"Molecular Biological investigations on viral diseases affecting farmed penaeid shrimps in Kerala"

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Declaration

I hereby declare that this thesis is a record of bonafide research carried out by me under the supervision and guidance of Dr. P. K Surendran, my supervising guide and Dr. Nirmala Thampuran, my Co-guide and it has not previously formed the basis for award of any degree, diploma, associateship, fellowship or other similar title or recognition to me, from this or any other University or Society

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CERTIFICATE

This is to certify that this thesis entitled "Molecular Biological investigations on viral diseases affecting farmed penaeid shrimps in Kerala" embodies the result of original work conducted by Sri. Toms C. Joseph, under our supervision from November 2004 to December 2009. We further certify that no part of this thesis has previously formed the basis for the award to the candidate, of any degree, diploma, associateship, fellowship or other similar titles of this or any other University or Society. He has passed the PhD qualifying examination of the Cochin University of Science and Technology, held in March 2006.

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Dedicated to my
beloved parents

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Toms C. Joseph

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Introduction

1. Introduction

Aquaculture is one of the world's fastest growing industry but disease outbreaks in cultured shrimp have caused serious economic losses. In Asia, mortalities of cultured shrimp because of white spot syndrome virus (WSSV) and yellow head virus (YHV) have resulted in economic losses of about $1 billion per year since 1994. Indian farmed shrimp production increased from about 30,000 tons in 1990 to around 1,15,000 tons during 2002–2003 (FAO, 2007). Due to the rapid expansion of shrimp aquaculture during early 1990s, the demand for post larvae (PL) could not be met by the hatcheries that existed in the country at that time. The import of PL from other Asian countries and poor management of the brood stock, the hatcheries and also the farms led to the outbreak of White Spot Syndrome Virus (WSSV) in 1994. The virus spread very rapidly, and the economic losses caused by mortalities were estimated at over US$ 200 million during 1999–2000 (FAO, 2007)

White Spot Syndrome Virus (WSSV) is the most serious pathogen of shrimp aquaculture in Asia. It first appeared in 1990s in Taiwan and China (Chou *et al.*, 1995). The virus can cause 100% mortality within 3-5 days in infected shrimp (Karunasagar *et al.*, 1997; Lotz & Soto, 2002). WSSV infects a wide range of wild crustaceans including crabs, lobsters and shrimp. The presence of WSSV has also been recorded from wild as well as hatchery reared post larvae. The simplest method to detect WSSV infection in shrimp is to observe for local lesions and white spots on the carapace. Sometimes a pink to reddish-brown coloration is seen on the shrimp due to the expansion of sub-cuticular chromatophores. Since the shrimps die after the appearance of symptoms, this method

cannot be used for diagnosis. Histological lesions include distinct hypertrophied nuclei containing marginated chromatin and amphophilic central inclusions in the cuticular epithelial cells, connective tissue cells and hemocytes. Molecular methods like polymerase chain reaction (PCR) and Real time PCR are sensitive for detection of WSSV infection even in carrier animals that do not show any gross symptoms of the disease.

Vembanad Lake is an estuarine system located on the south-west coast of India. Many crustaceans spent part of their lifecycle in the estuarine system before migrating to the sea. The borders of the Vembanad Lake support extensive as well as semi-intensive aquaculture. WSSV infection of penaeid shrimp has a devastating influence on the shrimp aquaculture in the Vembanad estuarine system. Interventions in the form of reclamation and discharge of pollutants to the Vembanad Lake also have an adverse impact on the potential of the aquatic ecosystem that used to support high levels of bioproductivity and biodiversity. There has been very limited study on the occurrence of WSSV in decapods in the Vembanad estuarine system.

WSSV is an enveloped ovoid virus with rod shaped nucleocapsid with flat ends. The virus belongs to the genus Whispovirus under the family Nimiviridae. The complete genome sequence of WSSV has been determined for three different isolates with Gene bank Accession numbers AF369029, AF440570, AF332093 for viruses isolated from Thailand, Taiwan, and China respectively. The genome of WSSV is approximately 300 kb in length with 180 putative Open Reading Frames. Most of the Open Reading Frames of WSSV encode certain structural genes. Structural proteins of viruses are classified as envelope and non-envelope proteins. Envelope proteins play a vital role in virus entry assembly and release. Neutralization experiments of antibodies against six WSSV

envelope proteins showed that the virus infection could be significantly delayed or neutralized by antibodies against three WSSV envelope proteins (VP68, VP281 and VP466) (Wu *et al.*, 2005). Very little genetic variation has been recorded in the structural genes of WSSV from different geographical locations. There had been very limited work on the nucleotide sequence of the structural genes of WSSV isolated from India.

Monodon Baculovirus (MBV) can cause mortalities in hatchery reared larvae. Infection with MBV can cause severe mortalities in post larvae of hatcheries. Although MBV is relatively well tolerated by *Penaeus monodon*, it has been implicated in mass mortalities in shrimp cultured at high densities. Although good culture practices may enhance the survival of MBV-infected stocks, growth, crop value and performance may significantly be reduced and MBV may render the infected shrimp susceptible to other pathogens with higher mortality rates (Bower, 1996).

Hepatopancreas Parvovirus (HPV) is distributed worldwide and infects several penaeid shrimp including *P. monodon* (Lightner, 1996). HPV infected *P. monodon* is found to grow slowly compared to uninfected ones. Early juvenile stages are reported to have high levels of HPV infection. Two strains of HPV have been characterized at the molecular level. They are the HPVchin from *P. chinensis* from Korea and HPVmon from *P. monodon* from Thailand. The DNA sequence of HPVmon differs from HPVchin by almost 30% by examining the partial sequences for HPVchin (AY008257) and HPVmon (AF456476) available at GenBank.

Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV) was first reported in juvenile *Litopenaeus stylirostris* in Hawaii (Lightner *et al.*, 1983a, b). The virus has been detected in many penaeid shrimp around the world. Even though IHHNV

infection does not cause mortality in *Litopenaeus vannamei* and *P. monodon*: it results in a disease called Runt Deformity Syndrome in both species and hence causes substantial economic losses (Kalagayan *et al.*, 1991; Browdy *et al.*, 1993; Primavera & Quinitio, 2000). There had been limited work on the simultaneous occurrence of viral pathogens in the post larvae of paenaid shrimp in hatcheries.

PCR is the method of choice for detection of viral pathogens affecting shrimp aquaculture. PCR has been widely used for routine detection of WSSV in broodstock and post larvae. Although PCR is the method of choice for detection of viral pathogens, it cannot detect very low numbers of the virus. The routine screening PCR test used for detection of WSSV are able to detect about 10-50 copies in a PCR reaction. Moreover the conventional PCR method cannot quantify the amount of virus, but only the presence or absence of virus. Real-time quantitative PCR using SYBR Green as the fluorescence dye is a rapid and highly sensitive method for detection as well as quantification of WSSV. SYBR Green is a minor-groove DNA dye with a high affinity for dsDNA. In Real-Time PCR, the fluorescence of the SYBR Green dye is monitored at the end of each PCR cycle. The increase of fluorescence is dependant on the initial template concentration. Real-Time PCR can be utilized to screen for virus low grade infection in postlarvae, for the development of a specific pathogen free shrimp, screening of shrimp broodstock and to implement effectively the sanitary and phyto-sanitary regulations. Real-time PCR has been successfully employed for quantification of WSSV in different shrimp species (Dhar *et al.*, 2001; Durand and Lightner, 2002; Durand *et al.*, 2003; Powell *et al.*, 2006; Sritunyalucksana *et al.*, 2006c; Jang *et al.*, 2009)

The objectives of this study were

- To determine the prevalence of WSSV among the crustaceans in the Vembanad estuary, the shrimp aquaculture farms surrounding the estuary, and the sea off Cochin coast, India.
- To compare the sequence of six major structural proteins of WSSV: vp28, vp26, vp19, vp68, vp281, vp466 from different geographical locations with that of an isolate from India.
- To monitor the simultaneous occurrence of HPV, IHHNV, MBV and WSSV in postlarvae of *P. monodon* from hatcheries in India by Polymerase Chain Reaction.
- Development of a quantitative assay for WSSV infection.
- To determine the viral load of postlarvae from hatcheries in Kerala meant for aquaculture

About this thesis

In this thesis, the investigation has been dealt in the following manner:

A study was done to determine the prevalence of WSSV among the crustaceans in the Vembanad estuary, the shrimp aquaculture farms surrounding the estuary, and the sea off Cochin coast, India using two sets of nested PCR primers.

An investigation was also done to compare the sequence of six major structural proteins of WSSV: vp28, vp26, vp19, vp68, vp281, vp466 from different geographical locations with that of an isolate from India.

A study was also done to monitor the simultaneous occurrence of HPV, IHHNV, MBV and WSSV in postlarvae of *P. monodon* from hatcheries in India by Polymerase Chain Reaction.

A real time PCR procedure was developed for the quantitative analysis of WSSV infection. The viral load of postlarvae from hatcheries in Kerala meant for aquaculture was also determined using the quantitative PCR.

The thesis is divided into five major chapters and each chapter is further divided into subheads. The first chapter highlights the identification of problem and with suitable objectives. The second chapter is the review of literature. The review includes a short description of the current status of shrimp aquaculture and the diseases affecting shrimp. A detailed review of spread of WSSV infection, genetic variability of WSSV strains, the structural proteins of WSSV, clinical signs and pathogenesis of WSSV infection, the host range, incidence of WSSV infection and diagnostic methods for WSSV. A review of the genome, the host range and detection methods for IHHNV, MBV and HPV infection is also included. In chapter 3, materials and methods are discussed. Details of sample analysed and all the methods employed in the investigation are presented. In chapter 4, results and discussion are presented. The findings are discussed in detail. The results are discussed in tables and relevant photographs are also included.

A summary of the entire work is presented in chapter 5. A detailed *bibliography* of all the citations made in the thesis is given at the end of the thesis.

Review Of Literature

2. Review of literature

2.1 Shrimp aquaculture

Global capture production in 2006 was about 92 million tonnes which is a decrease of 2.2 million tonnes in comparison with 2005. Nearly 19 per cent of the major commercial marine fish stocks monitored by the FAO are overexploited, 8 per cent are depleted and 1 per cent is ranked as recovering from depletion. This fact, together with ever increasing population growth, has provided impetus for rapid growth in fish and shellfish farming, or aquaculture. Global aquaculture production increased from 3.9 percent of total production by weight in 1970 to 36.0 percent in 2006 and accounts for 47 percent of the world's fish food supply (FAO, 2009). Aquaculture, which is sometimes referred to as the Blue Revolution, is in many ways analogous to the Green Revolution in modern agriculture. As the Green Revolution was acclaimed as the means to end world hunger, the Blue Revolution holds the promise of increasing income and assuring the availability of affordable protein to the poor in the developing countries. When the Food and Agriculture Organisation first compiled production statistics on shrimp in 1950, production came solely from wild catches (FAO, 1995). In Asia, shrimps had for centuries been traditionally grown in low-density monocultures, in polyculture with fish, or in rotation with rice in the *bheries* of West Bengal and *pokkalis* of Kerala in India (Shiva and Karir, 1997).

Global marine shrimp production reached about 4 million metric tons in 2005 with shrimp aquaculture contributing to two third of this production. It is one of the fastest growing agricultural industries, with more than 10% average growth in the last decade (FAO, 2005). This rapid increase in cultured shrimp production was achieved by

geographical expansion and technological advancements in reproduction, captivity, larval rearing, artificial diet and intensification in grow out systems. Shrimp grow out systems around the world are very diverse. They are generally divided into extensive, semi-intensive, intensive and ultra-intensive depending on characteristics including production per unit, stocking density, type and amount of feed used, percentage of water exchange, aeration, pond size, pond shape and water depth (Fast, 1992). Culture intensity, production rate and application of advanced technologies in grow out, greatly vary between shrimp farming countries. More than fifty countries, mainly from Asia, North, Central and South America, were involved in shrimp farming in 2005. China, Thailand, Vietnam and Indonesia produced 75% of the aquaculture production (FAO, 2005).

2.2 Diseases affecting shrimp

Aquaculture is one of the world's fastest growing industry but disease outbreaks in shrimp have caused serious economic losses. In Asia, mortalities of cultured shrimp because of white spot syndrome virus (WSSV) and yellow head virus (YHV) have resulted in economic losses of about $1 billion per year since 1994 (Lightner *et al.*, 1998). In 1996 and 1997 alone the lost export revenue due to WSSV was estimated at 1 billion US dollars (Flegel, 1997; Flegel and Alday-Sanz, 1998). In 1989 there existed only six world-wide shrimp viruses and by 1996 the number rose to over twenty (Hernandez-Rodriguez *et al.*, 2001). Thus, the most important threat to the long-term sustainability of any shrimp farming economy are these epizooitcs. In the mid-1990s, it was estimated that every year, over $3 billion US dollars were lost to various diseases, stunting 40% of world-wide production (Lundin and Gustaf, 1995).

The 1980s witnessed a remarkable growth in shrimp farming, particularly in tropical regions of the world. The monoculture being practiced in aquaculture ponds

along with high stocking densities render the population very susceptible to diseases (Moore and Brand, 1993). Indian farmed shrimp production increased from about 30,000 tonnes in 1990 to around 1,15.000 tonnes during 2002–2003 (FAO, 2007). Due to the rapid expansion of shrimp aquaculture during early 1990s, the demand for postlarvae (PL) could not be met by the hatcheries that existed in the country at that time. The import of PL from other Asian countries and poor management of the broodstock, the hatcheries and also the farms led to the outbreak of White Spot Syndrome Virus (WSSV) in 1994. The virus spread very rapidly, and the economic losses caused by mortalities were estimated at over US$ 200 million during 1999–2000 (FAO, 2007).

Shrimp diseases can be divided into non infectious and infectious in origin (Lightner and Redman, 1998). 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. The physico-chemical parameters of the pond including temperature, pH of pond water, dissolved oxygen content, alkalinity etc. play an important role on the susceptibility of shrimp to pathogens.

2.2.1 Viral diseases

Viruses are considered to be the most important pathogens in shrimp. Different life stages of shrimp may be susceptible to certain viral infections causing mortality, slow growth and deformations. More than 20 viruses have been reported as pathogenic to shrimp.

Table 2.2.1. Viral pathogens of penaeid shrimp

Family	Virus
DNA virus	
Parvoviridae	Infectious hypodermal and hematopoeitic necrosis virus (IHHNV)[1] Hepatopancreatic parvovirus (HPV)[1] Spawner-isolated mortality virus (SMV)[2] Lymphoidal parvo-like virus (LPV)[1]
Baculoviridae	Baculovirus penaei (BP)[1] Monodon baculovirus (MBV)[1] Baculovirus midgut gland necrosis virus (BMNV)[1] Type C baculovirus of *P. monodon*[3] Hemocyte infecting non-occluded baculo-like virus[4]
Iridovidae	Shrimp iridovirus (IRIDO)[5,6]
Nimaviridae	White spot syndrome virus (WSSV)[7]
RNA Virus	
Picornaviridae	Taura syndrome virus (TSV)[1]
Roniviridae	Yellow head virus (YHV)[8] Gill associated virus (GAV)[7] Lymphoid organ virus (LOV)[9]
Reoviridae	Reo-like virus (REO) type II and IV[1]

Rhabdoviridae	Rhabdovirus of penaeid shrimp (RPS)[1]
Togaviridae	Lymphoid organ vacuolization virus (LOVV)[1]
Totiviridae	Infectious myonecrosis virus (IMNV)[10]
Bunyaviridae	Mourilyan virus (MOV)[11]
unclassified	Monodon slow growth syndrome (MSGS)[12]

[1] Lightner, 1996; [2] Owens *et al.*, 1998; [3] Chang *et al.*, 1993, [4] Owens, 1993; [5] Lightner and Redman, 1993; [6] Tang *et al.*, 2007; [7] Mayo, 2002; [8] Soowannayan *et al.*, 2003; [9] Spann *et al.*, 1995. [10] Tang *et al.*, 2005; [11] Cowley *et al.*, 2005; [12] Sritunyalucksana *et al.*, 2006a

2.2.2 Bacterial diseases

Bacteria involved in shrimp disease can be pathogenic or opportunistic. Under unfavorable environmental circumstances for shrimp, opportunistic bacteria may cause disease. Bacterial infections in shrimp may cause mortality, cuticular lesions, necrosis, opacity of muscle, discoloration of gills, slow growth, loose cuticle, white gut, lethargia and reduced feed uptake. Major bacterial diseases in shrimp are vibriosis, filamentous bacterial disease, necrotizing hepatopancreatitis, mycobacteriosis, chitinolytic bacterial shell disease and rickettsial infection, (Lightner, 1996, Horowitz and Horowitz, 2001; Nunan *et al.*, 2005; Goarant *et al.*, 2006; Jayasree *et al.*, 2006). Vibriosis is caused by *Vibrio alginolyticus*, *V. anguillarum*, *V. campbelli*, *V. damsela*, *V. harveyi*, *V. parahaemolyticus*, *V. penaeicida*, *V. vulnificus*, *V. nereis*, *V. tubiashi*, *V. fluvialis*, *V. splendidus*, *V. nigripulchritudo*. The causative agents of filamentous bacterial disease are *Leucothrix mucor*, *Thiothrix* sp., *Flexibacter* sp., *Cytophaga*. The pathogen responsible for necrotizing hepatopancreatitis is an alpha proteobacterium. Mycobacteriosis is due to

Mycobacterium marinum, *Mycobacterium fortuitum* and other *Mycobacterium* sp. The chitinolytic bacterial shell diseases are caused by *Benekea*, *Pseudomonas*, *Aeromonas*, *Spirillum* and rickettsial infection by *Rickettsia* like organisms. Other gram positive bacteria such as *Aerococcus*, *Arthrobacter*, *Bacillus*, *Corynebacterium*, *Lactobacillus*, *Micrococcus*, *Staphylococcus* and gram negative bacteria *Acinetobacter*, *Alcaligenes*, *Flavobacterium*, *Moraxella*, *Chromobacterium*, *Spiroplasma penaei* sp. have also been described as the cause of disease in shrimp.

2.2.3 Fungal diseases

Fungal infections cause disease mainly in larval stages of shrimp in hatcheries. The major pathogenic species are *Lagenidium callinectes*, *L. marina*, *Sirolpidium* spp., *Phythium* spp., *Leptolegnia marina*, *Haliphthoros milfordensis*, *Fusarium solani*, *F. moniliformae* and *F. incarnatum*. (Lightner, 1996; Alday and Flegel, 1999; Khoa *et al.*, 2004).

2.2.4 Parasitic diseases

The diseases called cotton shrimp, gregarine disease and black gill/ brown gill disease are caused by infections with (i) protozoa-*Zoothamnium*, *Epistylis*, *Vorticella*, *Anophrys*, *Acineta* sp., *Lagenophrys*, *Ephelota*, (ii) ciliates-*Paranophrys* spp., *Parauronema* sp., (iii) flagellates-*Leptomonas* sp., (iv) annelid worms-*Nematopsis* spp., *N. litopenaeus*, *Paraphioidina scolecoide*, *Cephalobolus litopenaeus*, *C. petiti*, *Cephaloidophoridae stenai*, (v) microsporidian-*Ameson* sp., *Agmasoma* sp., *Pleistophora* sp., *Microspridium* sp., and (vi) haplosporidian (Lightner, 1996; Alday and Flegel, 1999; Gopalakrishnan and Parida, 2005).

Overall, pathogens are divided into three categories depending on the potential impact. The viruses WSSV, YHV, GAV, LOV, TSV, IHHNV, SMV are listed in the

category 1 (C-1); the viruses BP, MBV, BMN, HPV, the bacteria - α-proteobacterium and the protozoa microsporidians and haplosporidians in category 2 (C-2) and parasites causing gregarines in category 3 (C-3). C-1 pathogens may cause catastrophic losses of more than one species of shrimp. C-2 pathogens are less but still serious, while C-3 pathogens have minimal effects (Lightner, 2005).

2.3.1 White spot syndrome virus

2.3.1.1 Spread of WSSV infection

White spot syndrome virus (WSSV) first appeared in shrimp farms of northern Taiwan in 1992, causing disease and massive mortality (Chou *et al.*, 1995). The viral agent was first isolated from an outbreak in Japan in late 1993 and within a few years this new pathogenic agent spread to several shrimp farming countries (Inouye *et al.*, 1994; Flegel, 1997). WSSV was first reported in India in 1994 along the east coast and quickly spread to the west coast and all the culture areas of the country (Mohan *et al.*, 1998; Shankar & Mohan, 1998), causing severe economic losses. At first, it was thought that different viral agents had simultaneously appeared in different regions and each virus was given a specific name: hypodermal and haematopoietic necrosis baculovirus (HHNBV), *Penaeus monodon* non-occluded baculovirus (PmNOB III), rod shaped nuclear virus of *Marsupenaeus japonicus* (RV-PJ), penaeid rod-shaped DNA virus, systemic ectodermal and mesodermal baculovirus (SEMBV) or white spot baculovirus (WSBV) (Inouye *et al.*, 1994; Chou *et al.*, 1995; Wang *et al.*,1995; Wongteerasupaya *et al.*, 1995a; Durand *et al.*, 1996; Lightner 1996; Venegas *et al.*, 2000). Later, it was recognized that a single viral agent was responsible for these reports. Eventually an informal consensus was reached to call it White Spot Syndrome Virus (WSSV). This pathogen is now recognized as the most serious for shrimp aquaculture worldwide.

2.3.1.2 Morphology of WSSV

WSSV is a bacilliform, non-occluded enveloped virus (Chou *et al.*, 1995; Wongteerasupaya *et al.*, 1995a). Intact enveloped virions range between 210 and 380 nm in length and 70–167 nm in maximum width (Chang *et al.*, 1996; Flegel & Alday-Sanz, 1998; Rajendran *et al.*, 1999). A tail-like appendage at one end of the WSSV virion is sometimes observed in negatively stained electron micrographs (Durand *et al.*, 1996). The viral envelope is 6–7 nm thick and is a lipidic, trilaminar membranous structure with two electron-transparent layers divided by an electronopaque layer (Durand *et al.*, 1997; Nadala *et al.*, 1998). The nucleocapsid is located inside the envelope and is a stacked ring structure composed of globular protein subunits of 10 nm in diameter arranged in 14–15 vertical striations located every 22 nm along the long axis, giving it a cross-hatched appearance (Nadala & Loh, 1998). When released from the envelope, the nucleocapsid increases in length indicating that it is tightly packed within the virion.

2.3.1.3 Genome and classification

The WSSV genome is a circular, double stranded DNA molecule with an A+T content of 59% homogeneously distributed. The major differences among the three sequenced genomes are two polymorphic regions of about 14 kbp, which are not virulence related. Of the three isolates, WSSV-TH is the most virulent, with 50% mortality reported in *P. monodon* within 2 days (Marks *et al.*, 2005). A study of WSSV DNA samples from the isolates from China, Thailand and America shows similarities among the three isolates; however, the virulence from the American WSSV isolate has not been compared against that of the Asiatic ones (Lo *et al.*,1999).

WSSV viral genome is one of the largest sequenced so far, being only smaller than a huge virus (mimivirus) infecting an amoeba (1,181,404 bp), a canarypox (359,853

bp), a virus from the brown alga *Ectocarpus siliculosus* (335,593 bp) and a virus from *Paramecium bursaria* (PBCV-1) (330,743 bp) (van Hulten *et al.*, 2001a; Raoult *et al.*, 2004; http://www.giantvirus.org). Sequence analysis shows that the WSSV genome contains between 531 and 684 open reading frames (ORFs) with an ATG initiation codon. Of these, 181–184 ORFs are likely to encode functional proteins with sizes between 51 and 6077 amino acids, which represent 92% of the genetic information contained in the genome (van Hulten *et al.*, 2001a; Yang *et al.*, 2001). About 21–29% of such ORFs have been shown to encode WSSV proteins or share identity with other known proteins. These proteins include enzymes involved in nucleic acid metabolism and DNA replication such as DNA polymerase, a non-specific nuclease, a small and a large subunit of ribonucleotide reductase, thymidine kinase, thymidylate kinase, a chimeric thymidine-thymidylate kinase, a thymidylate synthase, a dUTPase and two PK (Tsai *et al.*, 2000a; Tsai *et al.*, 2000b; van Hulten & Vlak, 2001; Witteveldt *et al.*, 2001; Yang *et al.*, 2001; Chen *et al.*, 2002; Liu & Yang, 2005). Other proteins with a putative function include a collagen-like protein, flagellin, a chitinase, a pupal cuticle-like protein, a cell surface flocculin, a kunitz-like proteinase inhibitor, a class 1 cytokine receptor, a sno-like peptide and a chimeric anti-apoptotic protein (van Hulten *et al.*, 2001a; Yang *et al.*, 2001; Li *et al.*, 2004; Marks, 2005). Three ORFs (151, 366 and 427 of the Thailand isolate) may encode putative proteins involved in WSSV latency (Khadijah *et al.*, 2003). WSSV also has three immediate early (IE) genes (ORFs 126, 242 and 418 of the Taiwan isolate). These genes are transcribed independently of any viral protein synthesized de novo by the host cell machinery and are directly expressed in vitro. These IE genes may be important to determine host range and also can function as regulatory trans-acting factors during infection (Liu *et al.*, 2005). Transcriptional analysis of genes coding for proteins

required in DNA replication and nucleotide metabolism are synthesized early during virus replication. Early transcribed WSSV genes in general have a TATA box 20–30 nucleotides upstream of the transcription initiation site (TIS) (A/C)TCANT (Chen *et al.*, 2002; Liu *et al.*, 2005; Marks, 2005). Structural proteins are synthesized later during infection and generally have a degenerate TIS motif (A/TNAC/G) located 25 nucleotides downstream of an A/T rich region; which is similar to the TIS motif found in arthropods (Tsai *et al.*, 2004; Marks, 2005). WSSV has an internal ribosome entry site (IRES) element. WSSV IRES efficiently co-expressed a glutathione S-transferase and a GFP protein arranged in a dicistronic mRNA in vitro (Han & Zhang, 2006).

Sequence analysis of the DNA polymerase and the organization of several ORFs known to encode WSSV structural proteins were different from those of known baculoviruses, demonstrating that WSSV is not closely related to this virus group (Nadala *et al.*, 1998; van Hulten *et al.*, 2002; Zhu *et al.*, 2005). As WSSV is a distinct new virus, it has been assigned to its own virus family Nimaviridae (van Hulten & Vlak, 2002; Vlak *et al.*, 2005).

2.3.1.4 Genetic, antigenic and virulence variability in WSSV strains

The genome of three WSSV isolates has been fully sequenced: Thailand 293 kbp (van Hulten *et al.*, 2001a), China 305 kbp (Yang *et al.*, 2001) and Taiwan 307 kbp (Lo and Kou, 2001). The nucleotide identity between these isolates is 99.3% (Marks, 2005). WSSV isolates from China (*Fenneropenaeus chinensis*), India (*P. monodon*), Thailand (*P. monodon* and *Litopenaeus vannamei*) and the USA (crayfish, *Orconectes punctimanus* from Washington and *L. vannamei* from South Carolina and Texas) were compared by dot blot hybridization using a DNA probe from a Taiwanese isolate. With this method, negative results or a very faint signal were found in some samples from

India, Thailand and Texas. This finding suggests important differences between these isolates. Further, RFLP analysis of PCR products from 10 different primer sets showed that the Texas isolate was very different from the others (Lo *et al.*, 1999).

Different regions of the WSSV genome display important sequence variations which can be used to establish the origin of a WSSV isolate and its spread within a certain area and also to differentiate isolates in the field (Wongteerasupaya *et al.*, 2003; Dieu *et al.*, 2004). Such variability may also induce false negative results when using certain PCR primers (Claydon *et al.*, 2004; Kiatpathomchai *et al.*, 2005). An unstable region of 9.6 kbp of the Chinese WSSV genome appears to undergo spontaneous deletions of different sizes depending on the host species. This observation has led to the suggestion that such deletions may play an important function in WSSV virulence (Lan *et al.*, 2002).

