WHITE SPOT SYNDROME VIRUS IN PENAEIDS: HISTOPATHOLOGY, DEVELOPMENT OF POLYCLONAL ANTISERA AND A COCKTAIL VACCINE

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Certificate

This is to certify that the research work presented in this thesis entitled **'White Spot Syndrome Virus in Penaeids: Histopathology, Development of Polyclonal Antisera and a Cocktail Vaccine'** is based on the original work done by Ms. M. Manjusha under my guidance, in the Faculty of Marine Sciences, Microbiology & Biochemistry, Cochin University of Science and Technology, Kochi - 682 016, in partial fulfillment of the requirements for the degree of Doctor of Philosophy and that no part of this work has previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title or recognition.

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WHITE SPOT SYNDROME VIRUS: A REVIEW

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WHITE SPOT SYNDROME VIRUS: A REVIEW

1.1 History

The virus has a history starting from a decade. It was first reported in 1992 during the mass mortality of shrimps (*Penaeus japonicus* and *P.monodon*) in Taiwan (Chen 1992, Wang *etal* 1995, Lo and Kou 1998). Subsequently, the disease was noticed during 1993 in Japan (Nakano 1994, Takahashi *et al* ., 1994) and china. It appeared in Korea and Thailand during 1994 (Wongteerasupaya *et al.,* 1995, Huang *et al.,* 1994, Chanratchakool 1996, ASCC 1995, Nash 1995, Kasornchandra *et al.,*1995, 1997) Several disastrous outbreaks of white spot syndrome disease have also occurred in the Gulf of Mexico and on the South eastern coast of US (Lightner *et al.,* 1997, Lo *et al.,* 1999, Wang *et al.,* 1999, Lightner 1999, Lightner & Redman, 1993). In India the initial outbreak was in Andrapradesh and Tamilnadu during Nov- Dec 1994 (Anon, 1995, Krishna *et al.,* 1997). During Feb-Mar 1995 it spread to Orissa and later to West Bengal. Along the West coast the disease appeared during 1995 in Goa, Karnataka (Shankar and Mohan 1998, Karunasagar *et al.,* 1997) and Kerala. In Kerala the first reported outbreak occurred in 1995 at Model Shrimp Farm and Training Centre Poyya, Trichur. Subsequently it spread throughout Kerala. (Sen *et al.,* 1999, Jasmin and Mary 2000). The White Spot Virus was first isolated accidentally while studying Yellow Head Virus (Wongteerasupaya *et al.,* 1995).

1.2 Definition

The disease is caused by a rod shaped bacilliform virus generally termed as White Spot Syndrome Virus. The generally accepted name of the pathogen are Baculoviral hypodermal and mesodermal haematopoietic necrosis virus (HHNBV), Shrimp explosive epidemic disease (SEED) in China in 1993-94 (Cai *et al.,* 1995), China virus disease/ Chinese baculovirus (CBV) (Tapay *et al* ., 1997, Lu *et al* ., 1997), Rod shaped nuclear virus, Systemic ectodermal and Mesodermal baculovirus (SEMBV) (Takahashi *et al.,*

1996, Wongteerasupaya, 1995), Red disease (RD), White spot disease (WSD), White spot baculovirus (WSBV) and White spot syndrome virus (WSSV). Studies indicate that these viruses are identical although slight differences may exist among them causing the same disease with clinical manifestation (Wongteerasupaya *et al.,* 1996, Kasornchandra *et al.,* 1998, Nadala & Loh, 1998, Park *et al .,* 1998, Lo *et al .,* 1999, Wang *et al .,*1999).

1.3 Classification

Due to the morphology, size, site of assembly and nucleic acid of the virus, it has been proposed to be a member of the genes Non- occluded baculovirus, subfamily Nudibaculovirinae and family Baculoviridae. In 1995, the International Committee on Taxonomy of viruses (ICTV) deleted the genus Non-occluded baculovirus and the subfamily Nudibaculovirinae and left the viruses previously in this classification as unassinged invertebrate viruses. (Murphy *et al .,* 1995). Analysis of a 12kbp fragment of the 200kbp geneome of white spot syndrome virus of shrimp (WSSV) revealed that the virus resembled baculoviruses in morphology and pathology. Eight open reading frames were apparent including genes for the large and small subunits of ribonucleotide reductases phylogenetic analysis showed that these genes did not share an immediate common ancestor with the corresponding baculovirus genes. The data suggested that WSSV is either a member of a novel genus in the family baculoviridae, or a possible representative of the family. The name Whispovirus (a siglum for White spot) were proposed (Van Hulten, 1999).

