

# Incidence of White Spot Syndrome Virus (Wssv) in Indian Farmed Frozen Shrimp Products and Testing for Viability Through Bio-Inoculation Studies

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## Abstract

The incidence of white spot syndrome virus (WSSV) in the Indian farmed frozen shrimp products viz., whole, headless (HL) and peeled and undeveined (PUD) by polymerase chain reaction (PCR) was studied. The viability of WSSV in farmed frozen shrimps was investigated by conducting bio-inoculation studies. The products were screened for WSSV by both the single step (for the primer sets 1s5 & 1a16 and IK1 & IK2) and nested PCR (for the primers IK1 & IK2 – IK3 & IK4). Single step PCR detected WSSV in 18% of samples, while the nested PCR detected WSSV in 73% of samples. The frozen material, which gave positive results for WSSV by PCR, was further confirmed for the viability of WSSV by the bio-inoculation studies. Mortality (100%) was observed within the 45 h of intra-muscular post injection (P.I) into the healthy wild live WSSV-free shrimps (*Penaeus monodon*). These results show that the WSSV survived the freezing process and the cold storage and could cause infection in native shrimp farms if such products are reprocessed in the importing countries.

**Keywords:** WSSV; PCR; Farmed; Frozen; Shrimp products; Bio-inoculation

## Introduction

India earned about US \$ 1908.63 million as foreign exchange during 2008-09 through the export of fish and fishery products. Of the total exports, shrimps accounted for about 44% in terms of value. The contribution of farmed shrimps to export of total shrimps consisting of both the wild and farmed was about 75% by value [1]. Shrimps are mostly exported in frozen condition. Since shrimp and its products fetch a higher price in the international market, shrimp farming is a major industry in India. The spread of exotic viruses in farmed shrimps can be attributed to several reasons like transportation of infected stocks, by birds acting as vectors and carrying the diseases and by importation & processing of frozen food products [2,3]. Several authors studied the presence of white spot syndrome virus (WSSV) in shrimps by using PCR [4-6]. It has been reported that the transmission of exotic viral pathogens to farmed and native shrimp stocks may be possible via frozen prawn products prepared and packaged for human consumption [7]. During the last decades, this disease has spread worldwide and caused large-scale mortalities and severe damage to shrimp culture, particularly in Asia, leading to massive economic losses to the farmers, processors and the country. It is estimated that WSSV caused losses of 300,000 metric tons of shrimp, worth more than 1 billion US dollars [8].

Several developed countries like USA, Japan, EU and Australia import frozen shrimp products from India. Imports are typically repackaged at processing plants that are located in the importing countries. [2] Nunan et al. detected the WSSV in imported commodity shrimp in the USA. [3] Lightner reported the risk of spread of penaeid shrimp viruses in the Americas by the international movement of live and frozen shrimp. [9] (2000) reported that frozen commodity shrimp might be a potential avenue for the introduction of WSSV in USA.

Viral disease diagnostics on imported fresh and frozen stocks have been hardly implemented and have only included stock samples produced in Latin America or Asia [3,10,11,6]. It is listed as a non-exotic disease in EC directive 2006/88 [12,13] examined the populations of Australian crustaceans for evidence of White Spot

Syndrome virus (WSSV) and found neither mortalities, clinical signs of disease nor evidence of WSSV. Fresh/frozen shrimp products for human consumption imported into Australia have been subjected to mandatory testing since October 2007 using PCR technology for three major shrimp viruses viz., White Spot Syndrome Virus (WSSV), Yellow Head Virus (YHV) and Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV) at the level of 5% prevalence with 95% confidence. Batches that tested positive have to be destroyed or cooked in an approved facility [14,15]. As there are no reports on the prevalence of WSSV in Indian frozen shrimp products, the present study was undertaken to find out the incidence of WSSV in frozen shrimp products of India and testing for the viability of WSSV in frozen shrimps through bio-inoculation studies.