Several WSSV isolates from the USA (*F. setiferus* and *L. vannamei*), Panama, China (*F. chinensis* and *M. japonicus*), Indonesia (*P. monodon*), Japan (*M. japonicus*), Thailand, Malaysia, Taiwan or different isolates from India were shown to have low antigenic variability using polyclonal or monoclonal antibodies (from whole WSSV virions or raised against full or truncated recombinants of VP28) in different immunoassays such as immunodot assays, Western blot, indirect immunofluorescence (IIF), immunohistochemistry (IHC) or enzyme-linked immunosorbent assay (Nadala & Loh 2000; Poulos *et al.*, 2001; Shih *et al.*, 2001; Zhang *et al.*, 2001; Anil *et al.*, 2002)

Differences in virulence of six WSSV isolates were found in post-larvae of *L. vannamei* and juveniles of *Farfantepenaeus duorarum* inoculated per os. The Texas isolate was the most virulent while the Washington isolate (from crayfish) was the least virulent. The shrimp *F. duorarum* is known to be more resistant to WSSV infection. In

this species, cumulative mortality was 60% with the Texas isolate and 35% with the WSSV isolate from crayfish (Wang *et al.*, 1999a). Another study showed that differences in virulence and competitive fitness may be dependent on the genome size. A putative ancestral WSSV isolate (WSSV-TH-96-II) with the largest genome size recorded (312 kbp), showed a lower virulence [median lethal time (LT_{50}) = 14 days] and competitive fitness compared with another WSSV isolate (WSSV-TH) with a smaller genome size (292 kbp) (LT_{50} = 3.5 days). This study indicated that WSSV isolates with a smaller genome size may represent an advantage for virus replication (Marks, 2005).

2.3.1.5 Structural proteins

More than 40 viral proteins have been characterized in WSSV. Some non-structural proteins are probably involved in transcriptional regulation (VP9), virus proliferation (WSV021) and/or regulation of DNA replication (WSV477). At least 38 structural proteins have been located in the WSSV virion. Of these, 21 have been found in the envelope, 10 in the nucleocapsid and 5 in the tegument (a putative structure located between the envelope and nucleocapsid) (Liu *et al.*, 2006; Han *et al.*, 2007; Zhu *et al.*, 2007).

The external cover of the mature virus is called the envelope, which protects the virion from degradation during infection. The WSSV structural genes and their encoded proteins such as VP19 and VP28 have been identified and characterized (van Hulten *et al.*, 2000; Yang *et al.*, 2001; Shekhar & Ravichandran, 2007).VP28, the major envelope protein, has an important role in the infection process, and is required for the attachment of the virus to shrimp cells and its complete internalization (Yi *et al.*, 2004). The multiple glycosylation sites of VP28 are surmised to contribute in the recognition of receptors from the shrimp cell surface; however, this has not yet been demonstrated. The

development of polyclonal antibodies against VP19 and VP28 has helped in the detection of WSSV, and to stimulate the crustacean immune system (Li *et al.*, 2005).

The amino acid sequence of another structural protein, VP281, predicts a 31.5 kDa protein, which matches the ORF1050 (AF411634) from the WSSV genome encoded by vp281, which is homologous to vp292 from the same genome (Huang *et al.*, 2002). The primary structure of VP281 has been determined, and its location in the envelope elucidated by immuno gold-labelling and transmission electron microscopy (TEM). Recent studies show that VP281 binds to shrimp cell receptors and that it is used as a viral attachment protein (VAP) by WSSV (Liang *et al.*, 2005).

Another structural protein is VP26, which might be involved in virus penetration, due to its actin-binding motif, that facilitates the attachment of the virus to shrimp cell membranes. VP26 has been recently identified as a tegument protein, distinct from the envelope and nucleocapsid proteins (Xie & Yang, 2005; Tsai *et al.*, 2006).

In vivo neutralisation assays using antibodies against different structural proteins showed a significant delay of shrimp mortality, indicating that proteins such as VP28 might have an important role in virus penetration (van Hulten *et al.*, 2001b; Yi *et al.*, 2004; Wu *et al.*, 2005). Recently, a 25-kDa membrane protein from shrimp haemocytes was found to bind to recombinant VP28 or WSSV virions. This protein has high homology to the small GTP-binding protein Rab7. In vivo neutralization assays with anti-Rab7 antibody inhibited the binding of WSSV virions to the cells and significantly reduced mortality upon WSSV challenge (Sritunyalucksana *et al.*, 2006b). The role of different WSSV proteins in infection has recently been studied by RNA interference. In *L. vannamei*, long doublestranded (ds) RNA corresponding to VP19 induced a specific antiviral response that inhibited WSSV infection and significantly reduced mortality

(Robalino *et al.*, 2005). In *F. chinensis*, long ds RNA corresponding to VP28, VP281, WSSV protein kinases (PK) and an unrelated ds RNA from the green fluorescence protein (GFP) induced higher survival of WSSV-challenged shrimp. The highest survival rates were found in shrimp treated with ds RNA from VP28 and PK (Kim *et al.*, 2007). A complete inhibition of a WSSV infection in shrimp was achieved by three consecutive injections of small short interfering RNA (siRNA) against VP28 in *M. japonicus* (Xu *et al.*, 2007).

2.3.1.6 Clinical signs and pathology

Under culture conditions, many Asian and American penaeid species infected with WSSV display obvious white spots or patches of 0.5–3.0 mm in diameter embedded in the exoskeleton (Lo *et al.*, 1996a; Kasornchandra *et al.*, 1998; Wu *et al.*, 2001). The exact mechanism of white spot formation is not known. It is possible that a WSSV infection may induce the dysfunction of the integument resulting in the accumulation of calcium salts within the cuticle and giving rise to white spots (Wang *et al.*, 1999b). Other signs of disease include a reddish discolouration of body and appendages because of the expansion of chromatophores, a reduction in feed uptake, preening and response to stimulus, loose cuticle, swelling of branchiostegites because of accumulation of fluid, enlargement and yellowish discolouration of the hepatopancreas, and thinning and delayed clotting of haemolymph (Wongteerasupaya *et al.*, 1995a; Lo *et al.*, 1996b; Flegel 1997; Lightner *et al.*, 1998; Sahul-Hameed *et al.*, 1998; Otta *et al.*, 1999).

In the field, WSSV-infected shrimp gather near the pond edge and display clinical signs 1 or 2 days before the first mortalities occur. Cumulative mortality may reach 100% within 10 days after the onset of disease. In grow-out ponds, juvenile shrimp of all ages

and sizes are susceptible to the disease but massive mortality usually occurs 1 or 2 months after stocking (Karunasagar *et al.*, 1997; Lotz & Soto, 2002).

By histopathology, WSSV infection is characterized by cells with hypertrophied nuclei showing amphophilic intranuclear inclusions and marginated chromatin (Durand *et al.*, 1997; Wang *et al.*, 2000a). These intranuclear inclusions are markedly distinct and bigger than the Cowdry A-type inclusions characteristic of the infectious hypodermal and haematopoietic necrosis virus (IHHNV) (Wongteerasupaya *et al.*, 1995a). Infected nuclei become progressively more basophilic and enlarged (Chang *et al.*, 1996). In the late stages of infection, karyorrhexis and cellular disintegration may occur, leading to the formation of necrotic areas characterized by vacuolization (Karunasagar *et al.*, 1997).

2.3.1.7 Pathogenesis

The portals of WSSV entry into the shrimp have not yet been clearly identified. According to experimental data on feeding shrimp with WSSV-infected tissues, the primary sites of WSSV replication in early juvenile *P. monodon* are the subcuticular epithelial cells of the stomach and cells in the gills, in the integument and in connective tissue of the hepatopancreas as determined by in-situ hybridization (ISH) (Chang *et al.*, 1996).

Another study on *M. japonicus* indicated that epithelial cells in the midgut trunk may be a transient site of WSSV replication which would allow the virus to cross the underlying basal lamina (Di Leonardo *et al.*, 2005). In *P. monodon*, a WSSV challenge by immersion showed that haemocytes migrating to gills and midgut were WSSV-negative at late stages of infection [48–72 h post-inoculation (hpi)]. Many WSSV-positive cells were found in gills and only a few in midgut epithelium. Electron microscopy showed that epithelial cells in the midgut were VP28-positive in supranuclear

vacuoles early during infection (8 hpi), suggesting lysis of WSSV particles. VP28-positive nuclei were never seen in the epithelial cells of the midgut (Arts *et al.*, 2007). With a standardised oral inoculation procedure, the primary sites of WSSV replication as determined with IHC were the epithelial cells in the foregut, cells in the gills, and only with a high dose (10,000 SID_{50}), also cells in the antennal gland (Escobedo-Bonilla *et al.*, 2007).

The mechanism of viral spread from the primary replication sites to other target organs has been controversial. Some studies have indicated that WSSV infects haemocytes in crayfish and travels throughout the body in these cells to reach distant target organs (Wang *et al.*, 2002; Di Leonardo *et al.*, 2005). Other studies have shown by ISH, IHC and IIF that circulating haemocytes in freshwater prawns and shrimp are refractory to WSSV infection, thus indicating that WSSV might reach other target organs through haemolymph circulation in a cell-free form (van De Braak *et al.*, 2002; Shi *et al.*, 2005; Escobedo-Bonilla *et al.*, 2007). It is possible that these mechanisms of spread may be host species-dependent. WSSV targets cells of organs of ectodermal and mesodermal origin, including those of the epidermis, gills, foregut, hindgut, antennal gland, lymphoid organ, muscle, eye-stalk, heart, gonads, haematopoietic cells and cells associated with the nervous system. (Wongteerasupaya *et al.*, 1995a; Lo *et al.*, 1997; Chang *et al.*, 1998; Sahul-Hameed *et al.*, 1998; Rajendran *et al.*, 1999). In the late stages of infection, the epithelia of the stomach, gills and integument may become severely damaged (Chang *et al.*, 1996; Wang *et al.*, 1999b). This may cause multiple organ dysfunctions and probably leads to death. Molecules with important biological functions that showed variations in response to WSSV infection included those involved in energy production, nucleic acid synthesis, calcium homeostasis and/or cellular signaling. Many such molecules may be

useful as biomarkers and probably could be used to identify targets to control virus replication (Wang *et al.*, 2007).

Histopathological observations and PCR analysis of WSSV infected *Fenneropenaeus indicus* revealed the presence of intranuclear inclusion bodies in gill tissue, eyestalk, appendages and connective tissue at 36 h post-infection (p.i.) and in heart and stomach at 48 h p.i. The PCR analysis showed that hemolymph was positive for WSSV at 6 h p.i. and all other organs at 12 h p.i. (Yoganandhan *et al.*, 2003a). Nadala *et al.* (1997) conducted a time-course infectivity experiment with WSSV in *L. vannamei* to assess the Western blot technique for the detection of WSSV; their results revealed the detection of WSSV in hemolymph and gill of experimentally infected shrimp in 41 and 43 h p.i., respectively. A study carried out by Chang *et al.* (1996) using ISH revealed the detection of WSSV in the stomach, gill, cuticular epidermis and hepatopancreas at 16 h p.i.; and in the lymphoid organ, antennal gland, muscle tissue, heart, midgut and hindgut at 22 h.

Three distinct forms of white spot syndrome outbreaks associated with WSSV, among cultured penaeid shrimp *P. monodon* and *F. indicus* of India have been described by Sudha *et al.*, 1998. In a type I outbreak; acute to subacute, the tissue level severity of infection will be moderate to high, significant mortalities occur within 7–10 days, and the affected shrimp will have prominent white spots on the carapace as the principal clinical sign. In a type II outbreak; peracute, the affected shrimp display massive reddening, the tissue level severity of infection will be very high, and mass mortalities occur within 2–3 days. Type III outbreak; chronic, have low tissue level severity of infection, white spots and reddening will be absent, and the mortalities spread over a duration of 15–28 days. The peracute form is more common in juveniles while acute to subacute and chronic

forms were common in sub-adults and adults. Acute to subacute forms of the disease were observed in 60% of the outbreaks, while chronic and peracute forms were observed in 30 and 10% of the outbreaks, respectively.

2.3.1.8 Control of WSSV

The rapid and extensive spread of WSSV has been attributed to the movement of infected PL and frozen shrimps (Momoyama *et al.*, 1994, Nakano *et al.*, 1994, Nunan *et al.*, 1998b). Due to the pandemic nature of the disease, much emphasis has been given to prevention and early diagnosis of WSSV. For all these reasons screening of PL or broodstock for WSSV by PCR has been proposed to the shrimp-farming industry as a major component of disease-management strategies (Lo & Kou, 1998). Several WSSV diagnostic PCR methods have been developed for screening and early detection of the disease (Lightner, 1996; Kimura *et* al., 1996; Lo *et al.*, 1996a; Maeda *et al.*, 1998; Umesha *et al.*, 2006). At low viral loads, WSSV is latent without causing disease symptoms in the shrimps, and can only be detected by nested PCR (Lo *et al.*, 1996b, 1998; Kim *et al.*, 1998).

The screening of nauplii for WSSV was found to be an effective and practical screening strategy for commercially cultured *P. monodon* (Peng *et al.*, 2001). WSSV can be vertically transmitted from WSSV positive spawners to their offspring (Lo *et al.*, 1997, Tsai *et al.*, 1999), and screening and selecting WSSV-negative brooders markedly reduces the chances of a subsequent outbreak of WSSV (Satoh *et al.*, 1999). Hsu *et al.*, (1999) showed that most batches of eggs derived from WSSV-infected spawners were WSSV-positive and vice versa, have suggested that testing brooders for WSSV infection both before and after spawning, and testing samples of eggs for the presence of WSSV might be effective screening strategies.

The black tiger shrimp in the WSSV carrier state gives only nested PCR test results and that they may continue to do so for long periods of time without gross signs of disease. They may then convert to the patent infection state, giving a 1-step PCR positive reaction, within a few days or even hours (Peng *et al.*, 1998a, b; Lo *et al.*, 1998).

Larvae and post-larvae of *L. vannamei* (Boone) when submitted to primary challenge with IHHNV or formalin-inactivated WSSV were found to survive after secondary *per os* challenge with WSSV at post-larval stage 45 (PL45). This evidence suggests a protective role of IHHNV as an interfering virus, while protection obtained by inactivated WSSV might result from non-specific antiviral immune response (Melena *et al.*, 2006).

2.3.1.9 Host range

WSSV has a broad host range within decapod crustaceans. WSSV naturally infects all the major species of cultivated penaeid shrimp (Wongteerasupaya *et al.*, 1995a; Lo *et al.*, 1996a; Flegel, 1997; Nunan *et al.*, 1998b; Sahul-Hameed *et al.*, 1998). Both natural and experimental infections have been reported in caridean shrimp; *Exopalaemon orientalis*, *Macrobrachium rosenbergii*, *M. idella* and *M. lamerrae*, crayfish; *Cambarus clarki* and *P. leniusculus*, wild crabs; *Calappa lophos*, *Portunus sanguinolentus*, *Portunus pelagicus*, *Charybdis* sp., *Helice tridens* and *Scylla serrata*, wild lobsters; *Scyllarus arctus* and *Panulirus* sp. and planktonic copepods and pupae of an *Ephydridae* insect (Chang *et al.*, 1998; Kanchanaphum *et al.*, 1998; Peng *et al.*, 1998a; Wang *et al.*, 1998a; Chen *et al.*, 2000; Sahul-Hameed *et al.*, 2000; Corbel *et al.*, 2001; Huang *et al.*, 2001). Experimental WSSV infection has been demonstrated in freshwater crabs (*Paratelphusa hydrodomous* and *Paratelphusa pulvinata*), and *Artemia* sp. (Sahul-Hameed *et al.*, 2001, 2002). There are reports that freshwater species such as the crayfish;

Pacifastacus leniusculus and *M. rosenbergii* are also susceptible to infection (Jiravanichpaisal *et al.*, 2001; Chakraborty *et al.*, 2002).

Twenty species of Indian marine crabs were experimentally infected with WSSV, via the oral route and intramuscular injection, to determine their viral susceptibility, out of which 16 species (*Calappa philargius*, *Charybdis annulata*, *C. lucifera*, *Doclea hybrida*, *Grapsus albolineatus*, *Halimede ochtodes*, *Liagore rubronaculata*, *Lithodes maja*, *Matuta miersi*, *Paradorippe granulata*, *Parthenope prensor*, *Philyra syndactyla*, *Podophthalmus vigil*, *Portunus sanquinolentus*, *S. serrata* and *Thalamita danae*) were susceptible and 4 (*Atergatis integerrimus*, *Charybdis natator*, *Demania splendida* and *Menippe rumphii*) were refractive at 50 d post infection (p.i.). The presence of WSSV in these crabs was confirmed by PCR tests, histology and bioassay. WSSV was found in the gill, heart, eyestalks, striated muscle and cephalothoraxic tissue. The 4 WSSV-refractive species represent potential reservoirs or carriers of WSSV (Sahul-Hameed *et al.*, 2003).

Eight European marine and freshwater crustaceans were experimentally infected with diluted shrimp haemolymph infected with WSSV. High mortality rates were noted between 7 to 21 days post-infection for six crustaceans; *Liocarcinus depurator*, *Liocarcinus puber*, *Cancer pagurus*, *Astacus leptodactylus*, *Orconectes limosus*, *Palaemon adspersus* and *S. arctus* (Corbel *et al.*, 2001).

The susceptibility of *Macrobrachium idella*, *M. lamerrae* and *M. rosenbergii* to WSSV was tested by immersion challenge, oral route and intramuscular injection. Their susceptibility to WSSV was compared with that of *F. indicus* and *P. monodon*. The WSSV caused 43.3% and 53.3% mortality in *M. lamerrae* and *M. idella*, respectively, by immersion method and 53.3% and 66.7% mortality in *M. lamerrae* and *M. idella*, respectively, by oral route. This virus caused 100% mortality in *M. idella*, *M. lamerrae*,

F. indicus and *P. monodon* when the animals were injected WSSV intramuscularly. Moribund animals were screened for the presence of WSSV by western blot or histopathology. The results indicated the susceptibility of marine shrimp and freshwater prawn to this virus except *M. rosenbergii*. This virus failed to produce mortality with any of the methods of infection applied in *M. rosenbergii* (Sahul-Hameed *et al.*, 2000).

Wild-caught asymptomatic marine shrimp such as *Metapenaeus dobsoni*, *Parapenaeopsis stylifera*, *Solenocera indica* and *Squilla mantis* carry WSSV. This virus could be detected in apparently healthy marine crabs *Charybdis annulata*, *C. cruciata*, *Macrophthalmus sulcatus*, *Gelasimus marionis nitidus* and *Metopograpsus messor*. The virus could also be detected in asymptomatic *M. rosenbergii* cultured inland far away from coast (Hossain *et al.*, 2001a). The presence of the virus in wild crustaceans such as wild-caught shrimp; *M. japonicus*, *P. semisulcatus* and *P. penicillatus* and crabs; *C. feriatus*, *P. pelagicus* and *P. sanguinolentus* was reported by Lo *et al.*, 1996a. Otta *et al.*, 1999 noted that wild-caught marine crabs such as *C. cruciata* and *Matuta planipes* carried WSSV.

2.3.1.10 Incidence of WSSV infection

A study by Vaseeharan *et al.*, 2003 revealed that 34% of brooders tested from India were positive for WSSV by PCR. Magbanua *et al.*, (2000) reported nationwide screening indicated widespread occurrence of WSSV infection in the Philippines, both in hatcheries (50% postlarvae) and in growout ponds (79% of the juveniles). A study by Hossain *et al.* (2001a) revealed that the incidence of WSSV observed in brood stock in India was 50%. WSSV prevalence was also reported to be quite high in other wild decapod populations (Lo *et al.*, 1996b; Magbanua *et al.*, 2000). WSSV can be vertically transmitted from WSSV-positive spawners to their offspring (Lo *et al.*, 1996b; Lo *et al.*,

1997; Hossain *et al.*, 2001a), and screening and selecting WSSV-negative brooders markedly reduces the chances of a subsequent outbreak of WSSV. Hsu *et al.*, (1999) who showed that most batches of eggs derived from WSSV-infected spawners were WSSV-positive and vice versa, have suggested that testing of brooders for WSSV infection both before and after spawning and testing samples for the presence of WSSV might be effective screening strategies. Peng *et al.*, (2001) reported that even if a brooder has only a light infection after spawning, it might be better not to use for spawning. The presence of this virus in wild broodstock of *P. monodon* has been reported from Taiwan, Japan and India (Lo *et al.*, 1997; Itami *et al.*, 1998; Hossain *et al.*, 2001a) The incidence of the virus in wild caught crustaceans viz., shrimp (*M. japonicus*, *P. semisulcatus* and *P. pencillatus*) and crabs (*P. pelagicus* and *P. sanguinolentus*) was reported by Lo *et al.*, (1996b). Lo *et al.*, (1996a) noted that wild arthropods in shrimp culture ponds affected by WSSV water sources such as canals, creeks and estuaries near the shrimp farms in farming areas are being contaminated with WSSV. The nature of WSSV outbreaks and mortality pattern has become most unpredictable and health management effort has become arbitrary.

Hossain *et al.*, (2001b) reported that out of 42 cultured shrimp tested from Bangladesh tested for WSSV, 36 were positive for WSSV, with 18 by non-nested reaction and 18 by nested reaction. A total of 630 cultured samples consisting of 280 postlarvae collected from nine different hatcheries and 350 juvenile shrimps (40–60 day-old) collected from 18 different culture ponds from India were screened for WSSV. Of these cultured samples tested, 53% were found to be single-step PCR positive. A total of 419 samples of captured crustaceans viz., *P. monodon* brooders, *F. indicus* juveniles, *Metapenaeus* spp., crab *Scylla serrata* and *Squilla mantis* were also screened for WSSV by PCR, 23% of them were infected with WSSV (Vaseeharan *et al.*, 2003).

A maximum of 300 PL from each of the 73 batches of PL stocked at various farms in the west coast of India during September 1999 to January 2000 were tested for the presence of WSSV by 2-step nested PCR. Thirty-six (49%) of the 73 batches tested positive for WSSV either by 1-step alone (3 batches) or after 2-step nested PCR (33 batches). Sub-samples of 5 PL each or 1 PL each tested to quantify the proportion of infected PL within batches showed that WSSV prevalence was very high in 1-step PCR-positive batches and low in 2-step PCR-positive batches. The study also showed that appropriate sampling and sample size were major factors in determining the prevalence of WSSV in PL populations, underlining the need for testing large samples of PL to reduce errors from falsely negative results (Thakur *et al.*, 2002).

2.3.1.11 Variation of structural proteins among isolates

Chang *et al.*, (2002) observed C–T and C–G point mutations and attributed it to the noninfectious nature of the viral isolate due to the point mutation. A sequence deletion at hot spots in the WSSV genome, which accounted for the virulence of the isolates, has been reported (Lan *et al.*, 2002).

An Indian isolate of WSSV is very similar in external morphology to other WSSV geographical isolates (Mishra & Shekhar, 2005). At the protein level, the protein profiles by SDS-PAGE analysis revealed that the Indian WSSV isolate was very similar to the WSSV isolates from China, Thailand, South Carolina and Texas (Wang *et al.*, 2000a). At the genome level, WSSV isolates from China, India, Thailand and the US state of Texas and South Carolina was shown to have no distinctive differences by restriction analysis and Southern blot hybridization (Wang *et al.*, 2000b). However, in view of recent report of very high nucleotide similarity between the completely sequenced WSSV isolates (Marks *et al.*, 2004), the use of restriction fragment length polymorphism (RFLP) does

not seem to be appropriate to distinguish the WSSV isolates (Shekhar and Ravichandran, 2007). High variation in repetitive DNA fragments in different Thai WSSV isolates has been reported by Wongteerasupaya *et al.*, 2003. The variable loci located in some open reading frames of WSSV genome have been suggested for use in PCR based classification of WSSV isolates in epidemiological studies (Marks *et al.*, 2004).

Antigenic uniformity based on monoclonal antibodies (MAbs) produced against 28 and 18 kDa proteins have been reported for the WSSV isolates collected from the east and west coasts regions of India (Anil *et al.*, 2002). No serological differences using MAbs produced against 28 kDa WSSV protein could be detected among WSSV isolates from China, Thailand, India, Texas, South Carolina or Panama (Poulos *et al.*, 2001).

Genes encoding 5 viral structural proteins (VP15, 19, 24, 26 and 28) of nine isolates of WSSV from different geographical locations were found to be similar except the vp28 gene of the Korean isolate which has 1 base difference from that of other WSSV isolates, but this alteration did not cause a change in the amino acid sequence (Moon *et al.*, 2003). At the nucleotide level, VP19, VP28 and VP15 sequences of a Korean isolate of WSSV were, respectively, 99, 100 and 100% identical to those of China, Indonesia, Japan and the United States and the VP35 sequence was 100% identical to that of a Taiwanese isolate. The deduced amino acid sequences were 99 to 100% identical to those from other countries. In VP19, C and T in the foreign isolates were replaced by T and A in the Korean isolate at Positions 57 and 218 nt, respectively, downstream of A (+) of the VP19 start codon. The change at Position 218 nt resulted in valine in the foreign isolates being replaced by aspartate in the Korean isolate (Seok *et al.*, 2004).

The sequence of vp14, vp24 and vp26 of an Indian isolate of WSSV did not exhibit any variations in the sequences compared, vp19 gene sequence comparison

revealed major C–T and T–C point mutations at two loci each, while vp28 sequence had C–T transition in two loci. To examine whether this point mutation is also manifested at amino acid level, the genes were translated and compared. In vp19, at position 9, proline was substituted by serine, at position 15, alanine was substituted by valine, at position 66, serine was substituted by proline, and at position 96, methionine was substituted by threonine. In vp28, at position 18, alanine was substituted by cysteine, and at position 162, serine was substituted by threonine. The vp19 and vp28 at the amino acid level had a sequences identity of 96.7% and 98% with already published sequences, respectively (Sathish *et al.*, 2004). Genes encoding the major viral structural proteins VP28, VP26, VP24, VP19 and VP15 of 5 WSSV isolates collected from different shrimp species and/or geographical areas were sequenced and compared with those of 4 other WSSV isolate sequences in GenBank. For each of the viral structural protein genes compared, the nucleotide sequences were 100 to 99% identical among the 9 isolates. Hence gene probes or PCR primers based on the gene sequences of the WSSV structural proteins can be used for diagnoses and/or detection of WSSV infection (You *et al.*, 2004). Sequence comparison analysis of structural genes of an Indian isolate of WSSV with the three completely sequenced WSSV isolates indicated that the Indian isolate is similar to the Taiwanese isolate with respect to VP19 gene and similar to Chinese and Thai WSSV isolates with respect to VP24 gene. Based on these results, it is therefore difficult to suggest the proximity of Indian WSSV isolate to any of the three completely sequenced WSSV isolates of Taiwan, China and Thailand (Shekhar and Ravichandran, 2007).

Sequence of WSSV genes encoding nucleocapsid (VP26 and VP15) and envelope proteins (VP19 and VP28) of a Mexican isolate were compared that of WSSV isolates in GenBank. VP15 is highly conserved, and VP26 showed 99% homology to a Chinese

isolate. The VP28 fragment demonstrated 100% homology to the majority of the isolates analysed (UniProt accession no. Q91CB7), differing from two Indian WSSV and one Chinese WSSV isolates by two non-conserved and one conserved replacements, respectively. In contrast, VP19 of the Mexican isolate was distinguishable from almost all isolates tested, including an American strain of WSSV (US98/South Carolina, GenBank accession no. AAP14086). Although homology was found with isolates from Taiwan (GenBank accession no. AAL89341) and India (GenBank accession no. AAW67477), VP19 may have application as a genetic marker (Molina-Garza *et al.*, 2008).

2.3.1.12 Natural epidemics

Dead, infected shrimp are considered to be the source of subsequent infection for WSSV (Lotz & Soto, 2002), with the high density at which the shrimp are reared acting as a predisposing factor, resulting in high incidence and mortalities in culture sites. This is mainly due to a higher opportunity of horizontal transmission of the virus through cannibalism and the waterborne route (Wu *et al.*, 2001).

Natural epidemics of this disease have been reported throughout the world, especially in Asia. In mainland China, severe epizootics related to a WSSV infection that previously occurred in Taiwan in early 1992 have occurred every year since 1993, causing high mortalities in cultivated *F. chinensis* and resulting in great economic loss (Xu *et al.*, 2000). In Korea, massive mortalities have occurred among the penaeid shrimp *P. orientalis* since 1993, with an isolate originated from the same ancestor as the Taiwan, Thailand and China isolates (Park *et al.*, 1998; Moon *et al.*, 2003). Since 1994, WSSV has been detected in cultured *P. monodon* in peninsular Malaysia (Wang *et al.*, 1999b), while the Indian subcontinent has been affected by WSSV epizootics, with outbreaks occurring in postlarvae and cultured *P. monodon* (Selvin & Lipton, 2003; Manivannan *et*

al., 2002). In Thailand, epidemics with WSSV show a seasonal fluctuation in the percentage of individuals infected, ranging from a low percentage (0-6%) from January to May to a higher percentage (6-18%) for the rest of the year, with a peak from September to November (Withyachumnarnkul *et al.*, 2003).

In the Americas, mortalities of cultured *L. vannamei*, induced by WSSV, have occurred in Ecuador since 1999 (Rodriguez *et al.*, 2003). In Mexico, the first cases of this disease were also detected in 1999 (Galaviz-Silva *et al.*, 2004), when WSSV caused severe damage to the shrimp industries of both Central and South America (Global Aquaculture Alliance, 1999a, 1999b).