1.4 Geographical distribution

It emerged during the early 1990s in Taiwan (Chen 1992) and has caused a serious ongoing epizootic in the shrimp growing countries of Asia, including China, India, Thailand, Japan, Korea, Indonesia, Malaysia, Vietnam, Philippines, Australia (Inouye *et al .,* 1994, 1996, Momoyama *et al .,* 1994, Nakano *et al .,* 1994, Takahashi *et al .,* 1994, Chen 1995, Flegel *et al .,* 1995, Huang *et al .,* 1995, Wang *et al .,* 1995, Wongteerasupaya *et al .,* 1995, Kimura *et al.,* 1996, Mohan *et al .,* 1998, Magbanua *et al .,* 2000, Flegel 1996, Edgerton 1996). Many disastrous outbreaks occurred in Gulf of Mexico and South eastern coast of the United States (Lightner *et al .,* 1997, Lo *et al .,*

1999, Wang *et al* ., 1999) Aquaculture 1999 available at www.aphis.usda.gov/vs/aqua/wss.html.)

1.5 Species affected

Almost all the species of penaeid shrimp are susceptible to White Spot Syndrome Virus (WSSV) infection. The major species naturally infected by the virus include *Penaeus monodon, P.chinensis, P.indicus, P.pencillates, P.japonicus* (Inouye *et al.,* 1994, 1996, Nakano *et al.,* 1994, Takahashi *et al.,* 1994, Chou *et al.,* 1995, 1998, Flegel *et al.,*1995, Huang *et al.,* 1995, Wang *et al.,* 1995, Wongteerasupaya *et al.,* 1995, 1996, Chang *et al.,*1996, Kimura *et al.,* 1996, Lo *et al.,* 1996, Kasornchandra *et al.,* 1998, Mohan *et al* ., 1998, Nunan *et al* ., 1998, Park *et al* ., 1998). Mortality due to WSSV have also been observed in *P. setiferus* from the State of Texas and South Carolina in the USA (Lightner *et al.,* 1997, Lo *et al.,* 1999, Wang *et al.,* 1999). Other penaeid prawns infected with WSSV include *Metapenaeus ensis, P.aztecus, P.duorarum, P.merguiensis, P.semisulcatus, P.stylirostris, P.vannamei* and *Trachypenaeus curvirostris* (Cai *et al.,* 1995, Lightner *et al* ., 1997, 1998, Nunan and Lightner 1997, Tapay *et al* ., 1997, Chang *et al.,* 1998 (c), Nunan *et al.,* 1998, Wang *et al.,* 1998, Wang *et al.,* 1999). Non penaeid species infected include *Exopalaemon orientalis, Macrobrachium rosenbergii, Orconectes punctimanus and Procambarus sp* (Richman *et al.,* 1997, Chang *et al.,*1998 (c), Peng *et al.,* 1998, Wang *et al.,* 1998).

1.6 Structure of WSSV

Envelope: The morphology of the negatively stained intact WSSV virions was non occluded, largely rod shaped to somewhat elliptical, with an average size of 110-130nm in diameter and 260-350 nm in length. Each viral particle has a long tail like envelope extension at one extremity. The envelop was clearly trilaminar, consisting of 2 electron opaque layers separated by 1 electron lucent layer (Huang *et al.,* 2001, Wang *et al.,*1999, Durand *et al.,* 1997, Wang *et al.,* 2000(b), Wongteerasupaya *et al.,* 1995, Nadala *et al.,* 1998, Inouye *et al.,* 1994, Adams and Mc. Clintock 1991).

Capsid: They were cylindrical in shape with one end flat and the other end pointed. The capsid measured 244 +/- 28nm by 80+/-11 nm, the extended nucleocapsid showed a pattern of electron opaque bands (18nm) alternating with electron transparent bands 3nm arranged perpendicular to the long axis of the nucleocapsid. (Hameed *et al.,* 1998, Wang *et al.,* 1999, Durand *et al.,* 1997, Wang *et al* ., 2000(b), Takahashi *et al.,*1994, Hang *et al.,* 2001). Around 15 conspicuous vertical helices located along the long axis, of the rod shaped nucleocapsid core were also evident. Each helix with in the nucleocapsid has 2 parallel striations composed of 14 globular capsomers or sub units each of which are 8nm in diameter. The size of each helix and striation is 19 x 80 and 8 x 80 respectively. The spacing between each helix is 7nm, while the two striations with each helix is 3nm apart. (Huang *et al* ., 2001).