## Materials and Methods

### Sample collection

The commercially farmed frozen shrimp product samples to be exported were collected from various seafood processing plants located in and around Tuticorin, Tamil Nadu, India. About 99 samples were collected and tested for the incidence of WSSV. All the tests were done in triplicates. A preliminary observation was made on the collected samples for the symptoms of WSSV such as the dispersion of white spots and red discoloration over the body in whole shrimps [16].

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**Received** September 22, 2010; **Accepted** October 10, 2010; **Published** November 02, 2010

**Citation:** Reddy AD, Jeyasekaran G, Shakila JR (2010) Incidence of White Spot Syndrome Virus (Wssv) in Indian Farmed Frozen Shrimp Products and Testing for Viability Through Bio-Inoculation Studies. J Aquac Res Development 1:102. doi:10.4172/2155-9546.1000102

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## DNA extraction

The WSSV DNA for PCR was extracted by using standard phenol extraction procedure and ethanol precipitation [17]. Briefly, about 500 mg of homogenized tissue of farmed shrimp was taken in a 2.0 ml sterile microfuge tube. Then, 0.5 ml molecular grade water (Sartorius Stedim Biotech, Gottingen, Germany) was added to each tube and incubated for 15 min at -20°C. To each tube, 0.5 ml of buffered phenol (which was prepared by mixing 500 ml of phenol with 50 ml of 0.1M Tris (pH 8.0) and 100µl of β-Mercaptoethanol) was added. The tissue sample was then centrifuged at 10,000 rpm for 15 min at 4°C in a refrigerated microfuge (Eppendorf AG, Hamburg, Germany) and the supernatant was transferred to another sterile microfuge tube and 0.5 ml of buffered phenol was added and centrifuged. This process was repeated until the supernatant became clear. The supernatant was then transferred to another sterile microfuge tube and 0.5 ml of diethyl ether was added and centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was discarded and 50µl of 3 M sodium acetate and 1.5 ml of 100% ethanol were added and stored at -20°C overnight (or) at -80°C for 2 h. The extract was centrifuged at 10,000 rpm for 15 min at 4°C and the supernatant was discarded. The pellet was washed with 70% ethanol and dried at room temperature. The pellet was finally dissolved in 20µl molecular grade water and stored at -20°C until further use.

## Amplification of WSSV DNA

Oligonucleotide primers (1s5 & 1a16, IK1 & IK2, IK3 & IK4) chosen for the amplification of WSSV DNA fragments were based on the earlier reports of [18] East et al and [19] Pradeep et al. for single step and nested PCR, respectively (Table 1). The primers produced an amplicon size of 486 bp (for IK1 & IK2), 310 bp (for IK3 & IK4) and 198 bp (for 1s5 & 1a16).

## PCR for WSSV from farmed shrimp products

**First / Single step PCR:** The reaction mixture for the first/single step PCR contained crude DNA extract (template DNA), reaction buffer (100mM Tris with 15mM MgCl<sub>2</sub>), 0.4µg each of forward and reverse primer (1s5 & 1a16; IK1 & IK2), 100mM each dNTPs (deoxyribonucleotide triphosphates), 3U Taq DNA polymerase and molecular grade water. The components were mixed thoroughly and the PCR reaction was conducted in the Thermalcycler (GeneAmp 9700, ABI Systems, Rotkreuz, Switzerland). The PCR protocol comprised of 35 cycles of 60 sec at 94°C, 60 sec at 55°C and 90 sec at 72°C. The programme included an initial delay of 4 min at 94°C and final extension of 5 min at 72°C before and after 35 cycles, respectively.

**Second step/nested PCR:** In this case, an aliquot from the first

step PCR product was used as the DNA template together with the nested primer pair, IK3 & IK4. The rest of the PCR mixtures were the same as described above.