2.3.1.13 Transmission of virus

Infection of shrimp with WSSV arises from many sources; a classic mechanism of virus transfer between regions results from the movement of live shrimp or other hosts from infected to non-infected areas. Infections by WSSV have been reported in many species of cultured penaeids including: *P. monodon*, *L. vannamei*, *L. stylirostris*, *M. japonicus*, *F. chinensis*, *M. rosenbergii* and *Procambarus clarkii* (Chang *et al.*, 1998; Lightner & Redman, 1998; Lo *et al.*, 1999; Wang *et al.*, 1999a). Rapid transmission of WSSV on culture systems may occur from infected shrimp, through the water and by cannibalism of moribund shrimp (Chang *et al.*, 1996); however, the major source of infection for shrimp farms is from infected spawners and postlarvae. There are reports that show the relation between batches of infected postlarvae on a farm and the subsequent infection of a neighbour's pond and farm, leading to the general practice of batch-testing of postlarvae for WSSV using PCR before stocking. There are reports of a transovarial or vertical transmission of WSSV by infected gonads, oogonia and follicle cells in *P. monodon* ovarian tissues (Kou *et al.*, 1997; Mohan *et al.*, 1997; Lo & Kou,

1998). Hence, *P. monodon* PL are considered as a major entry route for WSSV into culture ponds (Flegel & AldaySanz, 1998, Mushiake *et al.*, 1999). Stocking of 1-step PCR-positive PL batches has also been associated with disease outbreaks and crop failure (Withyachumnarnkul, 1999).

Larvae (i.e. nauplii, protozoeae and mysis) and early postlarvae of *P. monodon* show no significant mortality after infection with WSSV by immersion and oral challenge. However, significant mortality was observed in postlarvae and juveniles (Yoganandhan *et al.*, 2003b). These results clearly indicate that susceptibility to WSSV disease increases with age of the host which was also observed by Venegas *et al.*, (1999) in *M. japonicus*. Infectivity studies carried out on *M. rosenbergii* with WSSV showed that the extent of infection was greater in larvae than in post-larvae and varied in larval, postlarval, juvenile and adult stages (Peng *et al.*, 1998a).

P. monodon populations are tolerant to WSSV at low infection levels (Withyachumnarnkul, 1999; Peng *et al.*, 2001). Tsai *et al.*, (1999) showed that *P. monodon* could be successfully cultured with light WSSV infection (nested PCR positive) for more than 13 months without any white spot syndrome disease outbreak.

Huang *et al.*, (1995a, b) reported that some zooplankton, such as copepods, were positive for WSSV by serological detection and might therefore be able to transmit the virus in shrimp ponds. Chang *et al.*, (2002) noted that Artemia were not infected with WSSV by immersion challenge and oral route. Sahul-Hameed *et al.*, (2002) observed no mortalities in the juveniles of *F. indicus* fed with Artemia exposed to WSSV by immersion challenge and oral route, whereas 100% mortality was observed in *F. indicus* fed with WSSV-infected shrimp meat. The PCR analysis was WSSV-positive for *F.*

indicus fed WSSV-infected shrimp meat, whereas it was negative for *F. indicus* fed Artemia exposed to WSSV. Histological studies agree with these observations.

Potential sources for WSSV transmission include human activities, seabirds or other animal's (insects, fish) migration, infected frozen food products, infected pond sediments, contaminated aquaculture tools or instruments and untreated infected shrimp by-products (liquid and solid wastes) from processing plants (Supamataya *et al.*, 1998; Sahul-Hameed *et al.*, 2002).

Birds, arthropods or other organisms exposed to contaminated effluent discharge from shrimp packing plants may transfer the pathogen to areas not contaminated with the virus. Polymerase chain reaction (PCR) results show that different arthropods, including copepods, and insects can act as vectors of WSSV virus (Chou *et al.*, 1996; Lo *et al.*, 1996b; Flegel 1997; Sahul-Hameed *et al.*, 2003). Furthermore, some of these arthropods, such as *P. pelagicus*, and Acetes sp., are common in shrimp culture areas and may transmit the WSSV (Supamataya *et al.*, 1998). Other vectors can enter shrimp ponds through pumped water, favouring management strategies that reduce water exchange rates or rely in closed cycles and recirculation (Sahul-Hameed *et al.*, 2003).

2.3.1.14 Diagnostic methods for WSSV

2.3.1.14.1 Field methods

There are not many field tests available for the detection of WSSV. A reverse passive latex agglutination assay (RPLA), which detects WSSV from stomach tissue homogenate, using high-density latex particles and specific polyclonal antibody after a 4-h incubation, has been proposed as a useful method for virus detection in the culture shrimp pond (Okumura *et al.*, 2004). The RPLA assay does not require biochemical expertise and all reagents can be provided as a kit. However, this method does not work

with haemolymph and gill homogenate. Other cost-effective diagnostic assays using fluoresceinated microspheres and latex beads coated with anti-WSSV serum have proved to detect the virus as early as 24 h post infection in shrimps (Sathish *et al.*, 2004).

2.3.1.14.2 Serological methods

Monoclonal antibodies (MAbs) have been produced against WSSV. A 28 kDa envelop protein of WSSV, encoded by the vp28 gene, and located on the surface of the virus particle, is involved in attachment and penetration into shrimp cells and has been extensively used for the preparation of MAbs (Liu *et al.*, 2002). These MAbs do not react with haemolymph from SPF shrimp or from shrimp infected with other virus, and do not detect any serological differences among WSSV isolates or between WSSV isolated from penaeid shrimp or from freshwater crayfish (Poulos *et al.*, 2001). These MAbs developed against WSSV are the basis of a number of detection methods. These methods have a detection limit of approximately 500 pg of the viral protein, comparing favourably with one-step PCR, and can be used to detect WSSV in symptomatic and asymptomatic shrimp (*P. monodon*). These tests are fast (3 h) and suitable for use as a field kit (Anil *et al.*, 2002). Monoclonal antibodies have also been used to develop an antigen-capture ELISA (Ac-ELISA) test, which can differentiate WSSV-infected shrimp from uninfected shrimp. The detection threshold is approximately 400 pg of purified WSSV, which is comparable in sensitivity to PCR but more sensitive than Western blot in the detection of purified virus in haemolymph and tissue homogenate samples (Liu *et al.*, 2002). Antiserum developed against the VP28 is able to neutralize WSSV infections of *P. monodon* in a concentration-dependent manner on intramuscular injection. Furthermore, the antiserum can detect WSSV in various organs such as the eyestalks, head muscle, gill tissue, heart tissue, haemolymph, tail tissue and appendages in experimentally infected *P.*

monodon and *F. indicus*, as early as 12 and 24 h post-infection. The detection threshold is 5 ng of WSSV in total haemolymph protein from WSSV infected shrimp (Yoganandhan *et al.*, 2004).

2.3.1.14.3 Polymerase chain reaction

The PCR has been used extensively for the detection of WSSV alone, or in multiplex reactions for the detection of the WSSV and other viruses such as Taura syndrome virus (TSV) (Tsai *et al.*, 2002) and Infectious hypodermal and haematopoietic necrosis virus (IHHNV) (Dhar *et al.*, 2001). Polymerase chain reaction is a sensitive and powerful method for the detection for WSSV, because it can detect as little as 5 fg of WSSV DNA (20 viral particles) in crude extracts of post larval samples, pleopods and haemolymph from larger shrimp (Kiatpathomchai *et al.*, 2001). A single-tube semi-nested PCR capable of simultaneously detecting and assessing the severity of WSSV infections in *P. monodon* was developed by Kiatpathomchai *et al.*, 2001. All this has made PCR a sensitive and specific alternative protocol for the detection of WSSV (Tapay *et al.*, 1999). A competitive PCR method for quantification of WSSV genome was developed and the presence of the WSSV genome in hemolymph was detected at 9 h post-injection, increased rapidly by 14 h post-injection, with only a small increase from 14 to 52 h post-injection (Tang and Lightner, 2000). PCR method was successfully used to find out the prevalence of dual and triple viral infections in black tiger shrimp ponds in India (Umesha *et al.*, 2006).

PCR has been used to detect the presence of WSSV in cultured and captured crustaceans (Lo *et al.*, 1996b; Otta *et al.*, 2003; Vaseeharan *et al.*, 2003), to estimate the prevalence of the virus in shrimp postlarvae at the time of stocking in shrimp farms (Thakur *et al.*, 2002; Uma *et al:* 2005), and to identify reservoirs for the virus in shrimp

ponds (Yan *et al.*, 2004). Furthermore, PCR can identify at least four geographic isolates of WSSV from both experimentally and naturally infected shrimp (Tapay *et al.*, 1999). PCR has also been used to assess the prevalence and geographic distribution of WSSV among cultured penaeid shrimp (Magbanua *et al.*, 2000).

2.3.1.14.4 Real time PCR

Real-time PCR has been successfully employed for quantification of WSSV in different shrimp species (Dhar *et al.*, 2001; Durand and Lightner, 2002; Durand *et al.*, 2003; Powell *et al.*, 2006; Sritunyalucksana *et al.*, 2006c; Jang *et al.*, 2009)

Dhar *et al.* (2001) demonstrated that SYBR Green PCR can be used as a rapid and highly sensitive detection and quantification method for shrimp viruses and that it is amenable to high-throughout assay. They observed that a linear relationship existed between the input copy number of the WSS virus template and the C_T values for the virus-specific product over 5 log10 dilutions, ranging from 2.37 X 10^5 copies (C_T =21.278 ± 0.037) to 1.48 copies (C_T =38.981 ± 0.528). Using genomic DNA as a template, SYBR Green PCR was found to be 100- to 2000-fold more sensitive than conventional PCR for the samples tested.

Durand and Lightner (2002) employed a real time PCR assay to quantify WSSV content in moribund shrimp (*L. stylirostris, P. monodon, L. vannamei*) and it was observed that WSSV levels ranged from 2.0×10^4 to 9.0×10^{10} copies μg^{-1} of total DNA (n=26). They observed 4.3×10^9 WSSV copies μg^{-1} of DNA in whole moribund post-larvae which is equivalent to 5.7×10^{10} WSSV copies g^{-1} of post-larvae. The comparison of WSSV content between different tissues showed that muscle and hepatopancreas tissues contained 10 times less virus than gills, pleopods and haemolymph.

Durand *et al.* (2003) established that quantitatively the head had a slightly higher WSSV load than did the tail using a real-time PCR. However, since the tail represents 58% of the total body weight, the total virus load on a per weight basis turns out to be similar in the head (49%) and tail (51%) of the same shrimp with acute phase WSSV infections.

Powell *et al.*, (2006) compared real-time PCR and Shrimple®, an immuno chromatographic diagnostic test kit for detection of WSSV in order to determine the range of sensitivity in which the diagnostic test kit is capable of detecting viral infection and the efficiency of the test kit when compared to the real-time PCR. The Shrimple® test kits fail to detect WSSV infection prior to 12 h post infection and demonstrate a significant reduction in detection efficiency during early onset of infection failing to detect any viral infection from 1 to 8 h p.i. compared to 100% with real-time PCR. False negative results were observed for specimens containing 4-1061 viral copies/ng genomic DNA. Faint positives were observed for specimens containing 36-1784 viral copies/ng genomic DNA. Although considerably less sensitive than real-time PCR, the Shrimple® test kits provide a useful tool for the detection of WSSV infections prior to development of gross signs of acute disease. While a faint Shrimple® band was observed in test kits for specimens measured as low as 36 viral copies/ng genomic DNA, the lowest true chromatographic positive test result was observed at 356 viral copies/ng genomic DNA. Disparity in the sensitivity of the test kit exists in that some specimens that tested negative with Shrimple® were determined to have infection levels as high as 1098 viral copies/ng genomic DNA.

Sritunyalucksana *et al.*, (2006c) demonstrated that real-time PCR could detect WSSV with certainty at dilutions of approximately 5 copies per reaction while 1000

copies were needed for a common one-step PCR method and 50 for a common single-tube nested PCR (1N-PCR) method. Of 2 two-tube nested PCR protocols tested, one required 100 and the other 1000 copies. In addition to these sensitivity tests, a triple-blind ring test was also carried out employing sets of 10 WSSV-infected DNA extracts sent to 12 commercial and public laboratories in Thailand, without specifying the PCR method to be used. Returned results included no false positives and two false negatives, the latter both from light infection vials. This translated into a test sensitivity of 97.3% and a specificity of 100%. Overall, this study confirmed the validity of PCR-based methods in Thailand for detection of WSSV in shrimp DNA extracts.

Jang *et al.*, (2009) developed a highly sensitive and specific TaqMan real-time PCR to quantify WSSV infections in wild broodstock and hatchery reared postlarvae of *F. chinensis* in South Korea. Out of 159 brooders assayed, 39 (24.5%) were negative and 120 (75.5%) were positive; 153 (96.2%) showed less than 100 copies (mean 10.2 copies), 111 (69.8%) showed less than 10 copies and only 6 individuals (3.8%) showed high infections with a range of 2.36×10^2 to 2.28×10^6 copies ng^{-1} of DNA. In 210 postlarvae, a range of 2.6 to 713.6 (with a mean of 220) copies g^{-1} of DNA was observed. The mean WSSV copy number in the postlarvae was 7.9×10^5, which was equivalent to 8.5×10^5 copies mg^{-1} of postlarvae weight.

2.3.1.14.5 Other diagnostic methods

A one-step immunochromatographic assay for detecting WSSV in shrimp was developed by Ko *et al* (2003). Other diagnostic methods for WSSV detection include in situ hybridization (Wang *et al.*, 1998b), and the mini array method, which allows one-step multiple detection of WSSV by hybridization of a PCR product onto a nylon membrane and the visualization of the hybrids by an antibody, increasing pathogen

detection considerably. The miniarray method was 10000 times more sensitive than the dot blot method and 100 times more sensitive than regular detection of PCR amplified products by electrophoresis (Quere *et al.*, 2002).

Another method is the loop-mediated isothermal amplification (LAMP), a novel, sensitive and rapid technique with a detection limit of up to 1fg, very sensitive when compared with the 10 fg detection threshold by nested PCR (Kono *et al.*, 2004).

2.3.2 Infectious hypodermal haematopoietic necrosis virus

The parvovirus, infectious hypodermal haematopoietic necrosis virus (IHHNV) first discovered in 1981 was found to cause high mortalities (up to 90%) in infected *L. stylirostris* that were being cultured in Hawaii (Lightner *et al.*, 1983a, b). The virus was found to cause acute disease in juvenile *L. stylirostris* (Bonami *et al.*, 1990). Since then, the virus has been detected in other life stages of a number of penaeids in the Americas, Oceania, east and south-east Asia (Lightner, 1996). Runt deformity syndrome (RDS) has been linked to chronic IHHNV in *L. vannamei* and *P. monodon* (Kalagayan *et al.*, 1991; Browdy *et al.*, 1993; Primavera & Quinitio, 2000). As such, OIE (2003b) has listed IHHNV in the Aquatic Animal Health Code. IHHNV infection can be transmitted vertically but does not seriously retard growth of *P. monodon* or affect fecundity of lightly infected broodstock (Withyachumnarnkul *et al.*, 2006).

2.3.2.1 IHHN symptoms in *L. stylirostris*

Symptoms of IHHNV infection in *L. stylirostris* have been described by Lightner *et al.*, (1987), Lightner, (1988) and Lightner, (1996). Gross signs are not IHHN specific, but juvenile *L. stylirostris* with acute IHHN show a marked reduction in food consumption, followed by changes in behaviour and appearance. Shrimp of this species with acute IHHN have been observed to rise slowly in culture tanks to the water surface,

there to become motionless and then to roll-over and slowly sink (ventral side up) to the tank bottom. Shrimp exhibiting this behaviour may repeat the process for several hours until they become too weak to continue, or until they are attacked and cannibalised by their healthier siblings. *L. stylirostris* at this stage of infection often have white or buff-coloured spots (which differ in appearance and location from the white spots that sometimes occur in shrimp with white spot syndrome virus infections) in the cuticular epidermis, especially at the junction of the tergal plates of the abdomen, giving such shrimp a mottled appearance. This mottling later fades in *L. stylirostris*. In *L. stylirostris* and in *P. monodon* with IHHN, moribund shrimp are often distinctly bluish in colour, with opaque abdominal musculature.

2.3.2.2 IHHN disease in *L. vannamei*

The chronic disease, runt deformity syndrome (RDS), occurs in *L. vannamei* as a result of IHHNV infection. The severity and prevalence of RDS in infected populations of juvenile or older *L. vannamei* may be related to infection during the larval or early postlarval stages (Lightner *et al.*, 1987; Lightner, 1988). RDS has also been reported in cultured stocks of *L. stylirostris*. Juvenile shrimp with RDS display bent or deformed rostrums, wrinkled antennal flagella, cuticular roughness, and other cuticular deformities. Populations of juvenile shrimp with RDS display a relatively wide distribution of sizes with many smaller than expected ('runted') shrimp. The coefficient of variation for populations with RDS is typically greater than 30% and may approach 90%, while IHHNV-free (and thus RDS-free) populations of juvenile *L. vannamei* and *L. stylirostris* usually show CVs of 10-30% (Lightner *et al.*, 1987; Castille *et al.*, 1993; Bray *et al.*, 1994).

2.3.2.3 Sequence variation

High sequence variations, particular in the open reading frame encoding non-structural protein 1, have been demonstrated amongst the various geographical IHHNV strains in samples of *P. monodon*. Up to 14% sequence divergence has been found, the highest between the Hawaii and Madagascar IHHNV isolates (Tang *et al.*, 2003). This contrasted with previous indications that IHHN parvovirus from different geographical regions showed little genetic drift and was highly stable (Nunan *et al.*, 2000, Tang & Lightner, 2002).

The sequence comparison of 14 isolates of IHHNV by Tang and Lightner, (2002) indicates that the IHHNV genome is very stable, with 99.6 to 100% similarity between the isolates. Only nucleotide substitutions were found. The percentage of substitution was higher in the putative capsid proteins region (1.3%) than in the putative non-structural proteins region (0.6%). Out of 25 substitutions found, 14 resulted in amino acid changes. There is no apparent association between clinical outcomes and particular amino acid substitutions. The results demonstrate that the sequence variation from the earliest isolates to the recent ones is very low, and there is no apparent association between the decrease in virulence of IHHNV and particular nucleotide substitutions. However, high mortalities have not been noticed in infected *L. stylirostris*. Even though this is an extremely virulent virus, individuals with asymptomatic IHHNV infections have been found among farm-raised *L. vannamei* (Lightner *et al.*, 1983a, Kalagayan *et al.*, 1991), and in wild-caught *L. stylirostris* (Pantoja *et al.*, 1999).

Nucleotide sequence variations of a 2.9 kb fragment of IHHNV isolated from samples of *P. monodon* were determined and compared with an isolate from Hawaii by Tang *et al.*, (2003). The infection characteristics of these isolates were examined by

histology, *in situ* hybridization, and laboratory challenge studies with *L. vannamei*. Isolates of IHHNV were obtained from samples collected from the SE Asian region (the Philippines, Thailand, and Taiwan). Isolates of putative IHHNV were obtained from African samples (Tanzania, Madagascar, and Mauritius). The Philippine isolate had a very high nucleotide sequence identity (99.8%) to Hawaii IHHNV. The Thailand isolate showed a slightly lower identity (96.2%). The putative IHHNV sequences collected from Tanzania and Madagascar showed greater divergence from Hawaii IHHNV, 8.2% difference for Tanzania and 14.1% difference for Madagascar. A phylogenetic analysis showed that the Philippine IHHNV clustered with IHHNV found in the western hemisphere. This supports the theory that the Philippines was the origin of IHHNV that was first detected in Hawaii. In the laboratory infection study, both the Philippine and Thailand IHHNV were passed into *L. vannamei*, and the infected shrimp did not suffer any mortalities.

2.3.2.4 Genome

IHHNV is a small, icosahedral, non-enveloped virus containing a single-stranded linear DNA genome approximately 4.1 kb in length (Bonami *et al.*, 1990, Mari *et al.*, 1993). Based on size, morphology and biochemical structure, IHHNV is considered to be a member of the family Parvoviridae (Bonami *et al.*, 1990). Nearly 100% of the IHHNV genomic sequence and its 3 large open reading frames (ORF1, 2 and 3) have been determined (Nunan *et al.*, 2000, GenBank AF218266). Among them, the ORF1 (nucleotide 816 to nucleotide 2813) encodes a 666 amino acid polypeptide, and this is predicted to be a non-structural protein based upon its degree of homology with *Aedes albopictus* densovirus and with *Aedes densonucleosis* virus (Tang and Lightner, 2001). ORF2 starts at 56 nucleotides upstream of ORF1 and overlaps with ORF1. It could

encode a 343 amino acid polypeptide, a putative nonstructural protein 2 (NS2). ORF3 also overlaps (59 nucleotides) with ORF1, and could encode a 329 amino acid polypeptide. At least 4 structural proteins, 74, 47, 39, and 37.5 kDa in size, are found in the purified virions (Bonami *et al.*, 1990).

2.3.2.5 Diagnostic methods

2.3.2.5.1 Histological method

Histological demonstration of prominent intranuclear, Cowdry type A inclusion bodies provides a provisional diagnosis of IHHNV infection. These characteristic IHHN inclusion bodies are eosinophilic and often haloed (with haematoxylin and eosin stains of tissues preserved with fixatives that contain acetic acid, such as Davidson's AFA and Bouin's solution), intranuclear inclusion bodies within chromatin-marginated, hypertrophied nuclei of cells in tissues of ectodermal (epidermis, hypodermal epithelium of fore- and hindgut, nerve cord and nerve ganglia) and mesodermal origin (haematopoietic organs, antennal gland, gonads, lymphoid organ, and connective tissue). Intranuclear inclusion bodies due to IHHNV may be easily confused with developing intranuclear inclusion bodies due to WSSV infection. *In-situ* hybridisation assay of such sections with a specific DNA probe to IHHNV provides a definitive diagnosis of IHHNV infection (Bell & Lightner, 1988; Kalagayan *et al.*, 1991).

2.3.2.5.2 Polymerase chain reaction

The PCR has been used extensively for the detection of IHHNV (Tang *et al.*, 2000; OIE 2000; OIE 2003a; Tang & Lightner, 2002; Nunan *et al.*, 2000; Tang *et al.*, 2003). Majority of published and available primers designed for PCR detection of IHHNV, including those provided by the Office International des Epizooties (OIE, 2003b), do not react with the Australian strain of IHHNV. Only the IHHNV392F/R

primers (Tang & Lightner, 2002), located in the non-structural protein 1 region of the IHHNV genome (Shike *et al.*, 2000), elicited a positive PCR for the Australian prawns.

The high nucleotide variation observed between the Australian and Hawaiian strains provides an explanation for the lack of IHHNV detection amongst Australian prawns with published IHHNV PCRs and commercially available gene probes as they are primarily designed on the basis of the Hawaiian strain (AF218266) (Krabsetsve *et al.*, 2004).

2.3.3 Monodon Baculo virus

Monodon Baculo virus (MBV) was first described in 1981 (Lightner & Redman, 1981). Several methods have been developed for diagnosis of MBV, including PCR of the polyhedrin gene (Chang *et al.*, 1993; Vickers *et al.*, 1993; Lu *et al.*, 1995). However polyhedrin gene exists in all nucleopolyhedroviruses including the shrimp viruses, BP and MBV (Couch, 1981; Lightner & Redman, 1981).

2.3.3.1 Pathogenicity and virulence

MBV infects epithelial cells of the hepatopancreas (HP) and midgut of *P. monodon* and other penaeid shrimp (Johnson & Lightner, 1988). MBV does not cause high mortality, but is believed to lead to a decrease in productivity (Fegan *et al.*, 1991; Flegel *et al.*, 1992). Virogenesis is characterized by *de novo* synthesis of virions and assembly of occlusion bodies in the nucleus (Lightner & Redman, 1981; Lightner *et al.*, 1983c).

MBV infections are characterised by the presence of prominent, spherical intranuclear occlusion bodies in affected epithelial cells of the hepatopancreas and midgut, or free within lysed cell debris in the faeces (Lightner, 1996; Bondad-Reantaso *et al.*, 2001). Although good culture practices may enhance the survival of MBV-infected

stocks, growth, crop value and performance may significantly be reduced and MBV may render the infected shrimp susceptible to other pathogens with higher mortality rates (Bower, 1996).

2.3.3.2 Incidence of infection

MBV was implicated in the collapse of the Taiwanese shrimp farming industry in 1987/88 (Lin, 1989). In India, larval mortalities in hatcheries due to MBV have been recorded by Ramasamy *et al.*, (1995), and similar mortalities have been recorded in Malaysia (Nash *et al.*, 1988). MBV has been associated with mortality of postlarvae, juveniles and brooders of black tiger shrimp in Asia (Chen *et al.*, 1989a, b). Though MBV is relatively well tolerated by *P. monodon*, it can cause mass mortalities in shrimp that are cultured at high densities (Fegan *et al.*, 1991; Fulks and Main, 1992).

Although, presently MBV is not considered a major threat to shrimp grow-out ponds, it can still cause serious mass mortalities in hatchery-reared larvae. In a histopathological and electron microscopic study, Ramasamy *et al.*, (1995) recorded 81% prevalence of MBV in *P. monodon* larvae in a hatchery in India with infected postlarvae showing 90% mortality. Using direct microscopic observation, Karunasagar *et al.*, (1998) noted an incidence of MBV ranging from 0% to 58% in different hatcheries in India.

Earlier reports on the incidence of MBV studied in Asia have been based on histological data. Hao *et al.*, (1999) noted less than 40% prevalence in wild shrimp seeds in Vietnam. MBV has been reported to occur frequently in *P. monodon* broodstock in Asia. Liao *et al.*, (1992) noted that the prevalence of MBV in female broodstock in Taiwan was only 33% in 1987, but it was 100% in 1989. Natividad and Lightner (1992) reported 85–100% prevalence of MBV in *P. monodon* postlarvae in the Philippines.

Presence of MBV need not necessarily result in disease and mortalities as this virus is well tolerated by *P. monodon* in light to moderate infections (Lightner, 1988). It has been suggested that the transmission of MBV occurs through oral route in water contaminated with MBV from fecal matter of broodstock. It has also been proposed that MBV infection of eggs/larvae can be avoided by washing the fertilised eggs in clean filtered seawater (Chen *et al.*, 1992).

2.3.3.3 Diagnosis of infection

Diagnosis of MBV infection is usually by microscopic examination of characteristic occlusion bodies produced by the virus. However, molecular methods for MBV surveillance are also available. These include an antibody-based enzyme linked immunosorbent assay (ELISA), gene probes for in situ hybridization and DNA-based PCR protocols (Vickers *et al.*, 1992; Belcher and Young, 1998; Lightner and Redman, 1998; Hsu *et al.*, 2000). Presence of MBV does not always result in disease and mortalities as this virus is well tolerated by *P. monodon* in light to moderate infections (Lightner, 1988). MBV can significantly retard larval growth and cause mortalities in *P. monodon* Fabricius and MBV-infected larvae varied more in body size than uninfected control shrimp, and were discoloured (Chang and Chen, 2008).

Typically, MBV-infected hepatopancreatic (or occasionally midgut) cells will present markedly hypertrophied nuclei with single or, more often, multiple occlusion bodies, chromatin diminution and margination. Occlusion bodies may be stained bright red with H& E stains, and intensely, but variably, with Gram's tissue stains. Brown and Brenn's histological Gram stain, although not specific for baculovirus occlusion bodies, tends to stain occlusions more intensely (either red or purple, depending on section

thickness, time of decolouration etc.) than the surrounding tissue, aiding in demonstrating their presence in low-grade infections (Lightner, 1988; Lightner & Redman, 1998).

2.3.4 Hepatopancreatic parvo virus

2.3.4.1 Occurrence

Hepatopancreatic parvo virus (HPV) in penaeid shrimp is a single-stranded DNA virus from the family *Parvoviridae* with wide occurrence and has been suggested to cause slow growth (Lightner, 1996). HPV infection was first reported in samples of penaeid shrimp species obtained from China, Kuwait, Singapore and the Philippines (Lightner & Redman, 1985) and it was subsequently suggested by Flegel *et al.*, 1995 that the virus could potentially cause economically significant losses to shrimp farmers from mortality in early phases of rearing. HPV has been isolated and characterized from *F. chinensis* (HPVchin) from Korea and from *P. monodon* (HPVmon) from Thailand (Bonami *et al.*, 1995; Sukhumsirichart *et al.*, 1999). Phromjai *et al.*, (2001) suggested that HPVchin and HPVmon (isolated from *P. monodon* cultured in Thailand) could be different varieties, based on the in situ hybridization results, their genome sizes and the sequence homology of PCR products.

