSV (control) animals were recorded 10^2 WSSV copies/μg, which was equivalent to severe or more of infection range.

The aspire of this study was to first record RT-PCR quantification from the SYBR Green chemistry assay of WSSV gene in a new imported candidate species of SPF *L. vannamei* and contrast investigational infection of captivity condition with application of commercial nucleotide antiviral drugs.

Conflict of interest statement

We declare that we have no conflict of interest.

References

[1] Vignesh R, Karthikeyan BS, Periyasamy N, Devanathan K. Antibiotics in aquaculture-an overview. *South Asian J Exp Biol* 2011; **3**: 114-120.

[2] Johnson KN, van Hulten MC, Barnes AC. "Vaccination" of shrimp against viral pathogens: phenomenology and underlying mechanisms. *Vaccine* 2008; **26**(486): 4885-4892.

[3] Lightner DV, Redman RM. A putative iridovirus from the penaeid shrimp *Protrachypene precipua* Burkenroad (Crustacea: Decapoda). *J Invertebr Pathol* 1993; **62**: 107-109.

[4] Horowitz A, Horowitz S. Disease control in shrimp aquaculture from a microbial ecology perspective. In: Browdy CL, Jory

DE. (eds). *The new wave, proceedings of the special session on sustainable shrimp culture*. Baton Rouge: World Aquaculture Society; 2001, p. 199-218.

[5] Rather MA, Sharma R, Aklakur M, Ahmad S, Kumar N, Khan M, et al. Nanotechnology: a novel tool for aquaculture and fisheries development. a prospective mini-review. *Fish Aqua* 2011; 1-5.

[6] Su Yongquan. *The health culture of shrimps*. Beijing: China Ocean Press; 1998, p. 32-38.

[7] Lo CF, Ho CH, Chen CH, Liu KF, Chiu YL, Yeh PY. Detection and tissue tropism of white spot syndrome baculovirus (WSSV) in captured brooders of *Penaeus monodon* with a special emphasis on reproductive organs. *Dis Aquat Organ* 1997; **30**: 53-72.

[8] Tsai JM, Wang HC, Leu JH, Wang AH, Zhuang Y, Walker PJ, et al. Identification of the nucleocapsid, tegument, and envelope proteins of the shrimp white spot syndrome virus virion. *J Virol* 2006; **80**(6): 3021-9.

[9] Flegel TW. Detection of major penaeid shrimp viruses in Asia, a historical perspective with emphasis on Thailand. *Aquaculture* 2006; **258**: 1-33.

[10] Umesha KR, Dass BKM, Naik BM, Venugopal MN, Karunasagar I, Karunasagar I. High prevalence of dual and triple viral infections in black tiger shrimp ponds in India. *Aquaculture* 2006; **258**: 91-96.

[11] Escobedo-Bonilla CM, Alday-Sanz V, Wille M, Sorgeloos P, Pensaert MB, Nauwynck HJ. A review on the morphology, molecular characterization, morphogenesis and pathogenesis of white spot syndrome virus. *J Fish Dis* 2008; **31**(1): 1-18.

[12] Chang YS, Liu WJ, Chou TL, Lee YT, Lee TL, Huang WT, et al. Characterization of white spot syndrome virus envelope protein VP51A and its interaction with viral tegument protein VP26. *J Virol* 2008; **82**: 12555-12564.

[13] Evensen O. Development in fish vaccinology with focus on delivery methodologies, adjuvants and formulations. *Options Mediterraneennes* 2009; **86**: 177-186.

[14] Lightner DV. *A handbook of shrimp pathology and diagnostic procedures for diseases of cultured penaeid shrimp*. Baton Rouge: World Aquaculture Society; 1996, p. 304.

[15] Chou HY, Huang CY, Wang CH, Chiang HC, Lo CF. Pathogenicity of a baculovirus infection causing white spot syndrome in cultured penaeid shrimp in Taiwan. *Dis Aquatic Org* 1995; **23**: 165-173.

[16] Syed Musthaq S, Madhan S, Sahul Hameed AS, Kwang J. Localization of VP28 on the baculovirus envelope and its immunogenicity against white spot syndrome virus in *Penaeus monodon*. *Virology* 2009; **391**: 315-324.

[17] Adams A, Aoki T, Berthe CJ, Grisez L, Karunasagar I. *Recent technological advancements on aquatic animal health and their contributions toward reducing disease risks – a review. In: Diseases in Asian Aquaculture VI*. Colombo, Sri Lanka: Fish Health Section, Asian Fisheries Society; 2008, p. 71-88.