## Electrophoresis

After completion of PCR, 5µl of PCR product was taken and mixed with 1µl of 6X loading buffer and subjected to electrophoresis (GE Healthcare Biosciences, Kowloon, Hong Kong) in 2% agarose gel incorporated with ethidium bromide at a concentration of 0.5µl/ml in 1X Tris-acetate – EDTA (TAE) buffer (pH 8.5) and the gel was analyzed under UV trans-illumination and photographed using Gel Documentation System (Alpha Innotech Corp. California, USA).

## Experimental animals for bio-inoculation studies

The healthy live WSSV-free shrimps (*P. monodon*) weighing 6-9 g obtained from the wild were used for the bio-inoculation studies. The shrimps were screened for WSSV prior to acclimatization in cement tanks. They were then maintained in 200 L of aged borewell seawater having salinity between 20-25 ppt in cement tanks with air-lift biological aerators at room temperature (24-29°C). They were fed with artificial pellet feed (CP Feed, Thailand). They were reared in laboratory conditions for 10 days prior to the bio-inoculation studies. They were divided into two groups of five animals each as per the earlier reports of [19,20]. One group was injected with the WSSV viral inoculum and the other group was treated as negative control in which the animals were injected with extracts from shrimp negative for WSSV.

## Preparation of viral inoculum

Viral inoculum was prepared by the method described by [21] (2009) with slight modification. Briefly, pleopods were removed from frozen shrimps separately, homogenized in TN buffer and centrifuged at 1000 g for 10 min at 4°C in a refrigerated microfuge. The supernatant fluid was diluted to 1/10 with 2% NaCl and filtered through 0.2µm membrane filter. About 100µl of inoculum was injected into the live healthy animals through the dorsal region of the third abdominal segment, as earlier workers have used 25 to 200µl inoculum for the bioassay studies [2,11,20].

## Results and Discussion

### Detection of WSSV in farmed frozen shrimp products

The incidence of WSSV analyzed by single and nested PCR with three sets of primers are given in Table 2. The incidence of WSSV was found to vary with primers as well as with single/nested PCR. The single step PCR detected WSSV in only 15% of product samples

Primer specific for	Primer name	Sequence (5'-3')	Product size
Single step PCR	1s5 1a16	CACTCTGGCAGAATCAGACCAGACCCCTGAC TTCCAGATATCTGGAGAGGAAATTCC	198 bp
Nested PCR 1 <sup>st</sup> step	IK-1	TGGCATGACAACGGCAGGAG	486 bp
2 <sup>nd</sup> step	IK-2 IK-3 IK-4	GGCTTCTGAGATGAGGACGG TGTCATCGCCAGCACGTGTGC AGAGGTCGTCAGAGCCTAGTC	310 bp