2.3.4.2 Sequence variation among HPV isolates

A segment of Madagascar HPV genomic sequence (5742 nucleotides) when determined through PCR and direct sequencing and was compared to isolates from Australia, Thailand, Korea, and Tanzania, and the mean distance was determined to be 17%. The Madagascar HPV is closest to the Tanzania isolate (12%), followed by isolates from Korea (15%), Australia (17%) and Thailand (20%). Analysis of the genomic structure revealed that this HPV sequence is comprised of one partial Left open reading frame (ORF) (349 amino acids, aa) and complete Mid (578 aa) and Right (820 aa) ORFs.

The amino acid sequences of the 3 ORFs were compared among isolates. The Right ORF was found to have the highest variation with a mean distance of 24%. This was followed by the Left and Mid ORF with distances of 13 and 7%, respectively. A phylogenetic analysis based on the amino acid sequence of the Right ORF divides 7 HPV isolates into 3 well-separated groups: Korea, Thailand, and Australia. The Madagascar HPV clustered with the Korea and Tanzania isolates. These results indicate that HPV displays a high degree of genetic diversity and is distributed worldwide among populations of penaeid shrimp (Tang *et al.*, 2008).

DNA sequence analysis has revealed that there are different geographical types of HPV. Genomes of HPV from *F. chinensis* (HPVchin) in Korea and HPV from *P. monodon* (HPVmon) in Thailand differ by approximately 1 kb and their DNA sequences differ by 30% as can be seen by examining the partial sequences for HPVchin (AY008257) and HPVmon (AF456476) available at GenBank. However, both of these types and HPV from Australian penaeids (*P. merquiensis* and *P. esculentis*) give positive in situ hybridization reactions with a commercial DNA probe designed from HPVchin. Thus, positive in situ hybridization does not mean high sequence identity. On the other hand, negative in situ hybridization results as obtained using the HPVchin probe with the HPV-like virus from *M. rosenbergii* does indicate a big genomic difference. The full sequence of Thai HPV is now available at GenBank (DQ002873) (Sukhumsirichart *et al.*, 2006).

2.3.4.3 Host range

The natural host range of HPV includes *L. vannamei*, *P. esculentus*, *F. indicus* and *P. penicillatus*, and its known geographic distribution corresponds to the Indo-Pacific area (China, Korea, Taiwan, Philippines, Malaysia, Singapore, Indonesia, Thailand and

Australia), Africa (Kenya), the Middle East (Israel and Kuwait), and the Americas (Brazil, Ecuador, Mexico and Hawaii) (Lightner & Redman, 1992; Lightner, 1993).

HPV has been reported in the postlarvae and juveniles of penaeid shrimp and can cause slow growth and death (Lightner, 1996; Flegel *et al.*, 1999). However, the virus has not yet been successfully transmitted in the laboratory and information about natural modes of transmission and possible reservoir species remain unanswered (Flegel, 1997). In addition to poor growth, it could cause significant mortality in juvenile stages, particularly in cases of multiple-agent infections (Lightner *et al.*, 1992). HPV was detected in samples of hatchery reared *P. monodon* postlarvae in the Philippines, the percentage of infection ranging from 20 to 100% (Catap *et al.*, 2003).

In India, mass mortalities in shrimp larvae infected with HPV along with MBV and WSSV has been recorded (Manivannan *et al.*, 2002). HPV in apparently healthy hatchery reared larvae and in wild shrimp in India has also been reported (Umesha *et al.*, 2003; Manjanaik *et al.*, 2005). The study by Umesha *et al.*, 2006 indicated that the strains of HPV present in India may be similar to that of HPVmon isolated from *P. monodon* from Thailand but different from HPVchin isolated from *P. monodon* in China

Previously, experimental work on HPV was hampered by the lack of an experimental transmission model. However, successful experimental infections by oral challenge in post-larvae of the black tiger shrimp *P. monodon* have been reported (Catap *et al.*, 2003) and this should open the way for precise analytical tests on the effects of HPV infection in shrimp.

The prevalence of HPV in wild penaeid shrimp samples from India was studied by nested PCR. The virus could be detected in 9 out of 119 samples by non-nested PCR. However, by nested PCR 69 out of 119 samples were positive. The PCR results were

confirmed by hybridization with digoxigenin-labelled DNA probe. Shrimp species positive by non-nested PCR included *P. monodon, F. indicus* and *P. semisulcatus* and by nested PCR, *P. stylifera, M. japonicus, Metapenaeus monoceros, M. affinis, M. elegans, M. dobsoni, M. ensis* and *Solenocera choprai*. This was the first report on the prevalence of HPV in captured wild shrimp from India (Manjanaik *et al.*, 2005).

2.3.4.4 Mode of transmission

Vertical transmission of HPV from parental broodstock was suggested when it was reported that F1 progeny of *F. chinensis* developed HPV infections (Lightner, 1996). This is supported by recent reports of HPV infections in hatchery larvae in India (Manivannan *et al.*, 2002; Umesha *et al.*, 2003) but not by experience in Thailand where infections have been reported only from post-larvae moved to outdoor nurseries (Flegel *et al.*, 2004). It is possible that horizontal transmission from unknown carriers is possible.

2.3.4.5 Symptoms of infection

Although HPV can cause considerable economic loss for shrimp farmers because of retarded growth, heavy infections result in no visible inflammatory response (Flegel *et al.*, 2004).

There is negative statistical correlation between severity of HPV infection and length of the shrimp examined (Flegel *et al.*, 1999). External signs of the disease are not specific for HPV and, frequently, diseased shrimp tend to be infected by other pathogens that may mask the actual effect of HPV infection (Lightner *et al.*, 1993).

2.3.4.6 Detection methods

Diagnosis of HPV historically depended on the histological evidence by hematoxylin and eosin staining of characteristic basophilic inclusions in enlarged nuclei of tubule epithelial cells of the hepatopancreas. As with other parvoviruses, it occurs

predominantly in the rapidly dividing E-cells at the distal end of the hepatopancreas tubules. Early HPV inclusions initially appear as small eosinophilic areas adjacent to the nucleolus. These enlarge to form giant. basophilic inclusions in hypertrophied nuclei often accompanied by an adjacent nucleolus that is compressed and crescent shaped (Lightner, 1996).

Since normal histopathological methods, rapid methods or TEM are destructive processes, PCR testing of broodstock specimens and pond reared shrimp has been developed using harmless samples of small appendages or feces (Pantoja and Lightner, 2000; Sukhumsirichart *et al.*, 2002). PCR methods are useful not only for screening cultured shrimp but also for rapid and easy screening of large numbers of potential hosts and life stages as potential carriers even if lightly infected. A rapid and sensitive PCR-ELISA has also been developed for detection of HPV in *P. monodon* with a sensitivity equivalent to three viral particles (Sukhumsirichart *et al.*, 2002).

2.3.5 Multiple infections

Due to higher sensitivity limits than most classical diagnostic methods, PCR has become the preferred method for diagnosis of most shrimp viruses (Tang and Lightner, 2000).

Dual infections of MBV and WSSV have been observed in wild black tiger shrimp from several sampling sites in the Philippines using PCR. Mixed infections of WSSV and MBV were also reported in other Asian countries like Vietnam and Thailand (Hao *et al.*, 1999; Flegel *et al.*, 2004; De la Pena *et al.*, 2005).

HPV infections alone or in combination with MBV have been reported to be associated with much reduced growth rates (Flegel *et al.*, 1999). PCR assays for HPV, WSSV and IHHNV were carried out on 240 shrimps from 6 ponds in Thailand where

visible lesions were apparent for MBV only. Surprisingly, 94% of the specimens gave a positive test for at least one of the four viruses. HPV and IHHNV alone or in combination were detected at high prevalence (approximately 60%) despite the absence of visible histological lesions and were confirmed by southern blot hybridization (Flegel *et al.*, 2004).

A duplex PCR protocol was developed for the simultaneous detection of two penaeid shrimp viruses, namely, WSSV and MBV infecting *P. monodon* in Philippines. An assessment of the sensitivity of the developed duplex PCR demonstrated the detection of both the amplicons up to 0.1 femtogram (fg) of plasmid DNA containing the target sequences equivalent to 15 copies of the viral target sequence. In addition to its high specificity and sensitivity, the developed duplex PCR offers an efficient and rapid tool for screening penaeid shrimp viruses since both WSSVand MBV can be diagnosed in a single reaction (Natividad *et al.*, 2006).

Simultaneous occurrence of WSSV, MBV and HPV in hatchery-reared postlarval *P. monodon* resulted in severe mortality (Manivannan *et al.*, 2002). The prevalence of HPV, MBV and WSSV in samples of *P. monodon* postlarvae (PL10 to PL20, 10 to 20 d old postlarvae) in India was studied by PCR. Samples collected from different hatcheries, and also samples submitted by farmers from different coastal states, were analyzed. HPV was detected in 34% of the hatchery samples and 31% of the samples submitted by farmers, using a primer set designed for detection of HPV from *P. monodon* in Thailand. However, none of these samples were positive using primers designed for detection of HPV from *F. chinensis* in Korea. This indicated that HPV from India was more closely related to HPV from *P. monodon* in Thailand. MBV was detected in 64% of the samples submitted by the farmers and 71% of the hatchery samples. A total of 84% of the samples

submitted by farmers, and 91% of the hatchery samples, were found positive for WSSV. Prevalence of concurrent infections by HPV, MBV and WSSV was 27% in hatchery samples and 29% in samples submitted by farmers. Only 8% of the hatchery samples and 16% of the samples submitted by farmers were negative for all 3 viruses (Umesha *et al.*, 2003). A survey of 18 ponds during 2 crops, 7 ponds showed simultaneous presence of infection by MBV and WSSV and 10 ponds (7 in first crop and 3 in second crop) showed the presence of MBV, WSSV and HPV (Umesha *et al.*, 2006).

The simultaneous presence of MBV and WSSV in apparently healthy postlarvae of *P. monodon* from different hatcheries in India was studied by nested PCR. MBV could be detected in 54% of the samples. However, only 15% of samples were positive by non-nested reaction. WSSV could be detected in 75% of samples, 19% being positive by non-nested reaction. The results show simultaneous presence of WSSV and MBV in many samples at various degrees of infection. Only 14% of the samples analysed were negative for both viruses (Otta *et al.*, 2003).

Prevalence of WSSV ranged from 25 to 50% in the broodstock and 10–13% in PL by nested PCR while MBV prevalence ranged from 25 to 60% in the broodstock and 34 to 39% in the PL by non-nested PCR (Uma *et al.*, 2005).

104 wild caught *Litopenaeus wannurnei* broodstock, captured off the Pacific coast of Panama, were screened for IHHNV and WSSV. The results from the dot blot assays indicated the prevalence of IHHNV in 28% and WSSV in 2% of the 104 hemolymph samples tested (Nunan *et al.*, 2001).

2.3.6 Yellow Head Virus

Yellow head virus (YHV) causes disease outbreaks resulting in mass mortality in farmed *P. monodon* in Asia since the early 1990s (Boonyaratpalin et al., 1993). YHV is

closely related to gill-associated virus (GAV), which causes disease and mortalities in *P. monodon* farmed in Australia (Cowley et al., 1999; Spann et al., 1997). GAV and YHV have been classified as type species of a genus *Okavirus* in a new family *Roniviridae* (Cowley et al., 2001). The okaviruses contain a long (GAV = 26.2 kb, YHV = 26.6 kb) positive-sense, single stranded RNA genome in which the 5'-terminal 20 kb region possesses two long overlapping reading frames (ORF1a and ORF1b) (Cowley and Walker, 2002; Sittidilokratna et al., 2008). Currently, there is no data implicating the involvement of any of the other YHV genotypes in disease (Wijegoonawardane et al., 2008).

2.3.6.1 Virus morphology and structure

YHV is a rod-shaped (bacilliform) virion of about 35-60 nm x 140- 200 nm with rounded ends (Boonyaratpalin et al., 1993; Chantanachookin et al., 1993). The enveloped virions are surrounded by a fringe of spike-like projections approximately 11 nm long (Wongteerasupaya et al., 1995b). The size of enveloped, rod-shaped GAV virions is about the same as that of yellow head virus (i.e., 183-200 x 34-42 nm). The virions are usually enclosed either tightly or loosely within cytoplasmic vesicles of infected cells (Spann et al., 1997).

2.3.6.2 Morphogenesis

Like most RNA viruses, YHV and GAV replication occurs in the cytoplasm of host cells. After encapsidation of viral RNA by the nucleocapsid protein, the long threadlike nucleocapsids obtain envelopes by budding through endoplasmic reticulum membrane. Acquisition of envelopes occurs when the nucleocapsids are still in the form of long filaments. Once enveloped, the nucleocapsid filaments appear to undergo fragmentation to give rise to shorter, mature virions. Enveloped virions can remain

densely packed in membrane-bound vesicles, while others occur freely in the cell cytoplasm (Chantanachookin et al., 1993; Cowley et al., 2004a). Some vesicles fuse with the plasma membrane so that virions are deposited into intercellular spaces (Chantanachookin et al., 1993).

2.3.6.3 Infection state

Like many other virus infections, GAV and YHV infections can occur in both chronic and acute forms. Several factors are believed to play roles in the transition between the two states of infection, including virus strains, viral loads and shrimp immune responses (Flegel, 2007).

2.3.6.4 Transmission

YHV and GAV can be transmitted both horizontally and vertically. Vertical transmission of YHV and GAV may occur commonly, since covert infections of both viruses and especially GAV are common in wild shrimp. Using PCR methodology, the prevalence of GAV in lymphoid organs and gonads of grossly healthy, wild broodstock captured on the eastern coast of Australia was found to approach 100% (Walker et al., 2001).

2.3.6.5 Host range

In addition to the black tiger shrimp *Penaeus monodon*, several other crustaceans can be infected with YHV, either naturally and/or experimentally. Species that have been reported to be infected naturally by YHV include *Penaeus merguiensis*, *Metapenaeus ensis*, *Euphasia superha* (krill), and *Palaemon styliferus* (Flegel, 1997). GAV also infects several penaeid shrimp species. Apart from the host, *P. monodon* originally reported in Australia, *Penaeus esculentus*, *Penaeus japonicus* and *Penaeus merguensis* have also

been shown to be susceptible to the virus in challenge experiments, and all displayed overt signs of disease with varying mortalities rates (Spann et al., 2000).

2.3.6.6 Detection and diagnosis

YHV and GAV infections can be detected at various level of assurance by several methods including the observation of gross signs of disease, histology, immunohistochemistry and molecular techniques.

Gross signs of the disease include a pale body colour accompanied by yellowish coloration of cephalothorax resulting from abnormal yellow color of the underlying hepatopancreas and gills showing through the translucent cuticle. In experimental laboratory infections, mortality caused by GAV occurs 6-8 days post challenge and this is 4-6 days slower than that for shrimp challenged with YHV that can cause initial mortality in less than 2 days (Spann et al., 1997; Spann and Lester, 1997). Gross signs of disease are not reliable for confirmation of YHV or GAV disease outbreaks (Flegel, 2006). Outbreak confirmation would require a minimum of additional histological analysis that could confirm the pathology characteristic of each virus.

2.3.6.7 Histopathology

Methods for rapid fixation and staining of gills or sub-cuticular epithelial tissue have been described for the detection of histopathology characteristic of yellow head disease in moribund shrimp from suspected outbreak ponds (Flegel, 2006). The confirmation of YHD can be done by demonstration of karyorrhectic and pyknotic nuclei in hemocyte smears from grossly normal shrimp from the same suspected outbreak pond (Nash et al., 1995)

2.3.6.8 Molecular diagnostic techniques

Molecular methods include utilization of nucleic acid probes as well as reverse transcription (RT) polymerase chain reaction (PCR) which is the gold standard for detection of YHV, GAV and other shrimp pathogens. The first RT-PCR method used for YHV detection was reported by Wongteerasupaya (1997). Subsequently, a number of RT-PCR methods were reported for both YHV and GAV, including multiplex RT-nested PCR methods that could differentiate between YHV and GAV (Cowley et al., 2004b; Cowley et al., 1999; OIE, 2006; Wijegoonawardane et al., 2008).

2.3.7 Taura Syndrome Virus

Taura syndrome was reported as a new disease in 1992 in commercial penaeid shrimp farms located near the mouth of River Taura in the Gulf of Guayaquil, Ecuador (Jimenez, 1992). Since its discovery, this lethal shrimp disease has spread into major shrimp growing region in the Americas by mid 1996 (Lightner, 1996; Lightner *et al.*, 1997). This disease represents a serious problem in the culture of *P. vannamei* due to the high level of mortality and the economic losses (Lightner *et al.*, 1997).

TSV particles are 32 nm, non-enveloped icosahedrons with a buoyant density of 1.338 g ml–1. The genome of TSV consists of a linear, positive-sense single-stranded RNA of 10,205 nucleotides, excluding the 3' poly-A tail, and it contains two large open reading frames (ORFs). ORF 1 contains the sequence motifs for nonstructural proteins, such as helicase, protease and RNA-dependent RNA polymerase. ORF 2 contains the sequences for TSV structural proteins, including the three major capsid proteins VP1, VP2 and VP3 (55, 40, and 24 kDa, respectively). The virus replicates in the cytoplasm of host cells (Bonami *et al.*, 1997; Mari *et al.*, 1998; Robles-Sikisaka *et al.*, 2001). TSV was

listed as an unassigned species in the Family Dicistroviridae by the International Committee on Taxonomy of Viruses (Fauquet *et al.*, 2005).

The principal host species for TSV are the Pacific white shrimp, *L. vannamei*, and the Pacific blue shrimp, *P. stylirostris*. While the principal host species for TSV all belong to the penaeid subgenus *Litopenaeus*, other penaeid species can be infected with TSV by direct challenge, although disease signs do not develop. Documented natural and experimental hosts for TSV include: *P. setiferus*, *P. schmitti*, *P. monodon*, *P. chinensis*, *P. japonicus*, *P. aztecus*, *P. duorarum* and *Metapenaeus ensis* (Bondad-Reantaso *et al.*, 2001; Brock, 1997; Overstreet *et al.*, 1997). TSV has been documented in all life stages (i.e. PL, juveniles and adults) of *L. vannamei* (the most economically significant of the two principal host species) except in eggs, zygotes and larvae (Lightner, 1996).

TSV causes 3 distinct disease phases in infected shrimp. The peracute/acute phase of the disease is characterized by moribund shrimp displaying an overall pale reddish coloration caused by the expansion of the red chromatophores. Shrimp in this phase usually die during the process of moulting. If the shrimp survive through the peracute/ acute phase, the recovery phase begins. Multifocal, melanized cuticular lesions are the major distinguishing characteristics of the recovery phase (Lightner, 1996). In the chronic phase of TSV infection, infected shrimp appear and behave normally, but remain persistently infected perhaps for life. The initial pointers to the cause of TS were two commonly used banana fungicides Tilt and Calixin and the condition was thought to be toxicity syndrome (Jimenez, 1992). Later transmission electron microscopy studies of infected shrimp demonstrated the presence of putative cytoplasmic virus particles named TSV (Brock *et al.*, 1995). Rapid spread of the TSV in pond populations occurs through cannibalization of infected moribund and dead shrimp by healthy members of the same

population (Brock *et al.*, 1995, Hasson *et al.*, 1995). TSV infected shrimp display histological lesions characteristic of the disease, which are necrosis, and nuclear pyknosis of the cuticular epithelium of the general body surface, appendages, gills, mouth, esophagus, stomach and hindgut (Brock *et al.*, 1995). The lesion is characterized by the presence of inclusion bodies that give TSV lesion a "peppered" or "buckshot" appearance which is considered to be pathognomonic for the disease (Brock *et al.*, 1995, Hasson *et al.*, 1997). For the detection of TSV, gene probes (Lightner, 1996, Hasson *et al.*, 1997, Mari *et al.*, 1998) and RT-PCR (Nunan *et al.*, 1998a) have been developed.

2.3.8 Whitish muscle disease (WMD)

Whitish muscle disease of *Macrobrachium rosenbergii*, also called "whitish disease" or "white tail disease", is a disease caused by *Macrobrachium rosenbergii* nodavirus (MrNV). It was first observed in a hatchery in Guadeloupe, French West Indies (Arcier *et al.*, 1999). It can cause immense economic losses in hatcheries and farms, with mortalities often reaching 100% in post-larval stages (Sahul-Hameed *et al.*, 2004; Notomi *et al.*, 2000). The specific host is giant freshwater prawn and the affected life stages are larvae, post-larvae and early juveniles (Sahul-Hameed, 2005).

2.3.8.1 Pathogen and mode of infection

The causative agents of WMD are *M. rosenbergii* nodavirus (MrNV) and associated extra small virus (XSV). MrNV is a small icosahedral non-enveloped virus, 26 to 27 nm in diameter that has been identified in the cytoplasm of connective tissue cells. The capsid contains a single polypeptide of 43 kDa (cp-43) (Bonami *et al.*, 2005). The nodaviruses are known to contain a genome consisting of two single-stranded positive-sense RNA segments, RNA1, which encodes the viral part of the RNA-dependent RNA polymerase (RdRp) and RNA2, which encodes the capsid protein. In infected cells,

RNA3, a subgenomic transcript of RNA1, is also present (Sommerset and Nerland, 2004). Viral replication takes place in the cytoplasm of connective tissue cells of most organs and tissues. XSV is also an icosahedral virus, 15 nm in diameter, its genome consisting of a linear single-stranded positive-sense RNA coding for a capsid protein, cp-17. Because of its extremely small size and absence of gene-encoding enzymes required for replication, it has been suggested that XSV may be a satellite virus, while MrNV plays the role of a helper virus (Sahul-Hameed *et al.*, 2004). The disease outbreak occurs in post-larvae 3 weeks after desalting. Incidence of post-larval mortalities of 30-100% within two or three days of appearance of the clinical sign of opaqueness was reported (Vijayan *et al.*, 2005). Very few post-larvae presenting these signs survive and survivors seem to grow normally in grow-out ponds (Sahul-Hameed, 2005). Bacteriological examination of affected PL showed the presence of *Staphylococcus* spp. as a predominant organism, while laboratory challenge of healthy PL with this bacterial isolate did not reproduce WMD (Vijayan *et al.*, 2005).

2.3.8.2 Transmission

Both vertical and horizontal transmission of MrNV has been observed (Sahul-Hameed, 2005). Infected broodstock serve as carriers and results in diseased postlarvae.

2.3.8.3 Signs and symptoms

The clinical signs of this disease include lethargy, anorexia and opaqueness of abdominal muscle in post-larvae and adults. Whitish appearance of the tail is the prominent clinical sign, and therefore, the disease is also named as white tail disease. This milky opaqueness gradually expands on both sides (anterior and posterior) and leads to degeneration of telson and uropods in severe cases. The tissues most affected in moribund PLs/early juveniles are striated muscles of the abdomen and cephalothorax and intratubular

connective tissue of the hepatopancreas (Sahul-Hameed, 2005). Histopathological examination of the infected animals reveals highly necrotic musculature. Multifocal areas of hyaline necrosis of muscle fibres are found in the striated muscle (Sahul-Hameed *et al.*, 2005). Degenerated muscle areas show aggregations of melanized nuclei, many of which look like inclusion bodies. The clinical signs and histopathology of WMD closely resemble to the idiopathic muscle necrosis (IMN) reported in *M. rosenbergii* (Nash *et al.*, 1987).

2.3.8.4 Diagnostic methods

The presumptive method for diagnosis includes gross observation of the presence of post-larvae with milky white colour abdomen followed by mortality, histopathological study of changes characterized by pale to darkly basophilic, reticulated cytoplasmic inclusions in the connective tissue cells of most organs and tissues (pryonin methyl green staining can be used to distinguish the characteristically green-stained MrNV viral inclusions from hemocyte nuclei) and virological studies.

2.3.8.5 Reverse-transcriptase polymerase chain reaction

RT-PCR is the most sensitive of all diagnostic method to detect MrNV. This method is used to synthesis and amplifiy cDNA copies from RNA viruses. Identification of virus can be done using amplification of cDNA by specific primers (Sri Widada *et al.*, 2003; Sahul-Hameed, 2005). More recently a single-tube, duplex RT-PCR method has been developed for simultaneous detection of MrNV and XSV (Yoganandhan *et al.*, 2005).

Materials and Methods

3. Materials and methods

3.1 Materials

3.1.1 Animals

To determine the prevalence of white spot syndrome virus in farmed and wild crustaceans, the samples were collected from ten aquaculture farms, Vembanad estuary that surrounds the farms and from sea landings off Cochin, Kerala state, India. Details of the samples are given in tables 3.1.1.1, 3.1.1.2 and 3.1.1.3. The specimens were checked for the presence of symptoms like white spots on the exoskeleton and reddish discoloration of body and appendage. One pleopod from each specimen was excised and stored at -70^0C until use.

Table 3.1.1.1: Details of crustacean samples from aquaculture farms tested for presence of WSSV

Common name	Scientific name	Number of samples
Tiger prawn	*Penaeus monodon*	112
White prawn	*F. indicus*	84
Brown shrimp	*Metapenaeus dobsoni*	22
Speckled shrimp	*M. monoceros*	31
Mud crab	*Scylla serrata*	15
Mud crab	*S. tranquebarica*	18
Fresh water prawn	*M. rosenbergii*	26
	Total	308

Table 3.1.1.2: Details of crustacean samples from estuary tested for presence of WSSV

Common name	Scientific name	Number of samples
Tiger prawn	*(Penaeus monodon)*	82
White prawn	*(F. indicus)*	43
Brown shrimp	*(Metapenaeus dobsoni)*	79
Speckled shrimp	*(M.monoceros)*	54
	Total	258

Table 3.1.1.3: Details of crustacean samples from sea landings tested for presence of WSSV

Common name	Scientific name	Number of samples
Marine Shrimp	*Parapenaeopsis stylifera*	78
Brown shrimp	*Metapenaeus dobsoni*	54
Tiger prawn	*Penaeus monodon*	52
White prawn	*F. indicus*	38
King prawn	*Metapenaeus affinis*	45
Indian nylon shrimp	*Heterocarpus woodmasoni*	23
	H. gibbosus	34
	Plesionika spinipes	21
Mud crab	*Scylla serrata*	14

Mud crab	*Scylla tranquebarica*	16
Blood spotted crab	*Portunus sanguinolentus*	43
Blue swimming crab	*Portunus pelagicus*	33
Sea Crab	*Charybdis cruciata*	18
Deep sea lobster	*Puuerulus* spp	17
Scalloped Spiny lobster	*Panulirus homarus*	18
	Total	504

To compare variations in structural protein sequences of an Indian isolate of WSSV with sequences in databank, black tiger shrimp (*Penaeus monodon*) naturally infected with WSSV were obtained from a shrimp farm in Cochin, India. The samples were immediately transferred to sterile polythene bags and transported to the laboratory in ice.

To determine the prevalence of multiple viral infections in *P. monodon* postlarvae, 104 samples with about 200 postlarvae each, were collected from hatcheries in Kerala and a subsample of 50 postlarvae was taken from each sample for extraction of DNA.

To quantify the WSSV load in hatchery-reared *P. monodon* postlarvae, 119 samples were obtained from rearing tanks of four hatcheries located in Kerala, India. A minimum of about 200 postlarvae per sample were collected from each hatchery tank. The animals were transported live to the laboratory and stored at -70C until analysis.

3.1.2 Molecular Biology Reagents

Taq DNA polymerase (MBI Fermentas, Germany)

Pfu DNA polymerase (MBI Fermentas, Germany)

T4 DNA Ligase (MBI Fermentas, Germany)

dNTP (Finnymes, Finland)

Primers (IDT, USA)

Min Elute Gel purification kit (Qiagen, Germany).