[18] Sun YD, Fu LD, Jia YP, Du XJ, Wang Q, Wang YH, et al. A hepatopancreas-specific C-type lectin from the Chinese shrimp *Fenneropenaeus chinensis* exhibits antimicrobial activity. *Mol Immunol* 2008; **45**: 348-361.

[19] Zhao ZY, Yin ZX, Xu XP, Weng SP, Rao XY, Dai ZX, et al. A novel C-type lectin from the shrimp *Litopenaeus vannamei* possesses anti-white spot syndrome virus activity. *J Virol* 2009; **83**(1): 347-356.

[20] Ji PF, Yao CL, Wang ZY. Immune response and gene expression in shrimp (*Litopenaeus vannamei*) hemocytes and hepatopancreas against some pathogen-associated molecular patterns. *Fish Shellfish Immunol* 2009; **27**: 563-570.

[21] Wang S, Zhao XF, Wang JX. Molecular cloning and characterization of the translationally controlled 131 tumor protein from *Fenneropenaeus chinensis*. *Mol Biol Rep* 2009; **36**: 1683-1693.

[22] Wang S, Liu N, Chen AJ, Zhao XF, Wang JX. TRBP homolog interacts with eukaryotic initiation factor 6 (eIF6) in *Fenneropenaeus chinensis*. *J Immunol* 2009; **182**: 5250-5258.

[23] Leu JH, Chang CC, Wu JL, Hsu CW, Hirono I, Aoki T. Comparative analysis of differentially expressed genes in normal and white spot syndrome virus infected *Penaeus monodon*. *BMC Genomics* 2007; **8**: 120.

[24] Soonthornchai W, Rungrassamee W, Karoonuthaisiri N, Jarayabhand P, Klinbunga S, Soderhall K, et al. Expression of immune-related genes in the digestive organ of shrimp, *Penaeus monodon*, after an oral infection by *Vibrio harveyi*. *Dev Comp Immunol* 2010; **34**: 19-28.

[25] Jaroenram W, Kiatpathomchai W, Flegel TW. Rapid and sensitive detection of white spot syndrome virus by loop-mediated isothermal amplification combined with a lateral flow dipstick. *Mol Cell Probes* 2009; **23:** 65-70.

[26] Kono T, Savan R, Sakai M, Itami T. Detection of white spot syndrome virus in shrimp by loop-mediated isothermal amplification. *J Virol Methods* 2004; **115**: 59-65.

[27] Mekata T, Sudhakaran R, Kono T, Supamattaya K, Linh NT, Sakai M, et al. Real-time quantitative loop-mediated isothermal amplification as a simple method for detecting white spot syndrome virus. *Lett Appl Microbiol* 2009; **48:** 25-32.

[28] Chen JY, K Anbarasu, CY Chen, YC Lee, FH Nan, CM Kuo. Passive immunity to white spot syndrome virus (WSSV) in *Penaeus monodon* treated with monoclonal antibiodies of the heterologously produced VP28 viral envelope protein. *J Mar Sci Technol* 2010; **18**: 145-152.

[29] Yang C, Zhang J, Ma FH, Zhang Q, Priya J, Zhang X, et al. A Toll receptor from Chinese shrimp *Fenneropenaeus chinensis* is responsive to *Vibrio anguillarum* infection. *Fish Shellfish Immunol* 2008; **24**: 564-574.

[30] Arts JAJ. Cornelissen FHJ, Cijsouw T, Hermsen T, Savelkoul HFJ, Stet RJM. Molecular cloning and expression of a toll receptor in the giant tiger shrimp, *Penaeus monodon*. *Fish Shellfish Immunol* 2007; **23**(3): 504-513.

[31] Choi, Mi Ran, Yeong Jin Kim, Ji-Suk Jang, Sung-Koo Kim. Transcriptional analysis for oral vaccination of recombinant viral proteins against white spot syndrome virus (WSSV) in *Litopenaeus vannamei*. *J Microbiol Biotechnol* 2011; **21**(2): 170-175.

[32] Ma TH, Benzie JA, He JG, Chan SM. PmLT, A C-type lectin specific to hepatopancreas is involved in the innate defense of the shrimp *Penaeus monodon*. *J Invertebr Pathol* 2008; **99**: 332-341.