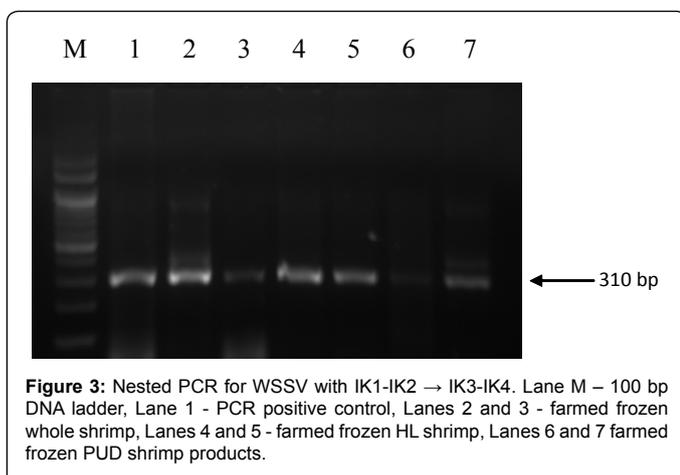
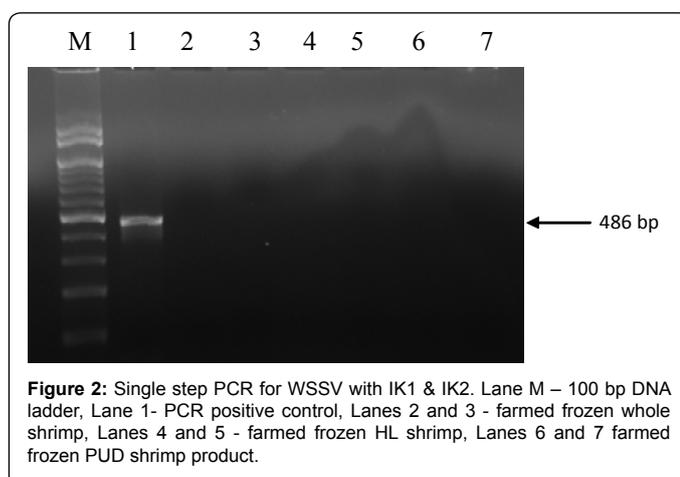
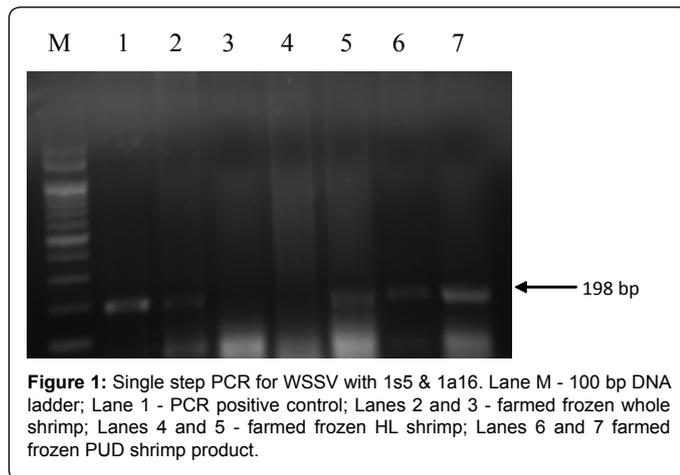
**Table 1:** Primers used for the detection of WSSV from farmed frozen shrimp products.

Farmed frozen shrimp products	No. of samples	No. positive by non-nested PCR		No. positive by nested PCR IK1-IK2→IK3-IK4
		1s5 -1a16	IK1- IK2	
Whole	75	6	3	57
Headless (HL)	15	6	-	9
Peeled and Undeined (PUD)	9	3	-	6
Total	99	15	3	72

**Table 2:** Incidence of White Spot Syndrome Virus (WSSV) in farmed frozen shrimp products using single step and nested PCR.

with 1s5 & 1a16 primers, while the detection was very low (3%) with the primers IK1 & IK2. On the other hand, nested PCR with IK3 & IK4 detected the incidence of WSSV in 73% of the samples tested. The incidence of WSSV was found to be high in frozen whole shrimp products with 76% showing positive in the nested PCR reaction. In the frozen HL shrimp products, the incidence was 60 %; while in frozen PUD shrimp products, it was 66% out of the total samples analyzed.

Of the two sets of primers used for the single step PCR in the present study, the primer set of 1s5 & 1a16 was found to be highly



sensitive, as evidenced by the detection of WSSV in more number (15%) of farmed shrimp products as against 3% by the other primer set of IK1 & IK2 (Table 2). The primer set of 1s5 & 1a16 yielded an amplicon of 198 bp (Figure 1) when compared to the other set of primer (IK1 & IK2) that yielded a product size of 486 bp (Figure 2). It has been earlier reported that the specificity and sensitivity of the reactions depend on the primers used and the sensitivity of PCR increases with decrease in size of amplicon yielded by a set of primer [22,23]. It is quite interesting to note that positive results for WSSV in the single step PCR could be obtained from the farmed frozen shrimp products with the primers 1s5 & 1a16. It shows that this primer set could even detect WSSV DNA that has been degraded due to freezing. The findings of [18] on the detection of degraded WSSV DNA by the primers 1s5 & 1a16 clearly supported the results of the present study. Primers IK1 & IK2 were amplified and showed positive results only for the shrimp products that exhibited prominent WSSV clinical signs.