InsTA clone PCR cloning kit (MBI Fermentas, USA)

SYBR Green PCR Master Mix (Invitrogen)

Triton-X 100, Sarcosyl, X-gal, IPTG, Tris-HCl, EDTA, Guanidine Hydrochloride, Phenol:Choloform isoamyl alcohol, Proteinase K (Sigma Aldrich, USA)

3.1.3 Agarose gel electrophoresis reagents

1. Agarose (Sigma Aldrich, USA)
2. Tris-Acetate EDTA buffer (Tris base 242g (Sigma Aldrich, USA), Glacial acetic acid 57.1 ml (Sigma Aldrich, USA), Ethylene diamine tetra acetate (EDTA) (Sigma Aldrich, USA)
3. Ethidium bromide (Sigma Aldrich) 10mg/ml
4. 6X gel loading buffer (Bangalore Genei, India)
5. Molecular Weight Marker (MBI Fermentas, Germany)

3.1.4 Equipments

The following equipments available in the Microbiology, Fermentation and biotechnology Division of the Central Institute of Fisheries Technology, Cochin were used in the studies

1. Refrigerated centrifuge 5804R (Eppendorf, Germany)
2. Cary100 Bio spectrophotometer (Cary Instruments, USA)
2. Horizontal gel electrophoresis system (Bangalore Genie, India)
3. PTC-150 Mini cycler (MJ Research, USA)
4. Chromo4 four colour Real-Time system (Bio-rad, USA)
5. Alpha imager 1220 gel documentation system (Alpha Innotech Corporation, USA)
6. Ultra Low Temperature freezer (Cryo Scientific, India)

3.2 Methods

3.2.1 Extraction of viral DNA

To determine the prevalence of white spot syndrome virus in farmed and wild crustaceans, fifty milligram of pleopod from animals were transferred to 1.5 ml microfuge tube and crushed well. To find out multiple viral infections in *P. monodon* postlarvae, DNA was extracted from 50 mg of *P. monodon* postlarvae. The viral DNA was extracted in both cases as described by Otta *et al.* 2003 with minor modifications. To the crushed tissue, 1 ml of guanidine hydrochloride buffer (10 mM Tris–HCl, pH 8.0, 0.1 M EDTA, pH 8.0, 6 M guanidine hydrochloride and 0.1 M sodium acetate) was added, mixed and allowed to react for 30 min. The homogenate was centrifuged at 5000 rpm for 5 min. Five hundred microliters of supernatant was transferred to a fresh microfuge tube

and 500 µl of cold ethanol was added. This was mixed few times by inverting and was subjected to centrifugation at 14,000 rpm for 20 min. The pellet obtained was washed once with 95% ethanol followed by one washing with 70% ethanol. The DNA pellet was dried in a vacuum drier and dissolved in 100 µl of sterile distilled water. Total DNA extracted from infected *P. monodon* was used as positive control.

Total DNA was extracted from pleopods of infected shrimp as described previously (Moon *et al.*, 2003) for amplification of structural protein of WSSV. Infected tissue was homogenized in TN buffer (20 mM Tris-HCl and 400 mM NaCl, pH 7.4). After centrifugation at 1700x*g* for 10 min, the supernatant was treated with proteinase K (0.2 mg/ml) and sarcosyl (1%) at 45°C for 3 h, followed by phenol/chloroform extraction and ethyl alcohol precipitation. The genomic DNA in the pellet was dissolved in TE (10 mM Tris-HCl and 1 mM EDTA, pH 7.5) and used as a template for PCR.

Extraction of total DNA from *P. monodon* postlarvae was done using a QIAmp DNA mini kit as per manufacturer's instruction for quantification of white spot syndrome virus (WSSV) in hatchery-reared postlarvae of tiger shrimp, *Penaeus monodon.*

The DNA was quantified by measuring OD260 and purity determined by $OD_{260/280}$ by using a Cary100 Bio spectrophotometer (Cary Instruments, USA).

3.2.2 Detection of WSSV in farmed and wild decapods by nested PCR

The occurrence of white spot syndrome virus in farmed and wild decapods was determined using two sets of primers. The first set of PCR was a nested PCR performed with primer pairs (IK1 and IK2, IK3 and IK4) as described by Umesha et al 2006 (Table 3.2.2). PCR reactions were carried out in 25 µl of reaction mixture that consisted of 2.5 µl of Taq Polymerase Assay Buffer, 0.5µM of each primer, 200 µM dNTP (Finnymes),

3.0 µl of template DNA and 2 units of Taq DNA polymerase (MBI Fermentas). For nested (2 step) PCR, 2.5 µl of the first step reaction mixture was added to the PCR cocktail. The PCR was performed in a thermocycler PTC-150 Mini cycler (MJ Research, USA) for 30 cycles each cycle consisting of three steps: denaturation of target DNA at 94^0C for 1 min, annealing of primers at 55^0C for 1 min and extension of primers at 72^0C for 2 min. The programme included an initial denaturation of 5 min at 94^0C and a final extension of 5 min at 72^0C. 20 µl of PCR product was mixed with 5.0 µl of loading buffer and subjected to electrophoresis in1.5% agarose gels.

Table 3.2.2:- Primers used for the detection of WSSV

Name of primer	Sequence	Size bp	Reference
IK1	5'TGG CAT GAC AAC GGC AGG AG 3'	486	Umesha *et al* 2006
IK2	5'GGC TTC TGA GAT GAG GAC GG3'		
IK3	5'TGT CAT CGC CAG CAC GTG TGC3'	310	
IK4	5'AGA GGT CGT CAG AGC CTA GTC3'		
WSSV1out	5'ATC ATG GCT GCT TCA CAG AC 3'	982	Kimura *et al* 1996
WSSV2out	5'GGC TGG AGA GGA CAA GAC AT3'		
WSSV1 in	5' TCT TCA TCA GAT GCT ACT GC3'	570	
WSSV2 in	5'TAA GGC TAT CCA GTA TCA CG3'		

A second set of primers was also used for the detection of WSSV using primers and PCR conditions as described by Kimura *et al* 1996 (Table3.2.2). Briefly, nested PCR amplifications were carried out with the second pair of primers (570F/570R) using the amplified product of the first pair (982F/982R) as a template. In both cases, PCR

amplification was carried out in a 25 µl reaction mixture containing 1.5 µl of template DNA(for the first step reaction) (c. 100 ng), 1X PCR buffer, 1.5 mM MgCl2, 0.2 µM of each primer, 200 µM of each deoxynucleotide triphosphate (Finnymes) and 2U of Taq DNA polymerase (MBI Fermentas). The PCR protocol comprised one start cycle at 94^0C for 1 min, 35 cycles at 94^0C for 0.5 min, 55^0C for 0.5 min and 72^0C for 1 min with a final extension at 72^0C for 5 min. For nested PCR, 2 µl of the one-step reaction mixture was added to the second PCR mixture. DNA from WSSV-negative postlarvae was used as negative control.

To eliminate the incidence of false reactions, a template free reagent control, a known negative control and a known positive control were run in all the reactions. The PCR amplified products were analysed in 1.5% agarose gels containing ethidium bromide at a concentration of 0.5µg/ml and photographed using gel documentation system (Alpha Imager 1220, Alpha Innotech, USA).

3.2.3 PCR Amplification of structural genes

The viral structural protein genes; VP28, VP26, VP19, VP68, VP281 and VP466 were amplified using the gene specific primer designed based on the sequences of a Taiwanese isolate (Yang et al. 2004) as given in Table 3.2.3. PCR amplification of VP28, VP26, VP68 and VP281 genes were performed in 25µl reaction volume containing 200 µM dNTPs, 1 X pfu buffer, 0.7 µM of forward and reverse gene specific primer, 2.5u of pfu DNA polymerase (MBI Fermentas, USA) and 40ng of WSSV genomic DNA. PCR was done for 30 cycles with initial denaturation of 94^0C for 3 min, denaturation at 94^0C for 30 sec; primer annealing at 57^0C for 1 min and extension at 70^0C for 3 min.

The VP 466 gene was PCR amplified in 25µl reaction volume containing 200 µM dNTPs, 1X pfu buffer, 0.7µM of forward and reverse gene specific primer, 2.5u of pfu DNA polymerase (MBI Fermentas, USA) and 40ng of WSSV genomic DNA. PCR was done for 30 cycles with initial denaturation of 94^0C for 3 min, denaturation at 94^0C for 30 sec; primer annealing at 57^0C for 1 min 30 sec and extension at 70^0C for 4 min and 30 sec.

Table 3.2.3: Primers used for amplification of the structural genes of WSSV

Sl.No	Gene	Sequence of primer
1	Vp28	**F**-5' TTC ACG AGG TTG TCA TCA CC 3' **R**-5' TGG TAT AAA TTT CCT CAA TTG TTT T3'
2	Vp26	**F**-5' TGG ATC CAA CCA ACA CGT AA 3' **R**-5'CTT GTA TTT TTA TTC AAA CAA AAC CTT 3'
3	Vp19	**F**-5' GGT GTC CTG ACA AAA ACC GTA 3' **R**-5' TTG TCC CTG ATG TTG TGT TTT C 3'
4	Vp68	**F**-5'AAC ACT TCT GGG TGA AAC CTA 3' **R**-5' TCG GAC AAA TAA AAG AAT TGG AA 3'
5	VP281	**F**-5' AGA AAC CCA AGG AAG GGT TG 3' **R**-5' TTT GTT TGC AAC ACC CTT TT 3'
6	VP466	**F**-5' TCA AGA CCA GTA CAC GTA ATT TGA T 3' **R**-5' TGA TGT CTG AGC CAT TTT TAT TAT G 3'

PCR amplification of VP19 gene was performed in 25µl reaction volume containing 200µM dNTPs, 1X pfu buffer, 0.7µM of forward and reverses gene specific

primer, 2.5u of pfu DNA polymerase (MBI Fermentas, USA) and 40ng of WSSV genomic DNA. PCR was done for 30 cycles with initial denaturation of 94^0C for 3 min, denaturation at 94^0C for 30 sec; primer annealing at 55^0C for 30 sec and extension at 70^0C for 1 min and 30 sec.

A final extension of 70^0C for 10 min was also included in all the reactions. The PCR reaction for amplification of all the structural genes were carried out in a final reaction volume of 50µl containing 0.5µM each of forward and reverse primer, 100ng of DNA, 200µM dNTP (MBI Fermentas, USA), 25mM magnesium sulphate, 1X Pfu buffer, 1.25U pfu DNA polymerase (MBI Fermentas, USA) and autoclaved Millipore water. The amplification was performed in a PTC-150 Mini cycler (MJ Research, USA). The size of the VP28, VP26, VP 68, VP 281, VP 19 and VP 466 genes were 615bp, 615bp, 207bp, 846bp, 366bp and 1401bp respectively. The PCR amplified product was analyzed on 1.5% agarose gel along with DNA molecular weight marker and documented using a gel documentation system (Alpha Imager 1220, Alpha Innotech, USA).

3.2.4 Cloning of PCR products in pTZ57R/T vector

An A overhang was added to the PCR product for cloning into TA vector. The reaction was carried at 72°C for 45 min in a reaction volume of 30µl containing 26µM dATP, 1X Taq buffer, 1.25 U Taq DNA polymerase (MBI Fermentas, USA) and the PCR product. The A overhang added PCR product was purified using Min Elute Gel purification kit (Qiagen, Germany).

InsTA clone PCR cloning kit (MBI Fermentas, USA) was used to clone the PCR product to pTZ57R/T 9vector. Ligation was set at 4°C overnight for a 30µl reaction volume using 5U of T4 DNA ligase (MBI Fermentas, USA). Competent *E.coli* JM109

cells were used as host system for transformation. The transformed cells were incubated at 37°C overnight on LB agar plates containing 40µl of 0.1M IPTG and 4µl of 20mg/ml X-gal in order to enhance blue white selection by α-complementation. Plasmids were isolated from the overnight culture by alkaline lysis method (Sambrook and Russell, 1989).

The recombinant clones having the specific insert in the forward orientation was then sequenced from both directions to get a contiguous sequence in an automated sequencer (ABI prism, model 377, Version 3.0). The nucleotide sequences obtained and the deduced amino acid sequences were analyzed using bioinformatics tools. Sequences were compared with database using BLAST (http://www.ncbi.nlm.nih.gov/BLAST) blastn and blastp (Altschul et al., 1990).

3.2.5 Polymerase Chain Reaction for detection of multiple viral infections

For detection of IHHNV two sets of primers were used (Table3.2.5). The PCR reactions were done as described in OIE Manual of Diagnosis for Aquatic Animals (2003). PCR reactions were carried out in 25µl of reaction mixture that consisted of 2.5 µl of Taq Polymerase Assay Buffer, 25 mM $MgCl_2$, 0.5µM of each primer, 200 µM dNTP (Finnymes), 2 units of Taq DNA polymerase (MBI Fermentas) and 100ng of template DNA.

The PCR was performed in a PTC-150 Mini cycler (MJ Research, USA) for 35 cycles each cycle consisting of three steps: denaturation of target DNA at 94^0C for 30 sec, annealing of primers at 55^0C for 30 sec and extension of primers at 72^0C for 1 min. The programme included an initial denaturation of 5 min at 94^0C and a final extension of 5 min at 72^0C. 20µl of PCR product was mixed with 5.0 µl of loading buffer and

Table 3.2.5: Primers used for detection IHHNV, HPV and MBV

Virus	Name	Sequence	Size (bp)	Reference
IHHNV	77112F	5'ATC GGT GCA CTA CTC GGA 3'	356	OIE, 2006
	77012R	5' TCG TAC TGG CTG TTC ATC 3'		
	389F	5' CGG AAC ACA ACC CGA CTT TA 3'	389	OIE, 2006
	389R	5' GGC CAA GAC CAA AAT ACG AA 3'		
HPV	H441F	5'GCA TTA CAA GAG CCA AGC AG 3'	441	Phromjai *et al.*, 2002
	H441R	5'ACA CTC AGC CTC TAC CTT GT 3'		
	HPVnF	5'ATA GAA CGC ATA GAA AAC GCT 3'	265	Umesha *et al.*, 2006
	HPVnR	5'CAG CGA TTC ATT CCA GCG CCA CC 3'		
	1120F	5'GGT GAT GTG GAG GAG AGA 3'	592	Pantoja and Lightner, 2000
	1120R	5'GTA ACT ATC GCC GCC AAC 3'		
MBV	MBV14F	5' CGA TTC CAT ATC GGC CGA ATA 3'	533	
	MBV14R	5' TTG GCA TGC ACT CCC TGA GAT 3'		
	MBV14NF	5' TCC AAT CGC GTC TGC GAT ACT 3'	361	Belcher and Young, 1998
	MBV14NR	5'CGC TAA ATG GGG CAC AAG TCT C 3'		

subjected to electrophoresis in1.5% agarose gels. Ethidium bromide was added at a concentration of 0.5µg/ml. The gels were observed and photographed using gel documentation system (Alpha Imager 1220, Alpha Innotech, USA). In the event that results were ambiguous using the 389F/R 'universal' primer set, a second reaction with primers 77012/77353 was used for confirmatory testing using the same reaction conditions.

For HPV detection, two sets of primers were employed. The first set utilizes a nested PCR with primers (H441F and H441R) for first PCR and primers (HPVnF and HPVnR) for nested PCR (Table 3.2.5). The PCR protocol for the first PCR was done as described by Phromjai *et al.*, 2002 and the nested PCR as described by Umesha *et al.*, 2006. PCR reagent for the first PCR included 1X PCR buffer with 1.5 mM of $MgCl_2$, 200 µM each of dATP, dTTP, dCTP and dGTP (Finnzymes), 0.2 µM each of primer H441F and H441R, 1.25 unit of *Taq* DNA polymerase (MBI Fermentas,USA), 1 to 10 ng DNA template and double distilled water to adjust the final volume to 50 µl before PCR with 1 cycle of 95°C for 5 min, and 35 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 1 min and a final extension at 72°C for 7 min. One microlitre of the reaction mixture from the first PCR was used as template for the nested step. The cycling conditions for the nested reaction comprised initial heating at 94 °C for 5 min followed by 30 cycles at 94 °C for 30 s, 55°C for 30 s and 72 °C for 30 s with a final extension at 72 °C for 5 min.

The second set utilized the PCR protocol and primers (1120F and 1120R) as described by Pantoja and Lightner (2000). A pair of PCR primers, 1120F and 1120R (Table 3.2.5), was designated from sequence information previously generated from a

HPV clone (clone HPV8; 2.136 kbp insert) constructed with purified DNA from a Korean isolate of HPV. Optimized conditions for the reaction are: primers (50 pmol each), dNTPs (200 µM each), Taq polymerase (1.25 U) (MBI Fermentas, USA), $MgCl_2$ (4.0 mM), in PCR buffer. A master mix was prepared by including all of the reagents, except the template to be amplified, and was dispensed into PCR tubes in portions of 25µl. The reaction mixture with the template was subjected to 40 cycles in a thermocycler (PT100 MJ Research, US) according to the following protocol: denaturation at 94°C for 1 min annealing at 60°C for 1 min; and polymerization at 72°C for 2 min. The second step of the nested PCR is accomplished with 0.5 µl of the primary PCR reaction used as template with the internal primers.

MBV was detected as described by Belcher and Young (1998). A total of 100 ng of extracted DNA was used as template. Each reaction tube contained 50 mM KCl, 10 mM Tris/HCl, pH 9, 0.1% Triton X-100, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 0.25 µM of each MBV14F and MBV14R, 2.5 U of *Taq*, and made up to a final volume of 50 µl. The conditions for the first round of amplification are: one cycle of 96°C for 5 minutes; 40 cycles of 94°C for 30 seconds, 65°C for 30 seconds, 72°C for 60 seconds; and one cycle of 72°C for 7 minutes. The second round of amplification reaction contains 50 mM KCl, 10 mM Tris/HCl, pH 9.0, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.25 µM of each of the primers MBV1.4NF and MBV1.4NR, and 2.5 U of Taq, and made up to a final volume of 50 µl. The conditions for the second round of amplification are: one cycle of 96°C for 5 minutes; 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 60 seconds; and one cycle of 72°C for 7 minutes. 20 µl of PCR product was mixed with 5.0 µl of loading buffer and subjected to electrophoresis

in1.5% agarose gels. Ethidium bromide was added at a concentration of 0.5 µg/ml. The gels were observed and photographed using gel documentation system (Alpha Imager 1220, Alpha Innotech, USA.

For amplification of WSSV, nested PCR were done with primers (IK1 and IK2, IK3 and IK4) as described by Umesha et al., (2006) in section 3.2.2. A second set of primers was also used for the detection of WSSV using primers and PCR conditions as described by Kimura et al., (1996) as in section 3.2.2. The amplified PCR products were analyzed in 1.5% agarose gel containing ethidium bromide at a concentration of 0.5µg/ml and analyzed using a gel documentation system (Alpha Imager, 1220, Alpha Innotech). A 100bp DNA ladder was included in the gel as the molecular weight marker.

3.2.6 Real-time SYBR Green PCR primer

The primers for SYBR Green real time PCR were designed using Primer 3 software based on nucleotitide sequence of ORF421 retrieved from GeneBank accession No. NC003225 (Yang *et al.*, 2001). Primers WSTIF 5'-GTA CCA CCT TTG GCG CAC-3' and WSTIR 5'-CGT GCA CGT ACA TGT CGA A-3' produced a fragment of 65bp after amplification.

3.2.7 Plasmid standard for quantification by SYBR Green PCR

DNA fragment of 65bp containing the forward and reverse primer was ligated in pGEM-T vector and cloned into *E. coli* JM109 vector. The recombinant clone containing the insert, designated as SGWSS1 was confirmed by sequencing. The copy number of the plasmid containing the 65-bp insert was determined and dilutions ranging from $2.35x10^7$ to $1.47x10^0$ viral copies μl^{-1} were made to use as standards for quantification.

3.2.8 SYBR Green PCR for quantification of WSSV

SYBR Green PCR amplifications were performed in a Chromo4 real time PCR (Biorad). The exact copy number of WSSV DNA in the DNA sample was determined by SYBR real time PCR in a reaction mixture containing SYBR Green PCR Master Mix (Invitrogen), 0.24μM of each WSTIF and WSTIR primer, 100 ng of total DNA. The thermal profile for all SYBR Green PCRs was 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 10 s and 60°C for 1 min. All reactions were repeated at least three times independently to ensure the reproducibility of the results.

3.4.9 Melting curve analysis of the PCR product

Specificity of the SYBR green PCR was determined by melting curve analysis of the PCR product. After SYBR Green PCR amplification, samples were heated to 95^0C and then brought down to 65^0C for 15s. The samples were then heated to 95^0C at a transition rate of 0.1^0C with constant monitoring of the change in fluorescence.

3.4.10 Analysis of data

After PCR cycle, data analysis was performed using the Chromo4 real time PCR system (Biorad, USA). A sample is considered positive when fluorescence exceeds an arbitrary threshold value. The cycle at which the fluorescence crosses the threshold value is considered the threshold cycle (C_T). The C_T values were exported into a Microsoft Excel Worksheet for further statistical analysis. A standard curve is prepared by plotting log of initial target copy numbers for a set of standards (dilutions of the recombinant clone carrying the insert) versus the C_T value. The viral copy number was calculated on mg tissue basis and on an individual basis. The amount of WSSV copies in the unknown samples were determined by extrapolating the C_T value in the standard curve to the viral copy number.

Results and Discussion

4. Results and discussion

4.1 Nested PCR method to determine incidence of white spot syndrome virus in farmed and wild crustaceans

4.1.1 Nested PCR for detection of WSSV

A nested PCR method as described by Umesha *et* al., 2006 was employed in this study for the detection of WSSV in farmed and wild crustaceans. Nested PCR method uses two sets of amplification primers. The target DNA sequence of one set of primers (termed "inner" primers) is located within the target sequence of the second set of primers (termed "outer" primers). In practice, a standard PCR reaction is first run with the template DNA using the "outer primers". Then a second PCR reaction is run with the "inner primers" using the product of the first reaction as the amplification target. This procedure increases the sensitivity of the assay by reamplifying the product of the first reaction in a second reaction. The specificity of the assay is increased because the inner primers amplify only if the first PCR reaction yielded a specific product. The nested PCR employed for the study uses an inner primer set; IK1 and IK2 and an outer primer set; IK3 and IK4. The inner primer set; IK1 and IK2 consistently yielded a PCR product of 486bp (fig.1) by first step PCR and outer primer set; IK3 and IK4 gave PCR product of 310bp (Fig.2) as anticipated. A second set of primers as described by Kimura *et al.*, 1996 was also used for detection of WSSV. The second primer set yielded PCR product of size 982bp and 570bp by first step PCR and nested PCR (Fig.3 and Fig.4) respectively. Lo *et al.* (1996a) noted that the sensitivity of the PCR assay could be increased 10^3 to 10^4 times

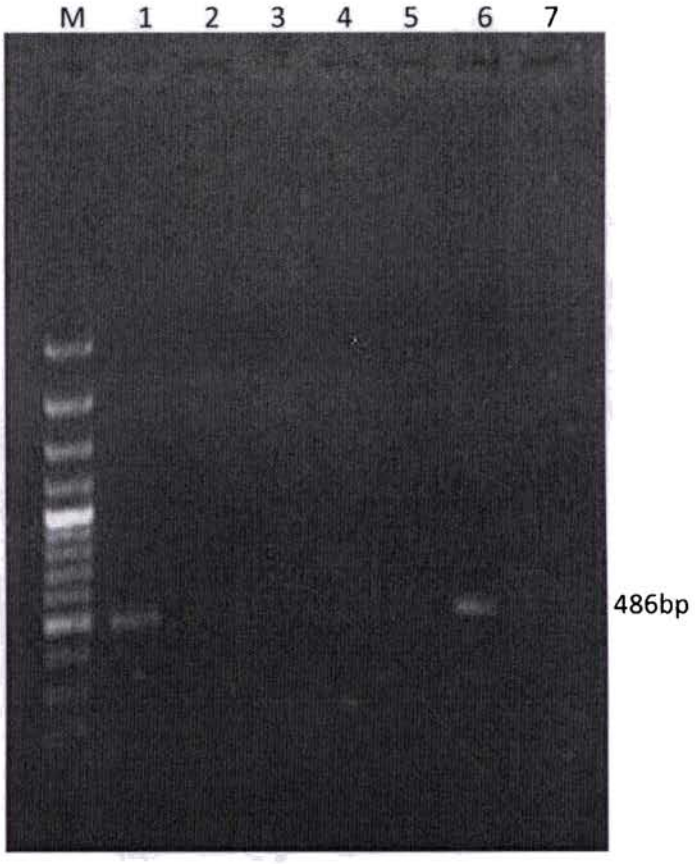

Fig1: First step PCR amplification for detection of WSSV in shrimp samples using IK1 and IK2 primers

Lane M; 100bp molecular weight marker, Lane 1: DNA template of WSSV infected *F. indicus* sample from an aquaculture pond. Lane6: DNA template of *P. monodon* sample from estuary. Lanes 2, 3, 4, 5 and 7: shrimp samples that are negative for WSSV by first step PCR. The size of the PCR product is 486 bp

Fig 2: Nested PCR amplification for detection of WSSV using IK3 and IK4 primers

Lane M: 100 bp Molecular weight marker; Lanes 1, 3, 4, 5 and 6: Shrimp samples from sea landings negative by nested PCR; Lane 2: DNA template of *M. dobsoni* sample from sea landing; Lane7: DNA template of *P. monodon* sample from estuary. The size of the nested PCR product is 310 bp

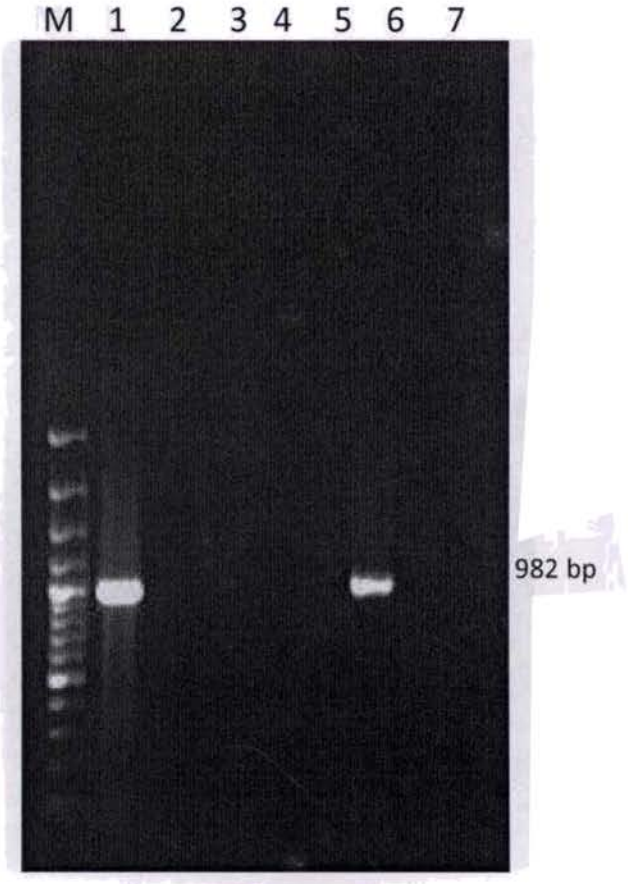

Fig3: First step PCR using WSSV1out and WSSV2out primers for detection of WSSV
Lane M; 100bp molecular weight marker, Lane 1: DNA template of WSSV infected *F. indicus* sample from an aquaculture pond. Lane6: DNA template from *P. monodon* sample from estuary. Lanes 2, 3, 4, 5 and 7: shrimp samples negative for WSSV by first step PCR. The size of the nested PCR product is 982 bp

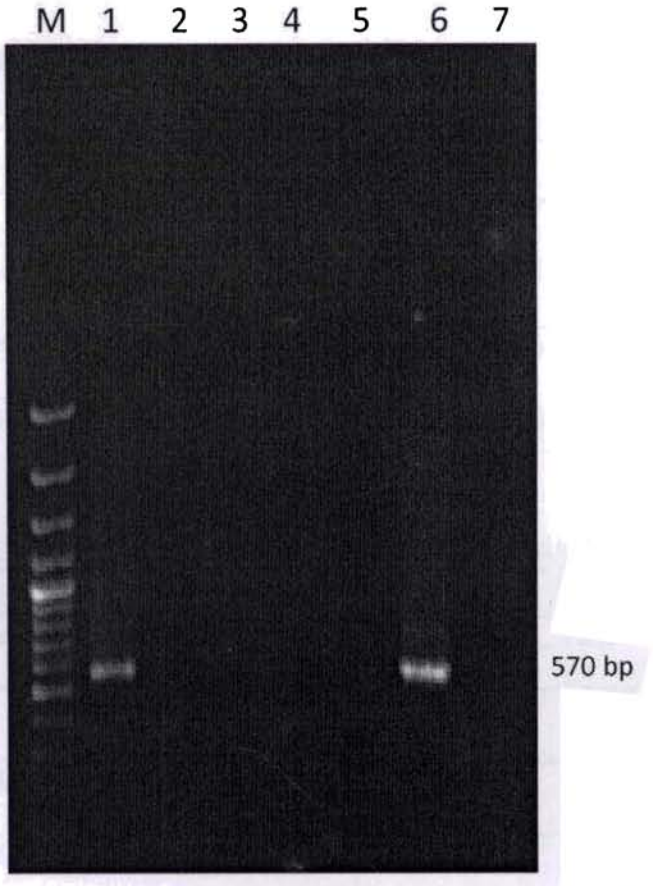

Fig4: Nested PCR using WSSV1in and WSSV2in primers for detection of WSSV
Lane M; 100bp molecular weight marker, Lane 1: DNA template of WSSV infected *F. indicus* sample from an aquaculture pond. Lane6: DNA template from *P. monodon* sample from estuary. Lanes 2, 3, 4, 5 and 7: Shrimp samples negative for WSSV by nested PCR. The size of the nested PCR product is 570 bp

by using a nested PCR wherein a second amplification step is employed using nested primers.