The detection level of WSSV by nested PCR was found to be very high with 73% incidence in the farmed frozen shrimp products when compared to single step PCR (Table 2). The primers (IK1-IK2 & IK3-IK4) used in nested PCR yielded an amplicon of 310 bp (Figure 3) Many authors have also suggested the use of nested PCR to increase the accuracy of viral diagnostics particularly WSSV from shrimps [6,11,24]. A normal harvest would not give any indication of infection status of shrimp products with respective important shrimp viral pathogens [15] and in such cases usually the nested PCR give positive results for the target viral pathogens.

Nunan et al. [2] reported a prevalence of WSSV in 50% of frozen shrimps sold in grocery store in Texas, America. Durand et al. (2000) reported that the incidence of WSSV in various retail outlets in Tucson, Arizona was 80%. It has also been found that WSSV was present in the frozen shrimp (*Penaeus monodon*) imported into Australia from Asian countries [4]. [25] Ostrowski found that 73% of the shrimp samples surveyed from grocery-store in Hawaii by PCR were positive for WSSV. The present results are in line with the above said earlier findings. But, [10] Reville et al. have detected WSSV in only 4.7% of shrimp products in supermarkets of Massachusetts, USA. In Mexico [6] Lopez et al. reported the incidence of WSSV in frozen shrimp products as 32%. The variation in prevalence of WSSV in shrimp products observed by different workers might be due to the differences in the pattern of outbreaks of WSSV in shrimp farms at different point of time of sampling.

The present result also showed the presence (66%) of WSSV in the frozen PUD shrimps. Since gross clinical signs of WSSV are not normally found in the HL and PUD shrimps, the shrimp processing plants process the whole shrimps to either HL or PUD. The present findings clearly indicated the presence of WSSV in farmed frozen shrimp products. Previous studies have also detected the presence of pathogenic viruses such as Infectious Hypodermal Hematopoietic Necrosis Virus (IHHNV), White spot syndrome virus (WSSV), Yellow head virus (YHV) and Taura syndrome virus (TSV) in frozen products from several shrimp species imported into the USA and Australia [2,3,9,4,10,11,6,15].

### Bio-inoculation studies

The bio-inoculation studies conducted on the healthy live WSSV-free shrimps (*P. monodon*) produced 100% mortality at 45 h of post injection (P.I.). None of the healthy shrimps died in negative control group. The WSSV viral inoculum prepared from the frozen cold stored shrimps showed 100% mortality at 45 h of post injection. The first

mortality occurred at 28 h and second mortality at 30 h of injection with clinical signs of pinkish red discoloration on the uropods. The third and fourth shrimps died at 36 h and 44 h, respectively during which time the clinical signs intensified further with pinkish red discoloration on the uropods as well as on the pleopods. The mortality of fifth shrimp occurred at 45 h and clinical signs were very intense. None of the shrimps showed white patches or spots on the carapace or muscle.

The shrimps inoculated with viral inoculum from frozen cold stored shrimps were tested for WSSV by PCR diagnostics after death. The shrimps that died after 28 and 30 h were found to be WSSV-positive only in nested PCR, while those died after 36 and 46 h showed WSSV-positive by single step PCR with 1s5 & 1a16 as well as by the nested PCR (Table 3 and Figure 4). All the three PCR primers tested gave WSSV positive results only in shrimps that died after 45 h.

The progress of WSSV infection in the bio-inoculated shrimps showed that WSSV first affected the pleopods followed by uropods. The injected shrimps showed gross clinical signs of WSSV infection. The WSSV infected animals on bio-inoculation exhibited pinkish red discoloration on the uropods and pleopods. [26] Sudha also reported that the shrimp with reddish discoloration without white spots was a preacute form of the disease caused by WSSV. The disease process may be completed in a short time period because of high viral load and therefore, the principal clinical sign of white spot appearance normally associated with WSSV syndrome may not develop at all. The successful WSSV infection on infectivity bioassays in all of the experimental animals caused rapid reduction in feed intake and lethargy [27], which was also observed in the present study. Of the 5 animals injected with the viral inoculum from frozen cold stored shrimps, the first mortality was observed at 28 h P.I. (Table 3 and Figure 4). No clinical signs of disease or mortality were observed among negative control shrimps during the experimental period. [28] Lightner also reported the appearance of reddish discoloration

or small white spots in the WSSV infected shrimps (*P. monodon*). [9] Durand observed 100% mortality in the specific pathogen free (SPF) indicator shrimps (*P. vannamei*) on injection of WSSV inoculum from frozen shrimp products. All the 5 animals in the present study died within 45 h after P.I, which showed that the viability of WSSV in shrimps was not affected by freezing and cold storage.

[2] Nunan reported that the bioassay using 15 indicator shrimps (*P. stylirostris*) produced 100% mortality within 8 day of P.I. of WSSV inoculum from frozen shrimp products. The series of experiments conducted by them also showed that frozen product imported from Asia contains the viral pathogens, WSSV and YHV and through the use of bioassay, they demonstrated that the viruses in the frozen imported product were infectious. [9] Durand observed that the challenge bioassay of WSSV by shrimp injection produced the first mortality in indicator shrimp on day 3, but, the entire indicator shrimps had died by day 4. [11] Hasson observed the clinical signs of disease including lethargy, anoxeria and chromatophore expansion resulting in dark body coloration and reddening of both the uropods and antennae, when SPF shrimps (*Litopenaeus vannamei*) juveniles were injected with WSSV PCR-positive shrimps (*Parapenaopsis sp.*) tissue homogenates resulting in 100% mortality in the experimental shrimps within 48 to 72 h of P.I. Pradeep [19] also reported that there was 100% mortality on day 3 when they intramuscularly injected WSSV extract into 5 live healthy animals of shrimps (*P. monodon*). It is inferred from the present study that the WSSV was virulent even in shrimps that were subjected to freezing and cold storage processes and infectious resulting in 100% mortality of healthy shrimps.

Shrimps infected with WSSV would still be fit for human consumption since this virus causes no known human health problems. However, several pathways have been identified by which imported and infected shrimps could become a source of infection to farmed and wild decapod crustaceans. The situation is further complicated by the broad host range of WSSV, which is known to be capable of infecting not only penaeid shrimps but also a number of other decapod crustaceans including crabs, lobsters and some other species of shrimps [23]. If the quality of a shipment of imported shrimps deteriorated (for any reason) such that they were no longer fit for human consumption, it is conceivable that their disposal, or use in other inappropriate ways, could indeed represent a threat to animal biosecurity [4], which supported the present findings.

## Conclusion

The detection level of WSSV by nested PCR was found to be very high with 73% incidence in the Indian farmed frozen shrimp products when compared to single step PCR. The present results of high incidence of WSSV in farmed frozen shrimp products and the resistance of WSSV to freezing process and cold storage based on bio-inoculation studies may alert the shrimp farmers, processors and exporters to be more vigilant on WSSV infection in farmed shrimps in order to prevent economic losses.