4.1.2 Detection of WSSV in shrimps and other decapods from farm environment

The list of crustaceans collected for presence of WSSV from aquaculture farms in and around Cochin are given in Tables 3.1.1.1 under the materials and methods section (Chapter 3). A total of three hundred and eight samples from farmed shrimp and other decapods from farm environment were analysed for presence of WSSV. The prevalence of WSSV by one step PCR and nested PCR are presented in Table 4.1.2.

Table 4.1.2:- Prevalence of WSSV in farmed shrimp and other decapods in aquaculture ponds

Common name	Scientific name	One step PCR	*Nested* PCR
Tiger prawn	*Penaeus monodon*	54/112(48)*	88/112(79)*
White prawn	*F. indicus*	29/84(35)	46/84(55)
Brown shrimp	*Metapenaeus dobsoni*	8/22(36)	15/22(68)
Speckled shrimp	*M. monoceros*	13/31(42)	22/31(71)
Mud crab	*Scylla serrata*	2/15(13)	5/15(33)
Mud crab	*S. tranquebarica*	3/18(17)	7/18(39)
Fresh water prawn	*M. rosenbergii*	9/26(35)	24/26(92)
	Total	118/308 (38)	207/308 (67)

** Figures indicate the number of animals positive for WSSV to number of animals tested.*
Figures in parenthesis indicate the percentage of animals tested positive for the virus.

The incidence of WSSV ranged from 33% in *Scylla serrata to* 92% in *M. rosenbergii* by nested PCR. In this study, the prevalence of WSSV in farmed *P. mondon* was 48% (54/112 samples) by first step PCR while it was 79% (88/112 samples) by

nested PCR. The incidence of WSSV was 35% (29/84 samples) by first step PCR and 55% (46/84 samples) by nested PCR in *F. indicus*. Brown shrimp (*M. dobsoni*) had WSSV prevalence rates of 36% (8/22 samples) by first step PCR while it was 68% (15/22 samples) by nested PCR. The prevalence of WSSV in *M. monoceros* was 42% (13/31 samples) by first step PCR while it was 71% (22/31 samples) by nested PCR. WSSV was present in 2/15 (13%) *S. serrata* samples by first step PCR while it was 5/15 (33%) by nested PCR. The incidence of WSSV was 17% (3/18 samples) and 39% (7/18 samples) in *S. tranquebarica* by first step PCR and nested PCR respectively. The fresh water prawn, *Macrobrachium rosebergii* had WSSV prevalence of 35% (9/26 samples) by first step PCR and 92% (24/26 samples) by nested PCR. The sensitivity of PCR improved considerably by nested PCR compared to the first step PCR. Vaseeharan *et al.* (2003) had screened 350 juvenile shrimps (40–60 day-old) collected from 18 different culture ponds from India for WSSV and it was found that 53% of the samples were single-step PCR positive. While in this study 38% of the samples were positive by one step PCR and 67% of the samples were positive by nested PCR. The cultured species; *P. monodon* and *F. indicus* had WSSV incidence levels of 79% and 55% respectively by nested PCR. Few samples from all the species screened had symptoms of the disease like white spots on the carapace and/or reddish discoloration of the body. Magbanua *et al.* (2000) reported that nationwide screening in the Philippines indicated widespread occurrence of WSSV infection, both in hatcheries (50% postlarvae) and in growout ponds (79% of the juveniles). Huang *et al.* (1995a, b) reported that some zooplankton, such as copepods, were positive for WSSV by serological detection and might therefore be able to transmit the virus in shrimp ponds. Sahul-Hameed *et al.* (2002) has reported that no mortalities

were observed in the juveniles of *P. indicus* fed with Artemia exposed to WSSV by immersion challenge and oral route, whereas 100% mortality was observed in *P. indicus* fed with WSSV-infected shrimp meat. They further reported that on PCR analysis, *F. indicus* fed with WSSV-infected shrimp meat was WSSV positive, whereas it was negative for *P. indicus* fed Artemia exposed to WSSV. It was further reported by them that histological studies agree with these observations.

Animals with symptoms of WSSV infection gave a PCR product in the first step itself. The fresh water prawns, *Macrobrachium rosenbergii* tested from an aquaculture pond had white spots on the carapace. These prawns had reached marketable size and did not show any mortality even after two weeks of the appearance of the symptoms. This could be because the freshwater prawns are more resistant to WSSV infection when compared with the marine shrimps. Similar results were also reported by Sahul-Hameed *et al.*, 2000 were they found that *M. rosenbergii* was resistant to WSSV infection.

There is evidence of WSSV infection in the reproductive tissue of male and female *P. monodon* broodstock. The postlarvae (PL) from infected *P. monodon* broodstock have been found to be infected with WSSV (Lo *et al.*, 1997; Mohan *et al.*, 1997; Lo & Kou, 1998). Hence, *P. monodon* PL are considered as a major entry route for WSSV into culture ponds (Flegel & Alday-Sanz 1998, Mushiake *et al.*, 1999). Stocking of 1-step PCR-positive PL batches has also been associated with disease outbreaks and crop failure (Withyachumnarnkul, 1999).

Peng *et al.* (1998b) revealed that black tiger shrimp, *P. monodon* in the WSSV carrier state gives positive results by nested PCR and that they may continue to do so for long periods of time without gross signs of disease (Lo *et al.*, 1998). They may then

convert to the patent infection state, giving a 1-step PCR positive reaction, within a few days or even hours (Peng *et al.*, 1998a, b; Lo *et al.*, 1998). However Tsai *et al.* (1999) showed that *P. monodon* could be successfully cultured with light WSSV infection (nested PCR positive) for more than 13 months without any white spot syndrome disease outbreak.

4.1.3 Detection of WSSV in shrimps from Vembanad estuary

A total of 258 samples from four species of shrimp; Tiger prawn (*Penaeus monodon)*- 82 Nos, White prawn (*F. indicus)*- 43 Nos, Brown shrimp (*Metapenaeus dobsoni)*- 79 Nos and Speckled shrimp (*M. monoceros)*- 54 Nos collected from the Vembanad estuary (Kerala State) were analysed for the presence of WSSV. The results are presented in Table 4.1.3

Table 4.1.3: - Prevalence of WSSV in shrimps from Vembanad estuary

Common name	Scientific name	One step PCR	*Nested* PCR
Tiger prawn	(*Penaeus monodon)*	36/82(44)*	59/82(72)*
White prawn	(*F. indicus)*	11/43(26)	19/43(44)
Brown shrimp	(*Metapenaeus dobsoni)*	25/79(32)	46/79(58)
Speckled shrimp	(*M. monoceros)*	21/54(39)	36/54(67)
	Total	93/258 (36%)	160/258 (62%)

**Figures indicate the number of animals positive for WSSV to number of animals tested.*
Figures in parenthesis indicate the percentage of animals tested positive for the virus.

The four species of shrimp tested had WSSV prevalence rates ranging from 44% to 72%. The incidence of WSSV is lowest in White prawn (*F. indicus)* at 44%. The proportion of shrimps infected by WSSV in estuarine waters is almost same as that in the aquaculture farms. Some of the specimens collected from estuaries had symptoms similar

to that of white spot disease. The prevalence of WSSV in *P. monodon* samples collected from Vembanad estuary was 44% (36/82 samples) by first step PCR while it was 72% (59/82 samples) by nested PCR. The incidence levels of WSSV in white prawn, *F. indicus* was 26% (11/43 samples) and 44% (19/43 samples) by first step PCR and nested PCR respectively. WSSV was present in 32% (25/79 samples) and 58% (46/79 samples) of *M. dobsoni* samples collected. The occurrence of WSSV in *M. monoceros* were 39% (21/54 samples) and 67% (36/54 samples) from Vembanad Estuary.

4.1.4 Detection of WSSV in wild captured decapods from sea landings

A total of 504 samples from 15 species of decapods from wild were tested for the presence of WSSV. The results are presented in table 4.1.3. Of the fifteen species decapods tested, twelve species had WSSV incidence levels ranging from 6-23%. WSSV was not detected from the three species of deep sea decapods tested; *H. gibbosus*, *Plesionika spinipes* and *Puuerulus spp.* The cultured species, *P. monodon* had the highest incidence of WSSV among the species from wild tested at 23%.

Table 4.1.4: - Prevalence of WSSV in shrimps and other decapods from sea landings

Common name	Scientific name	One step PCR	*Nested* PCR
Marine Shrimp	*Parapenaeopsis stylifera*	2/78(3)*	7/78(9)*
Brown shrimp	*Metapenaeus dobsoni*	4/54(7)	12/54(22)
Tiger prawn	*Penaeus monodon*	5/52(10)	12/52(23)
White prawn	*F. indicus*	3/38(7)	7/38(18)
King prawn	*Metapenaeus affinis*	2/45(4)	5/45(11)
Indian nylon shrimp	*Heterocarpus woodmasoni*	1/23(4)	3/23(13)
	H. gibbosus	0/34(0)	0/34(0)

	Plesionika spinipes	0/21(0)	0/21(0)
Mud crab	*Scylla serrata*	1/14(7)	3/14(21)
Mud crab	*Scylla tranquebarica*	0/16(0)	2/16(13)
Blood spotted crab	*Portunus sanguinolentus*	0/43(0)	4/43(9)
Blue swimming crab	*Portunus pelagicus*	2/33(6)	6/33(18)
Sea Crab	*Charybdis cruciata*	1/18(6)	3/18(17)
Deep sea lobster	*Puuerulus* spp	0/17(0)	0/17(0)
Scalloped Spiny lobster	*Panulirus homarus*	0/18(0)	1/18(6)
	Total	21/504 (4%)	65/504 (13%)

**Figures indicate the number of animals positive for WSSV to number of animals tested. Figures in parenthesis indicate the percentage of animals tested positive for the virus.*

None of the animals collected from wild showed symptoms of the disease. WSSV infection levels were lowest in specimens from the wild compared to the estuarine and farm environments. In this study, the prevalence of WSSV in *P. stylifera* collected from sea landings were 3% (2/78 samples) by first step PCR and 9% (7/78 samples) by nested PCR. The incidence of WSSV in Brown shrimp (*M.* dobsoni) from sea landings were 7% (4/54 samples) by first step PCR and 22% (12/54 samples) by nested PCR. Tiger prawn (*P.monodon*) had WSSV prevalence rates of 10% (5/52 samples) by first step PCR while it was 23% (12/52 samples) by nested PCR. The prevalence of WSSV in *F. indicus* was 7% (3/38 samples) by first step PCR while it was 18% (7/38 samples) by nested PCR. WSSV was present in 2/45 (4%) *M. affinis* samples by first step PCR while it was 5/45 (11%) by nested PCR. The incidence of WSSV was 4% (1/23 samples) and 13% (3/23 samples) in *H. woodmasoni* by first step PCR and nested PCR respectively. The

prevalence of WSSV in *S. serrata* samples collected from sea landings were 7% (1/14 samples) by first step PCR while it was 21% (3/14 samples) by nested PCR. The incidence levels of WSSV in *S. tranquebarica* was nil (0/16 samples) and 13% (2/16 samples) by first step PCR and nested PCR respectively. WSSV was not detected in forty three samples of blood spotted crab; *P. sanguinolentus* by first step PCR while four samples (9%) was positive by nested PCR. The prevalence of WSSV in *P. pelagic*us was 6% (1/18 samples) by first step PCR while it was 17% (3/18 samples) by nested PCR. WSSV was present in 1/15 (6%) *P. homarus* samples by nested PCR while the virus was not detected in any of the samples by first step PCR. WSSV was present in 6% (1/18 samples) in scalloped spiny lobster, *Panulirus homarus* by nested PCR while the virus was not detected in any of the samples by first step PCR. Vaseeharan *et al.,* (2003) screened a total of 419 samples of captured crustaceans viz., *P. monodon* brooders, *P. indicus* juveniles, *Metapenaeus* spp., crab; *Scylla serrata* and *Squilla mantis* for WSSV by PCR, 23% of them were infected with WSSV. While in the present study, WSSV was detected only in 13% of the samples tested. Hossain *et al.,* (2001a) reported that wild-caught asymptomatic marine shrimp such as *Metapenaeus dobsoni*, *Parapenaeopsis stylifera*, *Solenocera indica* and *Squilla mantis* was found to carry WSSV. They have reported that WSSV was detected in apparently healthy marine crabs *Charybdis annulata*, *C. cruciata*, *Macrophthalmus sulcatus*, *Gelasimus marionis nitidus* and *Metopograpsus messor* and in asymptomatic *M. rosenbergii* cultured inland far away from coast. Lo *et al.,* (1996a) reported the presence of virus in wild crustaceans such as wild-caught shrimp; *M. japonicus*, *P. semisulcatus* and *P. penicillatus* and crabs; *C.*

feriatus, *P. pelagicus* and *P. sanguinolentus*. Otta *et al.*, 1999 noted that wild-caught marine crabs such as *C. cruciata* and *Matuta planipes* carried WSSV.

PCR has become the method of choice for the detection of WSSV because it exceeds the sensitivity limits of other DNA based methods like dot blot or Southern blot hybridization. White spot syndrome is one of the major diseases affecting the global shrimp industry. The specificity of the WSSV PCR primers has already been established (Kimura *et al.*, 1996; Lo *et al.*, 1996a, b; Umesha *et al.*, 2006). As the industry relies on the use of wild brood stock for spawning purposes, it is important to determine the prevalence of WSSV in the wild as well as cultured shrimp. The virus has been isolated from a wide range of wild crustaceans living in marine and fresh water such as crabs, lobsters, shrimps, fresh water prawn and cray fish (Lo *et al.*, 1996 a, b Peng *et al.*, 1998a, Wang *et al.*, 1998a, Otta *et al.*, 1999). The present study indicated the presence of WSSV in wild population of shrimp. In this study the proportion of WSSV infected individuals among cultured species of shrimp in the natural waters viz. *P. monodon and F. indicus* is high compared to the non-cultivable species. This indicates that infected shrimps from the aquaculture farms may have found their way into the natural waters. The presence of this virus in wild crustaceans poses a potential threat to the very survival of their population as well as it could affect the shrimp cultivation.

The culture of *M. rosenbergii* is developing very fast as an alternative to the Penaeid culture in the tropics. This is due to the fact that the species is considered less susceptible to diseases than the marine shrimp. Peng *et al.*, (1998a) reported that since the post larval stage of *M. rosenbergii* is spent in brackish water, there is a chance of this species getting infected with viruses of the marine shrimp. In the present study it was

found that even though the clinical signs were suggestive of WSSV infection in the juveniles and adults of *M. rosenbergii* collected from fresh water farms, no mortality was reported. These infected animals may act as carriers of the virus especially so since a part of the life cycle of this prawn is completed in natural brackish water systems.

The presence of WSSV in wild population of decapods is a matter of great concern as they may act as carriers of the infection. Aquaculture farms in and around Cochin are situated adjacent to the backwaters. There is a possibility of the effluents from infected farms released directly without proper disinfection into the surrounding backwaters. The effluent water may carry the dead and diseased shrimp into the natural environment. The backwaters are the sites of breeding of many economically important shrimp species. Juveniles of these species migrate to seas for maturation and could carry the infection to the sea. Infection may not occur in the sea because of the absence of stress factors that induces multiplication of the virus in host. Ranching of hatchery reared post larvae into the coastal waters to replenish the stock may be avoided and the farmers should take care not to release farm reared shrimps during water exchange or during harvesting as it may have a negative impact from the disease point of view on the natural population of shrimps.

4.2 *Insilico* analysis to compare variations in structural protein sequences of an Indian isolate of WSSV with sequences in databank

The present study was undertaken to compare the sequence of six major structural proteins of WSSV; vp28, vp26, vp19, vp68, vp281, vp466 from different geographical locations available in the database with that of an isolate from India.

Black tiger shrimp (*Penaeus monodon*) naturally infected with WSSV obtained from a shrimp farm in Cochin, India was used in the study. DNA isolated from the infected shrimp was used as the template for amplification of the envelop proteins of WSSV. Primers were designed to amplify six envelop protein of WSSV as given in Table 3.2.5 under the Section Materials and Methods (Chapter 3). The envelop proteins; vp28, vp26, vp19, vp68, vp281 and vp466 were amplified using the gene specific primers and gave amplified products of expected size 615bp, 615bp, 366bp, 207bp, 846bp and 1401bp respectively (fig 5). These sequences were submitted to the GenBank and were assigned Accession Numbers EF534254, EF534253, EU012447, EF534252, EF534251 and EF534255 respectively. The gene sequences were compared with reported nucleotide and amino acid sequences.

4.2.1 Comparison of VP28 gene sequence of the Indian isolate of WSSV obtained in the study with that available from the GenBank

The vp28 gene sequence of the Indian isolate of WSSV (GenBank Acc. No. EF534254) obtained in the present study was compared with sequences in GenBank database. The results are presented in Table 4.2.1

Table 4.2.1: Variation in sequence of vp28 of an Indian isolate of WSSV obtained in this study with that of sequences in GenBank

Accession Nos.	Position of mutation	Base Substitution	Change in Amino acid
DQ013883	Nil	Nil	Nil
DQ902658, DQ681069, DQ098011, AY324881, AF332093*, AF272979, AY249443, AY249442, AY249441, AY249440, AF227911, AJ551447, DQ013882, DQ013881, DQ007315,	125	A→G	D→G

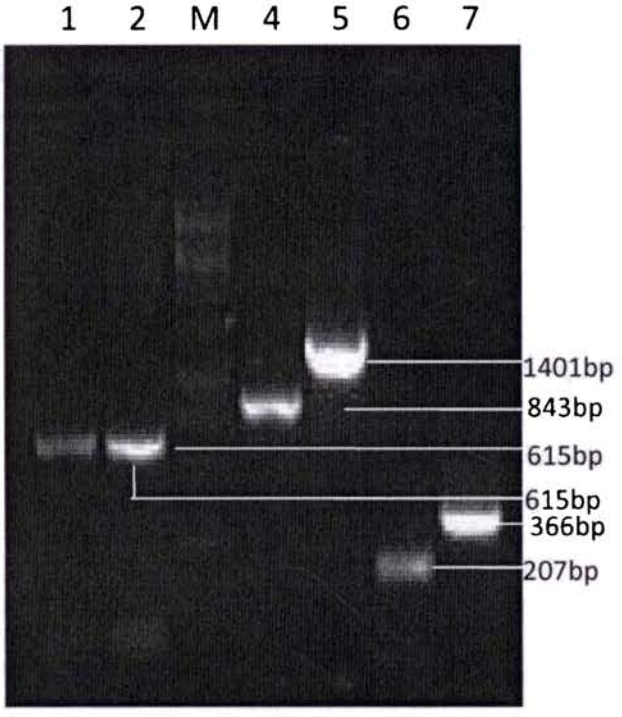

Fig 5: PCR amplification of viral protein genes of WSSV

Lane1:VP26, Lane2:VP28, Lane3: 1Kb DNA marker, Lane4:VP281, Lane5:VP466, Lane6:VP68, Lane 7:VP19

AF369029*, AY168644, AF440570*, AF173993, AY873785			
AY682926	125 234	A→G T→C	D→G Nil
AF502435, AY249434	125 119	A→G G→A	D→G R→H
AF380842	125 444	A→G T→C	D→G Nil
EF194079	125 113 434	A→G T→A T→C	D→G V→E L→P
DQ979320	125 306 536	A→G T→C T→C	D→G V→P V→A
AY422228	125 483 485	A→G T→C T→C	D→G F→S Nil

**Complete Genome sequence of WSSV*

The nucleotide sequence of the envelop protein; vp28 of the Indian isolate obtained in the study was compared with twenty-eight GenBank sequences from various geographical locations. Only one sequence reported from India (Accession No.DQ013883) had 100% homology with the isolate. There was an A→G nucleotide substitution at position 125 for all the other reported sequences, which translated into a substitution of Aspartic acid for Glycine. The nucleotide change at position 125 was present only in two WSSV isolates from India including the isolate reported in this thesis indicating that this mutation is restricted to India. Two sequences from China (AF502435 and AY249434) had an additional mutation at nucleotide position 119 from G→A which translated into an amino acid substitution from Arginine to Histidine. A Chinese isolate (Accession No.AY682926) and a Korean isolate (Accession No. AF380842) had a nucleotide substitution at position 234 and 444 respectively from T→C both of which did not translate to change of amino acid. A Thailand isolate (Accession No. EF194079), a

Chinese isolate (Accession No. DQ979320) and an Indian isolate (Accession No. AY422228) had three nucleotide substitutions including that at position 125. The Thailand isolate had nucleotide substitution at position 113 from T→A and at 434 from T→C which translated into change of amino acid from valine to glutamic acid and Leucine to proline respectively. The Chinese isolate had T→C nucleotide substitutions at positions 306 and 536 with change of amino acid from valine at both positions to proline and alanine respectively. The Indian isolate had substitutions from T→C at positions 483 and 485 with change of amino acid from phenylalanine to serine in the first position while the second position was conserved. There was nucleotide substitutions at ten different positions with the isolates studied, seven of which translated to a change of amino acid.

4.2.2 Comparison of VP26 gene sequence of the Indian isolate of WSSV obtained in the study with that available from the GenBank

The vp26 gene sequence of an Indian isolate of WSSV (GenBank Acc. No. EF534253) obtained in the present study was compared with sequences in GenBank database. The details of sequence compared and results are presented in Table 4.2.2

Table 4.2.2: Variation in sequence of vp26 of an Indian isolate of WSSV obtained in this study with that of sequences in GenBank

Accession No.	Position of mutation	Base substitution	Change in amino acid
AF308164, AF332093*, AF272980, AY249439, AY249438, AY249437, AY249436, AY249435, AY422230, AF380841, AF369029*, AF440570*, AF173992, DQ681070	Nil	Nil	Nil
AY220746	575	G→A	R→K
AJ551446	345	T→C	Nil

*Complete Genome sequence of WSSV

The nucleotide sequence of the gene encoding for envelope protein vp26 from an Indian isolate of WSSV obtained in this study was compared with 16 reported sequences and it was revealed that there was 100% similarity with fourteen of the reported sequences (Table 4.2.2). The G→A nucleotide substitution at the nucleotide position 575 for the Chinese isolate (Accession No. AY220746) resulted in a change of amino acid from Arginine to Lysine while a nucleotide change for the Vietnamese isolate (Accession No. AJ551446) at nucleotide position 345 from T→C was conserved and did not translate to change of amino acid. The VP 26 gene was found to be highly conserved among isolates from different geographical locations with just two nucleotide variations from the sixteen isolates compared.

4.2.3 Comparison of VP19 gene sequence of the Indian isolate of WSSV obtained in the study with that available from the GenBank

The vp19 gene sequence of the Indian isolate of WSSV obtained in this study (GenBank Acc. No. EU012447) was compared with sequences in GenBank database. The results are presented in Table 4.2.3. The vp19 sequence was compared with seventeen published sequences and it was found that there was 100% sequence similarity with four WSSV isolates reported from India, Vietnam, Taiwan and Mexico (Table 4.2.3). Point mutations; T→C, T→A and C→T were found at six different nucleotide positions with 13 reported sequences. To ascertain whether the variation at nucleotide level resulted in change in at amino acid level, the protein sequences of vp19 gene were also compared.

Table 4.2.3: Variation in sequence of vp19 of an Indian isolate of WSSV obtained in this study with that of sequences in GenBank

Accession No.	Position of mutation	Base substitution	Change in amino acid
DQ681071, AY160771, AF440570*, AJ937860	Nil	Nil	Nil
AY316119, AY873786	57	T→C	Nil
AF332093*, AF402997	196	C→T	P→S
AY249448, AY249447, Ay249446, AY248445, AY249444, AY220744, AF369029*	218	T→A	V→D
AY422227	25 44	T→C T→C	S→P V→A
DQ902655	25 44 287	T→C T→C C→T	S→P V→A T→M

*Complete Genome sequence of WSSV

The T→C point mutation at position 57(Accession No.AY316119 and AY873786) did not result in change in amino acid while a C→T mutation at position196 (Accession No. AF332093and AF402997) resulted in change amino acid from Proline to Serine. A T→A mutation at position 218 (Accession No. AY249444 to AY249448 and AF369029) had a change in amino acid from Valine to Aspartic acid. Two reported sequences from India, (Accession Nos. AY422227 and DQ902655), gave a T→C mutation at nucleotide positions 25 and 44 that translated into change of amino acids Serine to Proline and Valine to Alanine respectively. The WSSV isolate with Accession No. DQ902655 had an extra substitution at position 287 other than at nucleotide positions 25 and 44 that translated into Methionine, a change in the amino acid from Threonine.

4.2.4 Comparison of VP281 gene sequence of the Indian isolate of WSSV obtained in the study with that available from the GenBank

The nucleotide sequence of vp281 of an Indian isolate of WSSV (GenBank Acc. No. EF534251) obtained in this study was compared with sequences in GenBank database. The results are presented in Table 4.2.4.

Table 4.2.4: Variation in sequence of vp281 of an Indian isolate of WSSV obtained in this study with that of sequences in GenBank

Accession Nos.	Position of mutation	Base substitution	Change in amino acid
AF332093*, AF369029*, AF440570*, AF411634	807	A→T	Nil
DQ979321	807 841	A→T C→A	Nil P→T
AY517490	807 843	A→T G→A	Nil Nil

*Complete Genome sequence of WSSV

Comparison of vp281 sequence with six published sequences revealed A→T, C→A and G→A mutations at three different nucleotide positions (Table 4.2.4). The A→T mutation at nucleotide position 807 present in all the reported sequences and the G→A mutation at position 843 (Accession No. AY517490) did not translate into change of amino acid. While the C→T mutation at position 841(Accession No. DQ979321) translated into change in amino acid from Proline to Threonine.

4.2.5 Comparison of VP466 gene sequence of the Indian isolate of WSSV obtained in the study with that available from the GenBank

The vp466 gene sequence of the Indian isolate of WSSV (GenBank Acc. No. EF534255) obtained in the present study was compared with sequences in GenBank database. The results are presented in Table 4.2.5

Table 4.2.5: Variation in sequence of vp466 of an Indian isolate of WSSV obtained in this study with that of sequences in GenBank

Accession No.	Position of mutation	Base substitution	Change in amino acid
AF332093*, AF440570*, AF395545	Nil	Nil	Nil
AF369029*	1212	A→T	K→N

* Complete Genome sequence of WSSV

Three reported nucleotide sequence of vp466 had 100% homology with the sequence reported in the present study. There was an A→ T nucleotide substitution at position 1212 for a Thailand isolate (Accession No AF369029), which translated into a substitution of lysine to arginine.

The vp68 sequence of the Indian isolate of WSSV (GenBank Acc. No. EF534252) obtained in the study was 100% similar with the four reported sequences (AF332093*,AF369029*, AF440570*, AF4114664).

In the present study, the sequences of six envelope proteins of an Indian isolate of WSSV obtained in the study was compared with reported sequences of WSSV isolates. A common ancestor for the virus isolate could not be designated as there was variation in sequence with one envelope protein or other. Moon *et al.* (2003) reported that the vp26 gene of the Taiwanese, Thailand, Chinese and Korean isolates were identical at both nucleotide and amino acid levels while the vp28 gene was 99% identical at nucleotide level and 100% identical at amino acid level. You *et al.* (2004) reported that the sequence of five WSSV structural proteins (vp15, 19, 24, 26 and 28) from 5 different geographical WSSV isolates when compared with four WSSV isolates reported showed that the sequence of each gene were identical among the 9 isolates with the exception of VP28 gene of a Korean isolate with 1 base change from that of other WSSV isolates. However this base change did not result in change of amino acid sequence. Soek *et al.* (2004) reported that at the nucleotide level, VP19, VP28 and VP15 sequences of a Korean isolate of WSSV were, respectively, 99, 100 and 100% identical to those of China, Indonesia, Japan and the United States and the VP35 sequence was 100% identical to that of a Taiwanese isolate. The deduced amino-acid sequences were 99 to 100% identical to

those from other countries. In VP19, C and T in the foreign isolates were replaced by T and A in the Korean isolate at Positions 57 and 218 nt, respectively, downstream of A (+) of the VP19 start codon. The change at Position 218 nt resulted in valine in the foreign isolates being replaced by aspartate in the Korean isolate. Chang *et al.*, 2002 attributed the point mutations in the envelope proteins to the non-infectious nature of the virus isolate.