## Acknowledgements

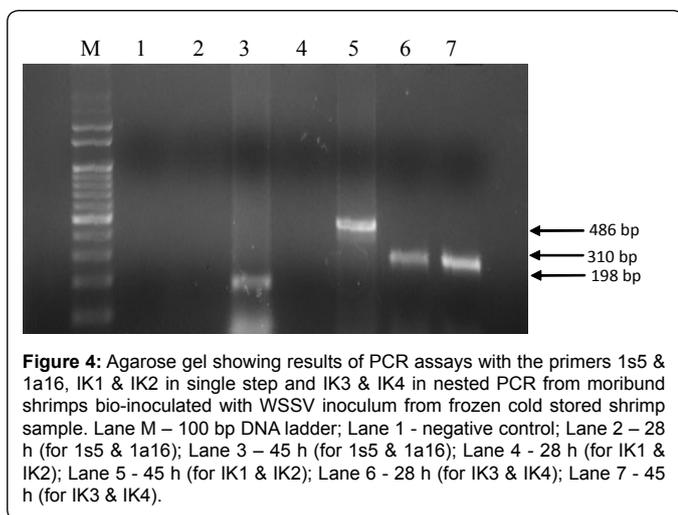
Authors wish to thank the Dean, Fisheries College and Research Institute, Tuticorin for providing all facilities and support to carryout this study. This study was a part of M.F.Sc thesis submitted by the first author to the Tamil Nadu Veterinary and Animal Sciences University, Chennai, India.

## References

1. MPEDA (2009) Accessed on 17<sup>th</sup> April, 2010.
2. Nunan LM, Poulos BT, Lightner DV (1998) The detection of white spot syndrome virus (WSSV) and yellow head virus (YHV) in imported commodity shrimp. *Aquaculture* 160: 19-30.

PCR diagnosis	Negative group	Time of mortality (P.I)				
		28 h	30 h	36 h	44 h	45 h
Single step PCR (1s5 - 1a16 primers)	-	-	-	+	+	+
Single step PCR (IK1 - IK2 primers)	-	-	-	-	-	+
Nested PCR (IK1-IK2 → IK3 - IK4 primers)	-	+	+	+	+	+

**Table 3:** Detection of WSSV by PCR from the tissues of shrimps (*P. monodon*) challenged by intramuscular (I.M.) injection of WSSV inoculum from frozen cold stored (5<sup>th</sup> month) shrimp sample.