Mishra & Shekhar (2005) reported that an Indian isolate of WSSV is very similar in external morphology to other WSSV geographical isolates. Wang *et al.* (2000a) reported that the protein profiles by SDS-PAGE that the Indian WSSV isolate was very similar to the WSSV isolates from China, Thailand, South Carolina and Texas. However at the genome level, WSSV isolates from China, India, Thailand and the US state of Texas and South Carolina was shown to have no distinctive differences by restriction analysis and Southern blot hybridization (Wang *et al.*, 2000b). However, Marks *et al.* (2004) reported very high nucleotide similarity between the completely sequenced WSSV isolates. Hence the use of restriction fragment length polymorphism (RFLP) does not seem to be appropriate to distinguish the WSSV isolates (Shekhar and Ravichandran, 2007). High variation in repetitive DNA fragments in different Thai WSSV isolates has been reported by Wongteerasupaya *et al.*, 2003. Hence, the variable loci located in some open reading frames of WSSV genome have been suggested for use in PCR based classification of WSSV isolates in epidemiological studies (Marks *et al.*, 2004).

Satish *et al* 2004) reported that the sequence of vp14, vp24 and vp26 of an Indian isolate of WSSV did not exhibit any variations in the sequences compared. Vp19 gene sequence comparison revealed major C–T and T–C point mutations at two loci each,

while vp28 sequence had C–T transition in two loci. To examine whether this point mutation is also manifested at amino acid level, the genes were translated and compared. In vp19, at position 9, proline was substituted by serine, at position 15, alanine was substituted by valine, at position 66, serine was substituted by proline, and at position 96, methionine was substituted by threonine. In vp28, at position 18, alanine was substituted by cysteine, and at position 162, serine was substituted by threonine. The vp19 and vp28 at the amino acid level had a sequences identity of 96.7% and 98% with already published sequences, respectively. Shekhar and Ravichandran (2007) reported that sequence comparison analysis of structural genes of an Indian isolate of WSSV with the three completely sequenced WSSV isolates indicated that the Indian isolate is similar to the Taiwanese isolate with respect to VP19 gene and similar to Chinese and Thai WSSV isolates with respect to VP24 gene. Based on these results, it is therefore difficult to suggest the proximity of Indian WSSV isolate to any of the three completely sequenced WSSV isolates of Taiwan, China and Thailand.

Molina-Garza *et al.* (2008) reported that when the sequence of WSSV genes encoding nucleocapsid (VP26 and VP15) and envelope proteins (VP19 and VP28) of a Mexican isolate were compared that of WSSV isolates in GenBank; VP15 was highly conserved, and VP26 showed 99% homology to a Chinese isolate. The VP28 fragment demonstrated 100% homology to the majority of the isolates analysed (UniProt accession no. Q91CB7), differing from two Indian WSSV and one Chinese WSSV isolates by two non-conserved and one conserved replacements, respectively. In contrast, VP19 of the Mexican isolate was distinguishable from almost all isolates tested, including an American strain of WSSV (US98/South Carolina, GenBank accession no. AAP14086).

Although homology was found with isolates from Taiwan (GenBank accession no. AAL89341) and India (GenBank accession no. AAW67477), VP19 may have application as a genetic marker.

Highly conserved DNA and amino acid sequence of the structural proteins of WSSV provide the basis for the development of sensitive nucleic acid and antibody based detection methodology for WSSV (You *et al.*, 2004). Recombinant vp28 and vp19 were found to have potential for use as injected vaccine or oral vaccine (Witteveldt *et al.*, 2004).

4.3 PCR amplification to determine the prevalence of multiple viral infections in penaeid postlarvae

Table 4.3: Status of viral infection in 104 *P. monodon* PL samples from hatcheries

Infection Status	**No. of Samples**	**Total Percentage (%)**
Uninfected	5	4.8
Infected	99	95.2
Single Infections		
HPV	12	11.5
IHHNV	24	23.1
MBV	0	0
WSSV	1	0.96
Double Infections		
HPV+WSSV	5	4.8
HPV+IHHNV	37	35.6
HPV+MBV	1	0.96
IHHNV+WSSV	1	0.96
IHHNV+MBV	6	5.8
WSSV+MBV	1	0.96
Triple Infections		
HPV+IHHNV+WSSV	5	4.8
HPV+IHNV+MBV	5	4.8
HPV+WSSV+MBV	0	0
IHHNV+MBV+WSSV	1	0.96

Total IHHNV	79	76
Total HPV	65	62.5
Total MBV	13	12.5
Total WSSV	13	12.5

On PCR analysis, it was found that 99 (95.2%) of the postlarval samples gave positive result for atleast one of the four viruses studied. Only 5 samples (4.8%) were negative for all the viruses studied. Out of the 104 post larval *P. monodon* samples tested, 79 (76%) were positive for IHHNV, 65 (62.5%) were positive for HPV, 13 (12.5%) were positive for MBV and 13 (12.5%) were positive for WSSV (Table 3.4).

4.3.1 Prevalence of WSSV in postlarvae from hatcheries

In this study, a nested PCR method as described by Umesha *et* al., 2006 was employed for the detection of WSSV in hatcheries. The primer set; IK1 and IK2 consistently gave PCR product of 486 bp (fig.1) by first step PCR and primer set; IK3 and IK4 gave PCR product of 310bp (fig.2) as anticipated. A second set of primers as described by Kimura *et al.,* 1996 was also used for detection of WSSV. The second set yielded PCR product of size 982bp and 570 bp by first step PCR and nested PCR (Fig.3 and fig.4) respectively. In the present study, WSSV was detected in 2.9% of the post larvae tested by first step PCR and 12.5% by nested PCR. One of the samples that were weakly positive by first step PCR by the primers of Umesha *et al.,* 2006 did not yield a product by the primers of Kimura *et al.,* 1996. However that sample was positive by nested PCR by both sets of primers. Results of all the other samples were similar with both sets of primers. WSSV has been found to be highly prevalent among post larvae samples from hatcheries of India. The prevalence of WSSV in post larvae reported in this study is comparatively lower than 75% prevalence reported by Otta *et al.,* (2003) but was similar to 12.4% prevalence reported by Uma *et al.,* (2005). Both one step methods

and nested PCR methods have been described for detection of WSSV. Lo *et al.*, (1996a, b) has reported that the nested PCR amplification procedure is 10^3-10^4 fold more sensitive than one step PCR method. Peng *et al.* (1998b) reported that the carrier state of WSSV gives only nested PCR test results. Introduction of stress to shrimp by environmental factors such as pH, salinity, temperature, water level (Hossain *et al.*, 2001b) may convert the pre-patent carrier state to the patent infecting state within few days or even hours (Itami *et al.*, 1998; Lo *et al.*, 1998; Peng *et al.*, 1998a, b) there by giving a first step PCR positive reaction. The presence of WSSV has been reported in wild broodstock from Taiwan, Japan and India (Lo *et al.*, 1997; Otta *et al.*, 1999; Uma *et al.*, 2005). The prevalence of WSSV in PL has been found to be much lower compared to the prevalence in broodstock (Lo *et al.*, 1997; Tsai *et al.*, 1999; Uma *et al.*, 2005). The presence of WSSV has been reported in apparently healthy post larvae by PCR (Lo *et al.*, 1996a; Otta *et al.*, 1999; Magbanua *et al.*, 2000; Otta *et al.*, 2003; Uma *et al.*, 2005).

4.3.2 Prevalence of MBV in postlarvae from hatcheries

In this study, a nested PCR method as described by Belcher and Young, 1998 was employed for the detection of WSSV in hatcheries. The PCR primers used for the detection of MBV gave an amplified product of 533bp (fig. 6) for the first reaction and a 361bp (fig. 7) fragment in the nested reaction. In the present study, MBV was detected in 3.8% of the post larvae tested by first step PCR and 12.5% by nested PCR. The results of this study indicates a low prevalence of MBV (12.5%) in postlarvae from hatcheries of India compared to 68-92% prevalence by wet squash method as reported by Ramasamy *et al.*, (1995) and 39-54% by PCR as reported by Otta *et al*, (2003) and Uma *et al.* (2005). Mortalities of shrimp larvae due to MBV have been reported in many countries

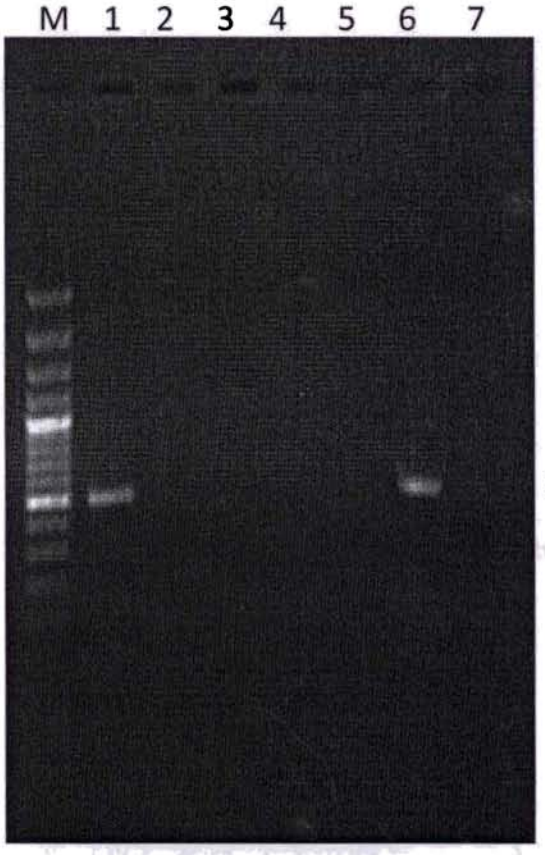

Fig 6: First step PCR amplification for detection of MBV in *P. monodon* postlarvae using MBV14F and MBV14R primers

Lane M; 100bp molecular weight marker, Lanes 1 and 6: DNA template of MBV infected *P. monodon* samples from hatcheries. Lane 2, 3, 4, 5 and 7: *P. monodon* postlarval sample that was negative by first step PCR for MBV. The size of the nested PCR product is 533 bp

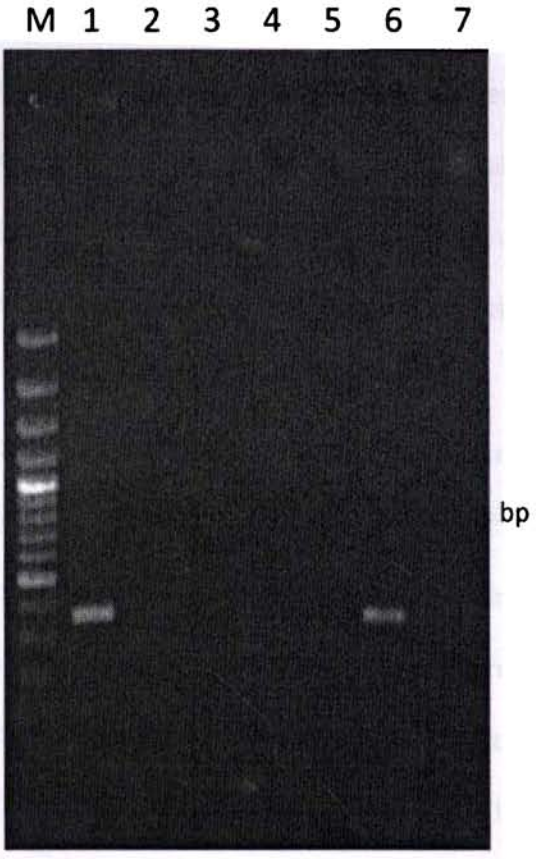

Fig 7: Nested PCR for detection of MBV in *P. monodon* postlarvae using MBV14NF and MBV14NR primers

Lane M; 100bp molecular weight marker, Lanes 1 and 6: DNA template of MBV infected *P. monodon* samples from hatcheries. Lane 2, 3, 4, 5 and 7: *P. monodon* postlarval samples negative for MBV. The size of the nested PCR product is 256 bp

(Lightner *et al.*, 1992; Manivannan *et al.*, 2002). MBV is reported to be well tolerated by *Penaeus monodon* (Lightner, 1988; Fegan *et al.*, 1991). Chen *et al.* (1992) reported that MBV is transmitted by oral route from water contaminated with virus from fecal matter of brood stock and that MBV infection of larvae can be prevented to a great extent by washing the fertilized eggs in filtered sea water. MBV was found to be prevalent in almost 40% of the wild shrimp seed in Vietnam by histological examination (Hao *et al.*, 1999). MBV has also been reported in female brood stock in Thailand with a prevalance of 33% in 1987 and 100% in 1989 (Liao *et al.*, 1992). The low levels of infection the PL in the present study may be due to improved hygiene practices in the hatchery.

4.3.3 Prevalence of HPV in postlarvae from hatcheries

In this study, a nested PCR method as described by Umesha *et al.*, 2006 was employed for the detection of WSSV in hatcheries. The PCR primers used for the detection of HPV gave an amplified product of 441bp for the first step reaction (fig.8) and a 265bp fragment in the nested reaction (fig.9). In the present study, HPV was present in 62.5% of the postlarval samples using nested PCR described by Umesha *et al* (2006). However all the postlarval samples were negative for HPV with the primers described by Pantoja and Lightner (2000). These results indicate that the strains of HPV present in the postlarval samples are similar to that of HPVmon isolated from *P. monodon* from Thailand and not HPVchin isolated from *P. monodon* in China. Umesha *et al.*, (2006) also reported the presence of only HPVmon in *P. monodon* from shrimp ponds from India. HPV is an emerging disease, which was first reported in 1984 in wild *P. merguiensis* and *F. indicus* from Singapore (Chong and Loh, 1984). HPV was reported in wild *P. monodon* and hatchery reared larvae with prevalence ranging from 31-62 %

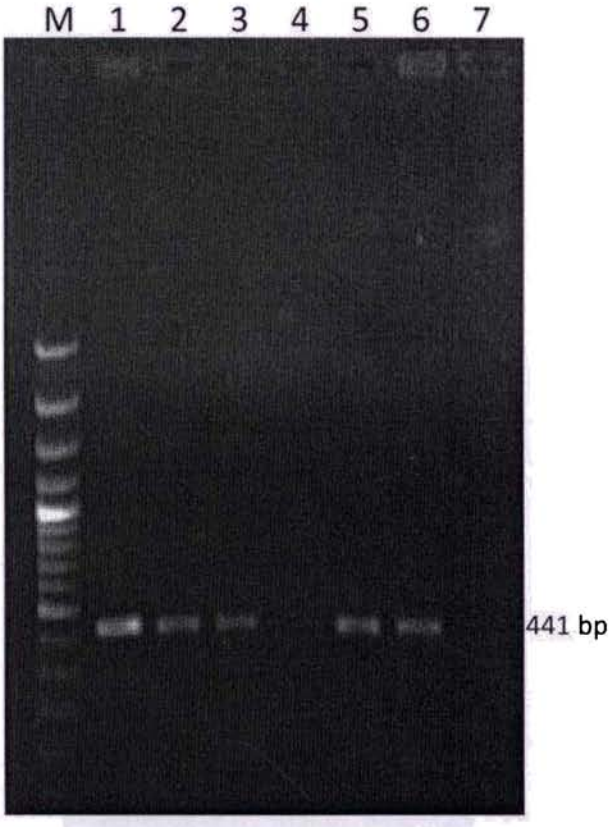

Fig 8: First step PCR amplification for detection of HPV in *P. monodon* postlarvae using H441F and H441R primers

Lane M; 100bp molecular weight marker, Lanes 1, 2, 3, 5 and 6: DNA template of HPV infected *P. monodon* samples from hatcheries. Lane 4 and 7: *P. monodon* postlarval sample negative for HPV. The size of the PCR product is 441 bp

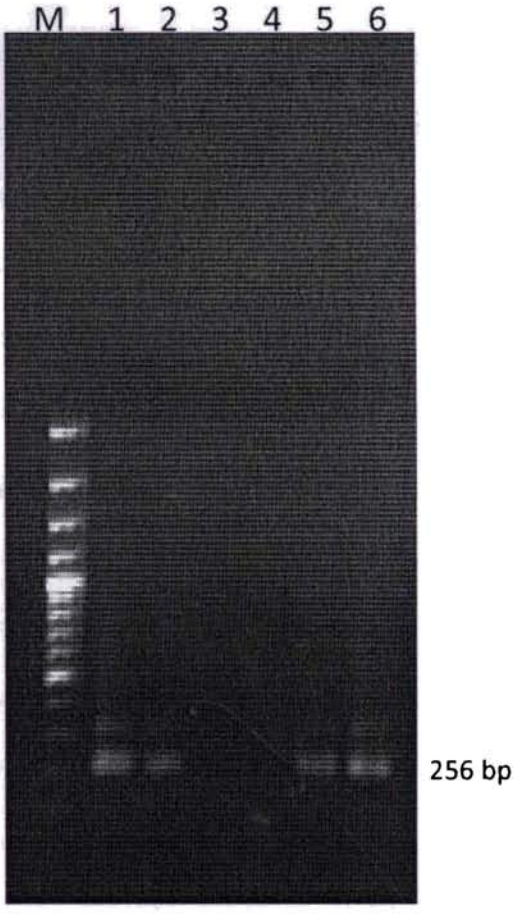

Fig 9: Nested PCR for detection of HPV in *P. monodon* postlarvae using HPVnF and HPVnR primers
Lane M; 100bp molecular weight marker, Lanes 1, 2, 5 and 6: DNA template of HPV infected *P. monodon* samples from hatcheries. Lane 3 and 4: *P. monodon* postlarval sample negative for HPV. The size of the nested PCR product is 256 bp

(Umesha *et al.*, 2003; Manjanik *et al.*, 2005; Umesha *et al.*, 2006). Sukhumsinchart *et al.*, (1999) reported that *P. monodon* infected with HPV rarely show gross signs of disease. Heavy infections due to HPV can cause poor growth in *P. monodon* that will result in economic losses (Sukhumsirichart *et al.*, 1999; Flegel *et al.*, 2004). However Umesha *et al.*, (2006) has noted no difference in production in HPV infected and HPV uninfected farms. Manivannan *et al.*, (2002) has implicated multiple virus infection with MBV, HPV and WSSV in *P. monodon* postlarvae in India as cause for mortality.

4.3.4 Prevalence of IHHNV in postlarvae from hatcheries

In this study, two sets of primers recommended by OIE (2006) were used for the detection of IHHNV. The first set of primers using 389F/R primers gave consistently a PCR product of 389 bp (fig.10) while the second set of primers (77012F and 77353R) gave a 356bp fragment (fig.11). In the present study, IHHNV was detected in 76% of *P. monodon* postlarval samples using the 389F/R primers while the primers 77012F and 77353R gave positive reaction with 53.8% postlarval samples. IHHNV infection is found to be well tolerated by *P. monodon* (Lightner, 1996; Flegel, 2001). However there is a possibility of transmitting the virus to species that are susceptible. Lightner and Redman (1998) reported that the economic losses caused by IHHNV on *L. vannamei* culture ranges between 10% and 50% in comparison with IHHNV free crops. Yang *et al.*, (2006) reported that IHHNV was found in 51.5 % of penaeid shrimp culture in China. Nunan *et al.*, (2001) reported that the prevalence of IHHNV from wild caught *L. vannamei* broodstock captured off the Pacific coast of Panama was 20% by dot blot assay. The virus can be transmitted horizontally through ingestion of infected and dead animals (Bell

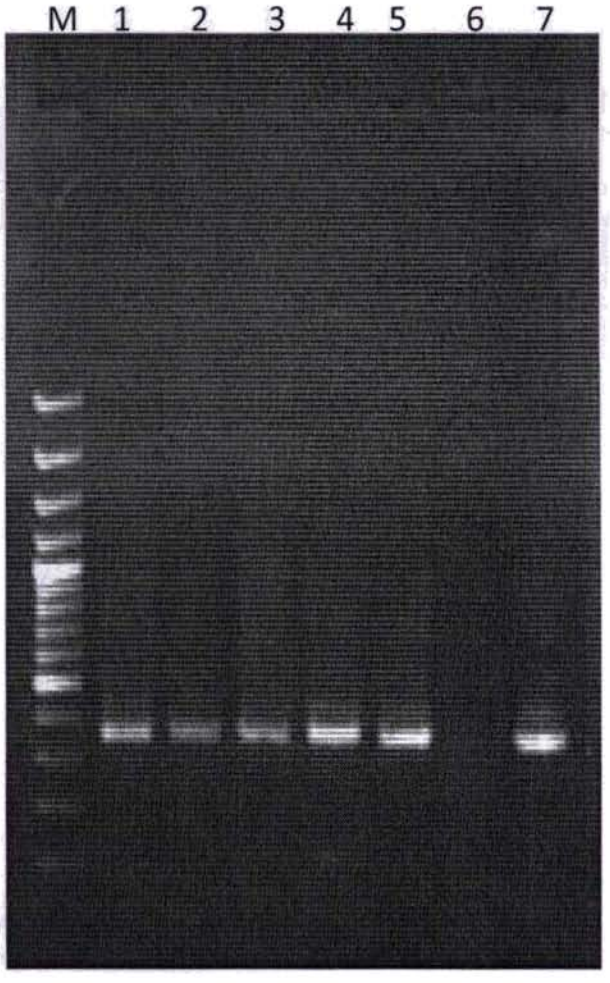

Fig 10: Detection of IHHNV from *P. monodon* postlarvae using 389F and 389R primers
Lane M; 100bp molecular weight marker, Lanes 1, 2, 3, 4, 5 and 7: DNA template of IHHNV infected *P. monodon* samples from hatcheries. Lane6: *P. monodon* sample negative for IHHNV. The size of the nested PCR product is 389 bp

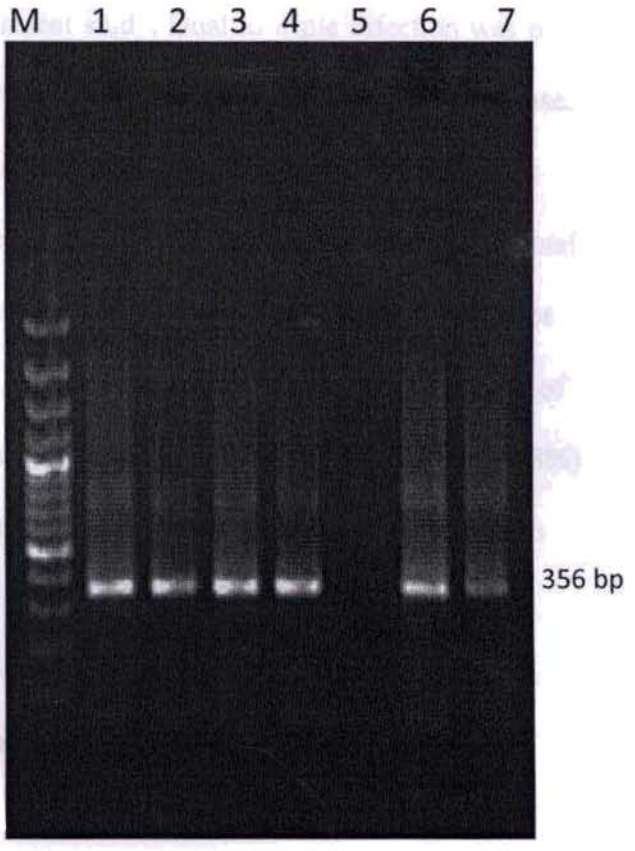

Fig 11: Detection of IHHNV from *P. monodon* postlarvae using 77112F and 77012R primers
Lane M; 100bp molecular weight marker, Lanes 1, 2, 3, 4, 6 and 7: DNA template of IHHNV infected *P. monodon* samples from hatcheries. Lane5: *P. monodon* sample negative for IHHNV. The size of the nested PCR product is 356 bp

and Lightner, 1984; Lotz, 1997). Motte *et al.* (2003) reported that the virus can be transmitted vertically also from infected females to the embryos.

4.3.5 Prevalence of multiple viral infections in postlarvae from hatcheries

In the present study, Dual to triple infection was present in 60.6% of the total postlarvae tested. Out of the 51 samples positive for two viruses, 50 (98%) included either HPV or IHHNV infection. HPV or IHHNV was present in all the post larval samples found positive 11 (100%) for triple viral infection. A total of 99/104 (95.2%) post larval samples were positive for HPV and IHHNV alone or in combination with other viruses. HPV or IHHNV was present alone in 36 (34.6%) of the total post larval samples tested. Out of the 99 samples infected with virus, 79 (79.8%) of the samples had IHHNV alone or in combination with other viruses while HPV was present in 65 (65.6%) of the samples.

Dual infections of MBV and WSSV have been observed in wild black tiger shrimp from several sampling sites in the Philippines using PCR. Mixed infections of WSSV and MBV were also reported in other Asian countries like Vietnam and Thailand (Hao *et al.*,1999; Flegel *et al.*, 2004; De la Pena *et al.*, 2005). In 1992, Natividad and Lightner reported an 85–100% prevalence of MBV in *P. monodon* postlarvae in the Philippines. On the other hand, WSSV was first reported in the Philippines in 1999, wherein 72% of the samples analyzed were positive for WSSV (Magbanua *et al.*, 2000).

Flegel *et al.* (1999) reported that HPV infections alone or in combination with MBV have been reported to be associated with much reduced growth rates. Flegel *et al.*, 2004 reported that when PCR assays for HPV, WSSV and IHHNV were carried out on 240 shrimp from 6 ponds in Thailand where visible lesions were apparent for MBV only,

94% of the specimens gave a positive test for at least one of the four viruses. HPV and IHHNV alone or in combination were detected at high prevalence (approximately 60%) despite the absence of visible histological lesions and were confirmed by southern blot hybridization.

Natividad *et al.,* (2006) reported a duplex PCR protocol for the simultaneous detection of two penaeid shrimp viruses, namely, WSSV and MBV infecting *P. monodon* in Philippines. An assessment of the sensitivity of the developed duplex PCR demonstrated the detection of both the amplicons up to 0.1 femtogram (fg) of plasmid DNA containing the target sequences equivalent to 15 copies of the viral target sequence. In addition to its high specificity and sensitivity, the developed duplex PCR offers an efficient and rapid tool for screening penaeid shrimp viruses since both WSSVand MBV can be diagnosed in a single reaction

Umesha *et al.* (2003) reported the prevalence of HPV, MBV and WSSV in samples of *P. monodon* postlarvae (PL10 to PL20, 10 to 20 d old postlarvae) in India was studied by PCR. Samples collected from different hatcheries, and also samples submitted by farmers from different coastal states, were analyzed. HPV was detected in 34% of the hatchery samples and 31% of the samples submitted by farmers, using a primer set designed for detection of HPV from *P. monodon* in Thailand. However, none of these samples were positive using primers designed for detection of HPV from *F. chinensis* in Korea. MBV was detected in 64% of the samples submitted by the farmers and 71% of the hatchery samples. A total of 84% of the samples submitted by farmers, and 91% of the hatchery samples, were found positive for WSSV. Prevalence of concurrent infections by HPV, MBV and WSSV was 27% in hatchery samples and 29% in samples submitted

by farmers. Only 8% of the hatchery samples and 16% of the samples submitted by farmers were negative for all 3 viruses. Umesha *et al.*, (2006) surveyed 18 ponds during 2 crops and reported that 7 ponds showed simultaneous presence of infection by MBV and WSSV and 10 ponds (7 in first crop and 3 in second crop) showed the presence of MBV, WSSV and HPV.

Manivannan *et al.* (2002) reported that simultaneous occurrence of WSSV, MBV and HPV in hatchery-reared postlarval *P. monodon* resulted in severe mortality. Otta *et al.*, (2003) reported the simultaneous presence of MBV and WSSV in apparently healthy postlarvae of *P. monodon* from different hatcheries in India by nested PCR. MBV and WSSV were detected in 54% and 75% of the samples respectively. Only 14% of the samples analysed were negative for both viruses.

Ramasamy *et al.*, (1995) recorded 81% prevalence of MBV in *P. monodon* larvae in a hatchery in India with infected postlarvae showing 90% mortality. Using direct microscopic observation, Karunasagar *et al.*, (1998) noted an incidence of MBV ranging from 0% to 58% in different hatcheries in India. Uma *et al.*, (2005) reported that the prevalence of WSSV ranged from 25 to 50% in the broodstock and 10-13% in PL by nested PCR while MBV prevalence ranged from 25 to 60% in the broodstock and 34 to 39% in the PL by non-nested PCR.

Nunan *et al.* (2001) screened 104 wild caught *Litopenaeus wannurnei* broodstock, captured off the Pacific coast of Panama, for IHHNV and WSSV by blot assay indicated a prevalence of IHHNV in 28% and WSSV in 2%.

There is very little data on the simultaneous presence of WSSV, MBV, HPV and IHHNV in *P. monodon* postlarvae meant for stocking in aquaculture ponds. In this study,

HPV and IHHNV alone or in combination was detected in 93.3% of the samples. It can be assumed that the very high rates of prevalence of HPV and IHHNV in samples are primarily due to lack of screening strategies for the presence of these viruses in India. Hence measures are yet to be initiated for control of HPV and IHHNV infection in shrimp. It is worth while to note that the percentage of hatchery reared postlarvae infected with WSSV and MBV is less. This is due to the stringent screening strategies initiated by hatcheries.