3. Lightner DV (1999) The penaeid shrimp viruses TSV, IHNV, WSSV and YHV. *Applied Aquaculture* 9: 27-52.
4. McColl K, Slater J, Jeyasekaran G, Hyatt A, Crane MS (2004) Detection of white spot syndrome virus and yellowhead virus in prawns imported to Australia. *Australian Vet J* 82: 69-74.
5. Pradeep B, Shekar M, Gudkous N, Karunasagar I, Karunasagar I (2008) Genotyping of white spot syndrome virus prevalent in shrimp farms of India. *Dis Aquat Org* 78: 189-198.
6. Reyes-López M A, Salazar-Marroquín E L, Oliva-Hernández A A, Salinas-López N, Narváez- Zapata J A (2009) White-spot syndrome virus diagnostics in frozen shrimp stocks imported to Mexico. *CyTA – Journal of Food* 7: 89-94.
7. Flegel TW (2009) Review of disease transmission risks from prawn products exported for human consumption. *Aquaculture* 290: 179-189.
8. Rosenberry B (2001) *World shrimp farming 2001*. San Diego: Shrimp News International.
9. Durand SV, Tang KFJ, Lightner DV (2000) Frozen commodity shrimp: potential avenue for introduction of white spot syndrome virus and yellow head virus. *J Aquat Anim Health* 12: 128-135.
10. Reville C, Al-beik J, Meehan-meola D, Xu Z, Goldsmith ML, et al. (2005) White spot syndrome virus in frozen shrimp sold at Massachusetts supermarkets. *Journal of Shellfish Research* 24: 285-290.
11. Hasson KW, Fan Y, Reisinger T, Venuti J, Varner PW (2006) White-spot syndrome virus (WSSV) introduction into the Gulf of Mexico and Texas freshwater systems through imported, frozen bait-shrimp. *Dis Aquat Organ* 71: 91-100.
12. Stentiford GD, Bonami J R, Alday-Sanz V (2009) A critical review of susceptibility of crustaceans to taura syndrome, yellowhead disease and white spot disease and implications of inclusion of these diseases in European legislation. *Aquaculture* 291: 1-17.
13. East IJ, Black PF, McColl KA, Hodgson R, Bernoth EM (2004) Survey for the presence of white spot syndrome virus in Australian crustaceans. *Australian Vet J* 82: 236-240.
14. Biosecurity Australia Policy Memorandum 2007/16, 24 July 2007.
15. Sritunyalucksana K, Srisala J, Wangnai W, Flegel TW (2010) Yellow head virus (YHV) transmission risk from commodity shrimp is reduced to negligible levels by normal processing. *Aquaculture* 300: 32-36.
16. Escobedo-Bonilla CM, Alday-Sanz V, Wille M., Sorgeloos P, Pensaert MB, et al. (2008) A review on the morphology, molecular characterization, morphogenesis and pathogenesis of white spot syndrome virus. *J Fish Dis* 31: 1-18.
17. Jeyasekaran G (2000) *A Report on Diagnostics in Fish diseases*. Australian Animal Health Laboratory Commonwealth Scientific and Industrial Research Organization, Geelong Australia pp 42.
18. East IJ, Black PF, Findlay VL, Bernoth EM (2005) A national survey to verify freedom from white spot syndrome virus and yellow head virus in Australian crustaceans. In: Walker P, Lester R, Bondad-Reantaso MG *Diseases in Asian Aquaculture V Fish Health Section*, Asian Fisheries Society, Manila, Philippines 15-26.
19. Pradeep B, Karunasagar I, Karunasagar I (2009) Fitness and virulence of different strains of white spot syndrome virus. *J Fish Dis* 32: 801-805.
20. Escobedo-Bonilla CM, Wille M, Alday-Sanz V, Sorgeloos P, Pensaert MB, et al. (2005) *In vivo* titration of white spot syndrome virus (WSSV) in specific pathogen-free *Litopenaeus vannamei* by intramuscular and oral routes. *Dis Aquat Org* 66: 163-170.
21. OIE (2009) *Manual of Diagnostic Tests for Aquatic Animals, White Spot Disease*, Office International des Epizooties, France, Chapter 2.2.5: 121-131.
22. Lo CF, Leu JH, Ho CH, Chen CH, Peng SE, et al. (1996) Detection of baculovirus associated with white spot syndrome (WSBV) in penaeid shrimps using polymerase chain reaction. *Dis Aquat Org* 25: 133-141.
23. Hossain MS, Otta SK, Chakraborty A, Sanath Kumar H, Karunasagar I, et al. (2004) Detection of WSSV in farmed shrimps, captured brooders, shrimp postlarvae and water samples in Bangladesh by PCR using different primers. *Aquaculture* 237: 59-71.
24. Peinado-Guevara LI, Lopez-Meyer M (2006) Detailed monitoring of white spot syndrome virus (WSSV) in shrimp commercial ponds in Sinaloa, Mexico by nested PCR. *Aquaculture* 251: 33-45.
25. Ostrowski AC (2004) Consortium research update FY2004. The latest report from the USMSFP consortium December 2004. Waimanolo, HI, 1-6.
26. Sudha PM, Mohan CV, Shankar KM, Hegde A (1998) Relationship between white spot syndrome virus infection and clinical manifestation in Indian farmed penaeid shrimp. *Aquaculture* 167: 95-101.
27. Corbel V, Zuprizalk Z, Shi C, Huang S, Arcierk JM, et al. (2001) Experimental infection of European crustaceans with white spot syndrome virus (WSSV). *J Fish Dis* 24: 377-382.
28. Lightner DV (1996) *A Handbook of shrimp pathology and diagnostic procedures for diseases of cultured penaeid Shrimp*. The World Aquaculture Society, USA pp305.