4.4 A Real-time SYBR Green PCR Assay for quantification of WSSV infection in postlarvae of *P. monodon*

4.4.1 Standard curve and limit of detection

A quantitative real time PCR method for detection of WSSV based on SYBR Green assay was developed. The primers were selected from ORF421 region of the WSSV genome. To determine the sensitivity of the real time PCR, a plasmid vector containing the cloned DNA fragment to be amplified was used as standard. Plasmid was serially diluted from 10^7 to 1.47 copies/ul. Four experiments were run in duplicates to prepare the standard curve. The lowest detection level of 1.47 copies of WSSV DNA was determined in four out of eight assays, while 2.94 copies were detected in 6 out of eight assays. This discrepancy in the results could be due to pippetting errors. Templates containing 5.88 copies were detected in all the assays. Thus the lower limit of detection was considered to be 5.88 copies. The average of Ct values versus copy number was plotted. Strong linear correlation (r2=0.9962 to 0.998) were obtained between the threshold cycles (Ct) and the target plasmid standard with a slope of (-) 3.488 ranging from $2.35X10^7$ to 1.47 copies of DNA (fig 3.5.1).

To determine the reproducibility of each reaction within each run (intra-assay) and between assays (inter-assay) 2.3X 10^7 to 1.47 WSSV copies were compared in independent reactions (Table 3.5.1). The correlations of variability ranged between 1.71 and 0.49 indicating that there is very little variation between the runs. With an optimal PCR mixture, 10^7 to 1.47 copies of WSSV can be detected from a specimen.

Table 4.4.1: Inter- and intra-assay reproducibility of four independent assays

Ct values at different copy numbers of WSSV							
Assay	1	2	3	4	Mean	S.D	CV(%)
23500000	14.75	14.88	14.22	14.92	14.63	0.25	1.71
	14.39	14.76	14.63	14.45			
2350000	17.70	17.94	18.16	18.50	18.12	0.24	1.30
	18.23	18.10	18.26	18.09			
235000	21.41	21.32	21.22	22.20	21.62	0.4	1.56
	21.43	21.64	21.94	21.78			
23500	24.70	24.83	24.32	25.40	24.80	0.36	1.45
	25.09	24.36	24.79	24.94			
2350	28.99	28.63	27.81	29.39	28.73	0.49	1.71
	29.14	28.75	28.82	28.34			
235	31.58	31.27	30.97	31.07	31.21	0.25	0.81
	31.26	30.82	31.23	31.47			
23.5	34.38	34.65	34.08	34.95	34.37	0.30	0.88
	34.22	34.37	34.05	34.26			
11.75	37.01	36.35	36.37	36.49	36.58	0.21	0.59
	36.48	36.69	36.69	36.56			
5.875	38.17	38.31	38.63	39.10	38.57	0.33	0.85
	38.24	38.26	38.47	38.57			
2.94	38.63	N.D	38.82	39.10	39.08	0.42	1.06
	38.78	39.03	N.D	38.90			
1.47	N.D	39.54	39.85	N.D	39.76	0.20	0.49
	N.D	39.98	N.D	39.67			

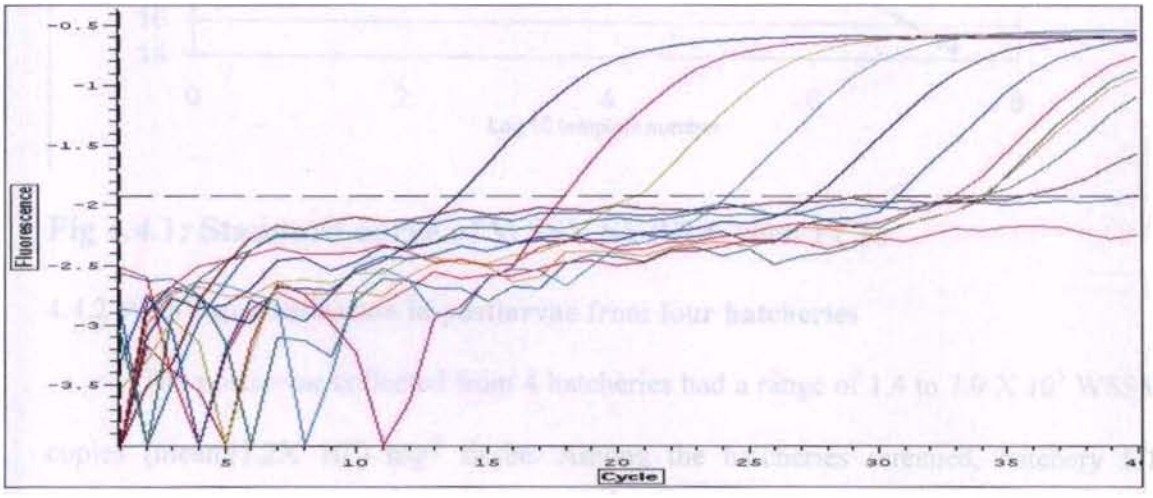

Fig 12: Amplification plot of fluorescence intensities (ΔRn) versus the PCR cycle numbers for serial 10-fold dilutions of standard plasmids for WSSV

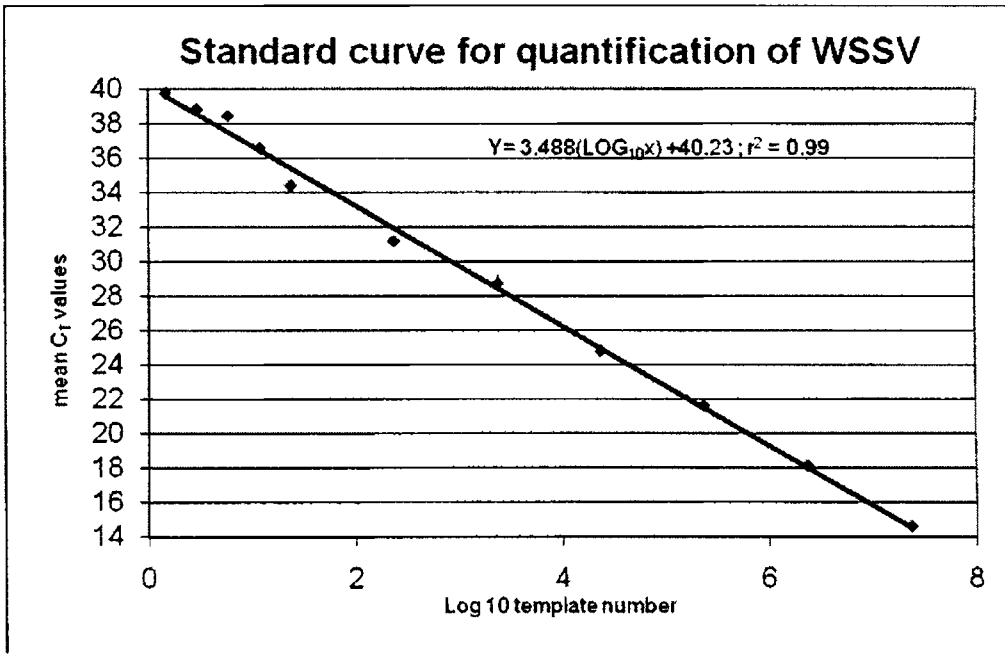

Fig 4.4.1: Standard curve of WSSV SYBR Green PCR

4.4.2 WSSV quantification in postlarvae from four hatcheries

The postlarvae collected from 4 hatcheries had a range of 1.4 to 7.9 X 10^5 WSSV copies (mean=1.2X 10^4) mg^{-1} tissue. Among the hatcheries screened, hatchery H1 showed the lowest degree of WSSV infection with mean copy number of 2.73 X 10^2 copies mg^{-1} tissue followed by H2 with 3.55X10^2 copies mg^{-1} tissue. The highest degree of WSSV infection was from H4 with 2.8X10^3 copies mg^{-1} tissue. Out of the 119 post larval samples, 72% were infected with WSSV. Hatchery H2 has the lowest degree of WSSV infection with 53.6% (15/31) samples negative for the virus while hatchery H3 and H4 had the highest degree of WSSV infection with 83.3% (25/30) and 80.6% (25/31) infection rates respectively. The level of WSSV was less than 100 mg^{-1} tissue in seventy samples (58.8%) and 12 (10.1%) samples showed higher levels of infection ranging from 8.67X10^2 to 7.9X10^5 WSSV copies mg^{-1} tissue with three samples having more than 10^5

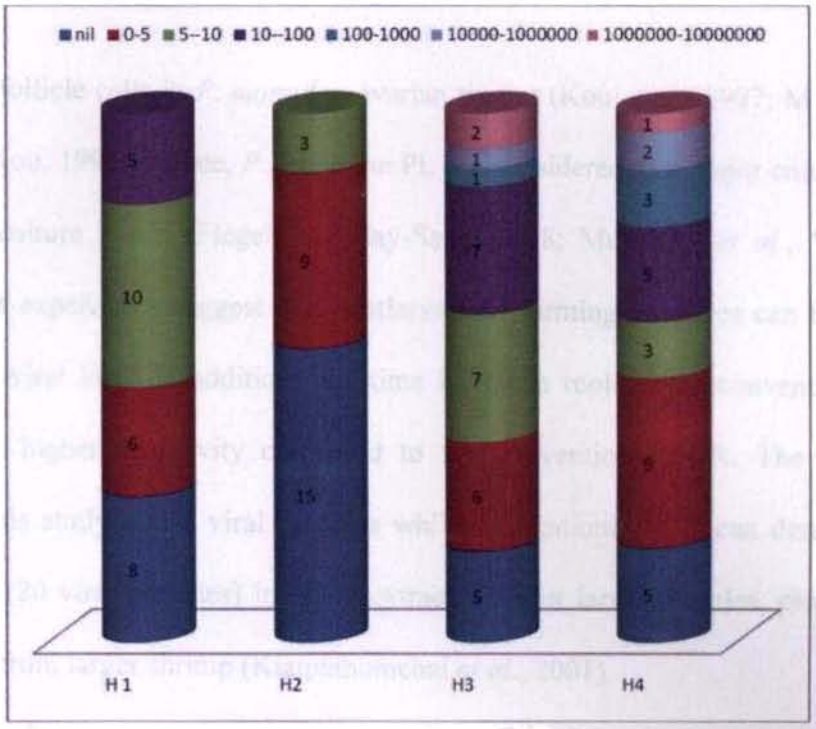

Fig 4.4.2: *P. monodon* postlarvae infected with WSSV. The figures on the bar indicates the number of samples infected with WSSV

copies, one from H4 and two from H3.

The higher mean value of WSSV infection in H4 and H3 is due to higher loads of virus in few batches of postlarvae from these hatcheries. Jang *et al.* (2009) reported a range of 2.6 to 713.6 (with a mean of 220) copies g^{-1} of WSSV DNA in 210 postlarvae obtained from seven commercial hatcheries in South Korea. The mean WSSV copy number in the postlarvae was 7.9×10^5, which was equivalent to 8.5×10^5 copies mg^{-1} of postlarvae weight.

Rapid transmission of WSSV on culture systems may occur from infected shrimp, through the water and by cannibalism of moribund shrimp (Chang *et al.*, 1996); however, the major source of infection for shrimp farms is from infected spawners and postlarvae. There are reports of a transovarial or vertical transmission of WSSV by infected gonads,

oogonia and follicle cells in *P. monodon* ovarian tissues (Kou *et al.*, 1997; Mohan *et al.*, 1997; Lo & Kou, 1998). Hence, *P. monodon* PL are considered as a major entry route for WSSV into culture ponds (Flegel & Alday-Sanz, 1998; Mushiake *et al.*, 1999). The results of this experiment suggest that postlarvae for farming purposes can be selected based on the viral load. In addition, real-time PCR can replace the conventional PCR based on the higher sensitivity compared to the conventional PCR. The sensitivity obtained in this study was 5 viral particles while conventional PCR can detect 5 fg of WSSV DNA (20 viral particles) in crude extracts of post larval samples, pleopods and haemolymph from larger shrimp (Kiatpathomchai *et al.*, 2001).

Withyachumnarnkul (1999) reported that PCR-positive postlarval batches have been associated with disease outbreaks and crop failure. Hence screening of PL or broodstock for WSSV by PCR has been proposed to the shrimp-farming industry as a major component of disease-management strategies (Lo & Kou, 1998). The findings of this study indicates a very high level of WSSV infection (72%) among postlarvae of four hatcheries tested and hence proper screening strategies should be in place before stocking of postlarvae in rearing ponds.

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Summary and Conclusions

5. Summary and Conclusions

This thesis covers various aspects of viral diseases affecting shrimp aquaculture. The research component of this thesis can be divided into four areas. The areas covered are: 1) A study to determine the prevalence of WSSV among the crustaceans in the Vembanad estuary, the shrimp aquaculture farms surrounding the estuary, and the sea off Cochin coast, India using two sets of nested PCR primers. 2) An investigation to compare the sequence of six major structural proteins of WSSV; vp28, vp26, vp19, vp68, vp281, vp466 from different geographical locations with that of an isolate from India. 3) Simultaneous occurrence of HPV, IHHNV, MBV and WSSV in postlarvae of *P. monodon* from hatcheries in India was monitored by Polymerase Chain Reaction. 4) A real time PCR procedure was developed for the quantitative analysis of WSSV infection. The viral load of postlarvae from hatcheries in Kerala meant for aquaculture was also determined using the quantitative PCR.

5.1 Detection of WSSV in shrimps and other decapods from farm environment

A nested PCR method as described by Umesha *et* al., 2006 and Kimura *et al.*, 1996 were employed in this study for the detection of WSSV in farmed and wild crustaceans. A total of three hundred and eight samples from farmed shrimp and other decapods from farm environment were analysed for presence of WSSV. The prevalence of WSSV in farmed *P. mondon* was 48% (54/112 samples) by first step PCR while it was 79% (88/112 samples) by nested PCR. The incidence of WSSV was 35% (29/84 samples) by first step PCR and 55% (46/84 samples) by nested PCR in *F. indicus*. Brown shrimp (*M. dobsoni*) had WSSV prevalence rates of 36% (8/22 samples) by first step PCR while it was 68% (15/22 samples) by nested PCR. The prevalence of WSSV in *M. monoceros* was 42% (13/31 samples) by first step PCR while it was 71% (22/31

samples) by nested PCR. WSSV was present in 2/15 (13%) *S. serrata* samples by first step PCR while it was 5/15 (33%) by nested PCR. The incidence of WSSV was 17% (3/18 samples) and 39% (7/18 samples) in *S. tranquebarica* by first step PCR and nested PCR respectively. The fresh water prawn, *Macrobrachium rosebergii* had WSSV prevalence of 35% (9/26 samples) by first step PCR and 92% (24/26 samples) by nested PCR.

5.2 Detection of WSSV in shrimps from Vembanad estuary

A total of 258 samples from four species of shrimp; Tiger prawn (*Penaeus monodon)*- 82 Nos, White prawn (*F. indicus)*- 43 Nos, Brown shrimp (*Metapenaeus dobsoni)*- 79 Nos and Speckled shrimp (*M. monoceros)*- 54 Nos collected from the Vembanad estuary (Kerala State) were analysed for the presence of WSSV. The prevalence of WSSV in *P. monodon* samples collected from Vembanad estuary was 44% (36/82 samples) by first step PCR while it was 72% (59/82 samples) by nested PCR. The incidence levels of WSSV in white prawn, *F. indicus* was 26% (11/43 samples) and 44% (19/43 samples) by first step PCR and nested PCR respectively. WSSV was present in 32% (25/79 samples) and 58% (46/79 samples) of *M. dobsoni* samples collected. The occurrence of WSSV in *M. monoceros* were 39% (21/54 samples) and 67% (36/54 samples) from Vembanad Estuary.

5.3 Detection of WSSV in wild captured decapods from sea landings

A total of 504 samples from 15 species of decapods from wild were tested for the presence of WSSV. Of the fifteen species decapods tested, twelve species had WSSV incidence levels ranging from 6-23%. WSSV was not detected from the three species of deep sea decapods tested; *H. gibbosus*, *Plesionika spinipes* and *Puuerulus spp.* The cultured species, *P. monodon* had the highest incidence of WSSV among the species from wild tested at 23%. The prevalence

of WSSV in *P. stylifera* collected from sea landings were 3% (2/78 samples) by first step PCR and 9% (7/78 samples) by nested PCR. The incidence of WSSV in Brown shrimp (*M.* dobsoni) from sea landings were 7% (4/54 samples) by first step PCR and 22% (12/54 samples) by nested PCR. Tiger prawn (*P.monodon*) had WSSV prevalence rates of 10% (5/52 samples) by first step PCR while it was 23% (12/52 samples) by nested PCR. The prevalence of WSSV in *F. indicus* was 7% (3/38 samples) by first step PCR while it was 18% (7/38 samples) by nested PCR. WSSV was present in 2/45 (4%) *M. affinis* samples by first step PCR while it was 5/45 (11%) by nested PCR. The incidence of WSSV was 4% (1/23 samples) and 13% (3/23 samples) in *H. woodmasoni* by first step PCR and nested PCR respectively. The prevalence of WSSV in *S. serrata* samples collected from sea landings were 7% (1/14 samples) by first step PCR while it was 21% (3/14 samples) by nested PCR. The incidence levels of WSSV in *S. tranquebarica* was nil (0/16 samples) and 13% (2/16 samples) by first step PCR and nested PCR respectively. WSSV was not detected in forty three samples of blood spotted crab; *P. sanguinolentus* by first step PCR while four samples (9%) was positive by nested PCR. The prevalence of WSSV in *P. pelagic*us was 6% (1/18 samples) by first step PCR while it was 17% (3/18 samples) by nested PCR. WSSV was present in 1/15 (6%) *P. homarus* samples by nested PCR while the virus was not detected in any of the samples by first step PCR. WSSV was present in 6% (1/18 samples) in scalloped spiny lobster, *Panulirus homarus* by nested PCR while the virus was not detected in any of the samples by first step PCR. The present study indicated the presence of WSSV in wild population of shrimp. The presence of WSSV in wild population of decapods is a matter of great concern as they may act as carriers of the infection.

5.4 *Insilico* analysis to compare variations in structural protein sequences of an Indian isolate of WSSV with sequences in databank

Six envelope protein genes of WSSV; vp28, vp26, vp19 vp68, vp281 and vp466 on PCR amplification from the viral genomic DNA gave amplified products of expected size 615bp, 615bp, 366bp, 207bp, 846bp and 1401bp respectively. These sequences were submitted to the GenBank were assigned accession numbers EF534254, EF534253, EU012447, EF534252, EF534251 and EF534255 respectively. The vp28 gene sequence of the Indian isolate of WSSV (GenBank Acc. No. EF534254) obtained in the present study was compared with sequences in GenBank database. In the present study, the sequences of six envelope proteins were compared with reported sequences of WSSV isolates. A common ancestor for the virus isolate could not be designated as there was variation in sequence with one envelope protein or other.

5.5 Comparison of VP28 gene sequence of the Indian isolate of WSSV obtained in the study with that available from the GenBank

The nucleotide sequence of the envelop protein; vp28 of the Indian isolate obtained in the study was compared with twenty-eight GenBank sequences from various geographical locations. Only one sequence reported from India (Accession No.DQ013883) had 100% homology with the isolate. There was an A→G nucleotide substitution at position 125 for all the other reported sequences, which translated into a substitution of Aspartic acid for Glycine. The nucleotide change at position 125 was present only in two WSSV isolates from India including the isolate reported in this thesis indicating that this mutation is restricted to India. Two sequences from China (AF502435 and AY249434) had an additional mutation at nucleotide position 119 from G→A which translated into an amino acid substitution from Arginine to Histidine. A Chinese isolate (Accession No.AY682926) and a Korean isolate (Accession No. AF380842) had a nucleotide substitution at position 234 and 444 respectively from T→C both of which did not translate to change of amino acid. A Thailand isolate (Accession No. EF194079), a Chinese

isolate (Accession No. DQ979320) and an Indian isolate (Accession No. AY422228) had three nucleotide substitutions including that at position 125. The Thailand isolate had nucleotide substitution at position 113 from T→A and at 434 from T→C which translated into change of amino acid from valine to glutamic acid and Leucine to proline respectively. The Chinese isolate had T→C nucleotide substitutions at positions 306 and 536 with change of amino acid from valine at both positions to proline and alanine respectively. The Indian isolate had substitutions from T→C at positions 483 and 485 with change of amino acid from phenylalanine to serine in the first position while the second position was conserved. There was nucleotide substitutions at ten different positions with the isolates studied, seven of which translated to a change of amino acid.

5.6 Comparison of VP26 gene sequence of the Indian isolate of WSSV obtained in the study with that available from the GenBank

The vp26 gene sequence of an Indian isolate of WSSV obtained in this study (GenBank Acc. No. EF534253) obtained in the present study was compared with sequences in GenBank database. The nucleotide sequence of the gene encoding for envelope protein vp26 from an Indian isolate of WSSV obtained in this study was compared with 16 reported sequences and it was revealed that there was 100% similarity with fourteen of the reported sequences. The G→A nucleotide substitution at the nucleotide position 575 for the Chinese isolate (Accession No. AY220746) resulted in a change of amino acid from Arginine to Lysine while a nucleotide change for the Vietnamese isolate (Accession No. AJ551446) at nucleotide position 345 from T→C was conserved and did not translate to change of amino acid. The VP 26 gene was found to be highly conserved among isolates from different geographical locations with just two nucleotide variations from the sixteen isolates compared.

5.7 Comparison of VP19 gene sequence of the Indian isolate of WSSV obtained in the study with that available from the GenBank

The vp19 gene sequence of the Indian isolate of WSSV obtained in this study (GenBank Acc. No. EU012447) was compared with sequences in GenBank database. The vp19 sequence was compared with seventeen published sequences and it was found that there was 100% sequence similarity with four WSSV isolates reported from India, Vietnam, Taiwan and Mexico. Point mutations; T→C, T→A and C→T were found at six different nucleotide positions with 13 reported sequences. To ascertain whether the variation at nucleotide level resulted in change in at amino acid level, the protein sequences of vp19 gene were also compared. The T→C point mutation at position 57(Accession No.AY316119 and AY873786) did not result in change in amino acid while a C→T mutation at position196 (Accession No. AF332093and AF402997) resulted in change amino acid from Proline to Serine. A T→A mutation at position 218 (Accession No. AY249444 to AY249448 and AF369029) had a change in amino acid from Valine to Aspartic acid. Two reported sequences from India, (Accession Nos. AY422227 and DQ902655), gave a T→C mutation at nucleotide positions 25 and 44 that translated into change of amino acids Serine to Proline and Valine to Alanine respectively. The WSSV isolate with Accession No. DQ902655 had an extra substitution at position 287 other than at nucleotide positions 25 and 44 that translated into Methionine, a change in the amino acid from Threonine.

5.8 Variation in sequence of vp281 of an Indian isolate of WSSV obtained in this study with that of sequences in GenBank

The nucleotide sequence of vp281 of an Indian isolate of WSSV (GenBank Acc. No. EF534251) obtained in this study was compared with sequences in GenBank database.

Comparison of vp281 sequence with six published sequences revealed A→T, C→A and G→A mutations at three different nucleotide positions . The A→T mutation at nucleotide position 807 present in all the reported sequences and the G→A mutation at position 843 (Accession No. AY517490) did not translate into change of amino acid. While the C→T mutation at position 841(Accession No. DQ979321) translated into change in amino acid from Proline to Threonine.

5.9 Comparison of VP466 and vp68 gene sequence of the Indian isolate of WSSV obtained in the study with that available from the GenBank

The vp466 gene sequence of the Indian isolate of WSSV (GenBank Acc. No. EF534255) obtained in the present study was compared with sequences in GenBank database. Three reported nucleotide sequence of vp466 had 100% homology with the sequence reported in the present study. There was an A→ T nucleotide substitution at position 1212 for a Thailand isolate (Accession No AF369029), which translated into a substitution of lysine to arginine.

The vp68 sequence of the Indian isolate of WSSV (GenBank Acc. No. EF534252) obtained in the study was 100% similar with the four reported sequences (AF332093*,AF369029*, AF440570*, AF4114664).

5.10 PCR amplification to determine the prevalence of multiple viral infections in penaeid postlarvae

PCR examination 99 (95.2%) of the postlarval samples obtained from different hatcheries in Kerala gave positive result for at least one of the four viruses studied. Out of the 104 post larval *P. monodon* samples tested, 79 (76%) were positive for IHHNV, 65 (62.5%) were positive for HPV, 13 (12.5%) were positive for MBV and 13 (12.5%) were positive for WSSV. Dual to triple infection was present in 60.6% of the total postlarvae tested. Out of the 51 double positives

50 (98%) included either HPV or IHHNV infection. HPV or IHHNV was present in 11 (100%) post larval samples found positive for triple viral infection. A total of 99/104 (95.2%) post larval samples were positive for HPV and IHHNV alone or in combination with other viruses. HPV or IHHNV was present alone in 36 (34.6%) of the total post larval samples tested. Out of the 99 samples infected with virus, 79 (79.8%) of the samples had IHHNV alone or in combination with other viruses while HPV was present in 65 (65.6%) of the samples.

There is very little data on the simultaneous presence of WSSV, MBV, HPV and IHHNV in *P. monodon* postlarvae meant for stocking in aquaculture ponds. In this study, HPV and IHHNV alone or in combination was detected in 93.3% of the samples. It can be assumed that the very high rates of prevalence of HPV and IHHNV in samples are primarily due to lack of screening strategies for the presence of these viruses in India. Hence measures are yet to be initiated for control of HPV and IHHNV infection in shrimp. It is worth while to note that the percentage of hatchery reared postlarvae infected with WSSV and MBV is less. This is due to the stringent screening strategies initiated by hatcheries.

5.11 A Real-time SYBR Green PCR Assay for quantification of WSSV infection in postlarvae of *P. monodon*

A quantitative real time PCR method for detection of WSSV based on SYBR Green assay was developed. The primers were selected from ORF421 region of the WSSV genome. To determine the sensitivity of the real time PCR, a plasmid vector containing the cloned DNA fragment to be amplified was used as standard. Plasmid was serially diluted from 10^7 to 1.47 copies/ul. Strong linear correlation (r2=0.9962 to 0.998) were obtained between the threshold

cycles (Ct) and the target plasmid standard with a slope of (-) 3.488 ranging from $2.35X10^7$ to 1.47 copies of DNA

To determine the reproducibility of each reaction within each run (intra-assay) and between assays (inter-assay) $2.3X\ 10^7$ to 1.47 WSSV copies were compared in independent reactions. The correlations of variability ranged between 1.71 and 0.49 indicating that there is very little variation between the runs. With an optimal PCR mixture, 10^7 to 1.47 copies of WSSV can be detected from a specimen.

5.12 WSSV quantification in postlarvae from four hatcheries

The postlarvae collected from 4 hatcheries had a range of 1.4 to 7.9 X 10^5 WSSV copies (mean=$1.2X\ 10^4$) mg^{-1} tissue. Among the hatcheries screened, hatchery H1 showed the lowest degree of WSSV infection with mean copy number of 2.73 X 10^2 copies mg^{-1} tissue followed by H2 with $3.55X10^2$ copies mg^{-1} tissue. The highest degree of WSSV infection was from H4 with $2.8X10^3$ copies mg^{-1} tissue. Out of the 119 post larval samples, 72% were infected with WSSV. Hatchery H2 has the lowest degree of WSSV infection with 53.6% (15/31) samples negative for the virus while hatchery H3 and H4 had the highest degree of WSSV infection with 83.3% (25/30) and 80.6% (25/31) infection rates respectively. The level of WSSV was less than 100 mg^{-1} tissue in seventy samples (58.8%) and 12 (10.1%) samples showed higher levels of infection ranging from $8.67X10^2$ to $7.9X10^5$ WSSV copies mg^{-1} tissue with three samples having more than 10^5 copies, one from H4 and two from H3. The findings of this study indicates a very high level of WSSV infection (72%) among postlarvae of four hatcheries tested and hence proper screening strategies should be in place before stocking of postlarvae in rearing ponds.

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List of publications based on this thesis

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Toms C. Joseph., Roswin,J., Anbu Rajan,L. and Thampuran,N. Analysis of Two Major Viral Protein Genes of an Indian Isolate of White Spot Syndrome Virus. Presented at the 8th Asian Fisheries Forum 20-23 Nov 2007.

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- Toms C Joseph*, Roswin James, L Anburajan, P.K Surendran and Nirmala Thampuran. White Spot Syndrome Virus infection: A threat to crustacean biodiversity in Vembanad Lake, Kerala, Biodiversity and Conservation (Communicated)
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