Genome: A double stranded circular DNA molecule longer than 150kbp (Wang *et al.,* 1995), 305kbp (Zhang *et al.,* 2001(b), 200Kbp (Yang *et al.,* 1997). Characterization and partial cloning of the genomic DNA of the baculovirus from *P.japonicus* was carried out (Arimoto, 1995). The genomic variations among geographical isolates of White Spot Syndrome virus using restriction analysis and southern blot hybridization was carried out and found that only slight variation exist between them (Wang *et al.,* 2000(a). The WSSV genomic DNA was sequenced (Zhang *et al.,* 2001(b), Yang *et al.,* 2001) and several genes encoding for the basic proteins have been identified (Zhang *et al.,* 2001(b), Van Hulten *et al.,* 2000). Initially the virus was thought to have only 3 structural proteins such as 27, 22 and 18KDa (Hameed *et al.,* 1998), and later four proteins such as 19, 23.5, 27.5 and 75Kda (Nadala *et al.,* 1998, Nadala and Loh (1998) 28, 26, 24, and 19Kda (Van Halten *et al.,* 2000, 2002), 19, 23, 25 Kda (Wang *et al* ., 2000). Later around 13 consistent protein bands ranging from 16 K Da to 190 K Da were identified (Huang *et al* ., 2001). The morphogenesis of WSSV have been described by several researchers (Durand *et al* ., 1997(a), Wang *et al* ., 1997, Takahashi *et al* ., 1994, Wang *et al* ., 2000, Wang *et al* .,1999).

1.7 Clinical signs

General clinical signs of the disease are reddish discoloration with white spots on the exoskeleton and epidermis with muscle opacity, lethargy, surfacing frequently, loss of balance, reduced feeding and preening activity, molting inhibition (in certain cases) and reddening of uropod, telson, and periopods (Takahashi *et al.,* 1994, Nakano *et al.,* 1994, Chen and Kou 1994, Rajan *et al .,* 2000, Kasornchandra *et al.,* 1994, Hameed *et al.,* 1998, Momoyama *et al.,* 1994, Chou *et al.,* 1995, Wang *et al.,* 1995, Lightner 1996, Peng *et al.,* 1998). A preliminary study on the developing mechanism of the characteristic white spots on the shell in *P.monodon* was carried out (Wang *et al.,* 1996). One of the features of this virus is the transformation of latent to patent stage. It has been noticed that the latent stage persists for longer – months together, and the transformation of latent to patent takes within hours under stressful conditions. Stresses could be crowding, high temperature, oxygen depletion, ammonia toxicity at high pH, hydrogen sulphide, very high and very low salinity and even periopod excision. (Peng and Lo, 1998, Peng *et al.,* 1997, Kasornchandra *et al.,* 1998, Kou and Lu, 1997, Hameed *et al.,* 1998).

1.8 Mode of transmission

Mode of transmission of the virus can be through various ways. It has been noticed that frozen products exported from Asian countries contained infectious virus particles. Principally this is transmitted through water and natural feed (Rajan *et al.,* 2000, Nakano *et al.,* 1994, Shankar and Mohan 1998, Chou *et al.,*1995, Andres Soto *et al.,* 2001). The presence of WSSV was detected in frozen commodity shrimp imported to US (Overstreet *et al.,* 1998). Massive transmission is through death and disintegration of the infected animals. Meanwhile vertical transmission also has been demonstrated. (Mohan *et al.,* 1997, Tsai *et al.,* 1999, Lo *et al.,* 1997 Bootland *et al.,*1991). Various lab experiments to study the mode of transmission of WSSV have been carried out (Supamattaya *et al.,* 1998, Chang *et al.,* 2001, Kanchanaphum *et al.,* 1998).

1.9 Carriers/ reservoirs

The virus has a wide range of potential hosts (Flegel, 1997). It infects not only several species of penaeid shrimp including those cultivated in the Western hemisphere

(Lu *et al.,* 1997(b) and also a wide range of other decapods including crabs and other related crustaceans (Chen *et al.,* 2000). In Taiwan, (Peng *et al.,* 1998, Chang *et al.,* 1998 (c), and Wang *et al.,* 1998, Wang *et al.,* 1997(b) polymerase chain reaction (PCR) analysis along with detailed histology including TEM and *in situ* hybridization, confirmed that many of the suspected carriers are indeed infected. Some carriers have been shown to transmit the virus to *P.monodon.* These carriers include penaeid shrimps, other shrimps, crabs, lobsters, copepods and insect larvae. Certain prawns such as *Metapenaeus dobsoni*, *Parapenaeopsis stylifera*, *Solenocera indica*, *Squilla mantis* and certain crabs like *Charybdis annulata*, *C.cruciata*, *Macropthalmus sulcatus, Gelasimus marionis nitidus, Metopograpsus messor* were also detected as the new hosts of WSSV (Hossain *et al .,* 2001). Similar studies in Thailand have confirmed that local crabs can be carriers. One of the studies by Supamattaya *et al .,* 1998, showed that the swimming crab *Portunus pelagicus* and the mud crab, *Scylla serrata* could be infected with white spot disease virus by injection or feeding. Moreover, these crabs subsequently showed typical white spot viral disease histopathology by light and electron microscopy. Leisons were positive by *in situ* hybridization with a DNA probe specific for white spot disease virus (Wongteerasupaya 1996). Two fresh water crabs (*Paratelphusa hydrodomous* and *P.pulvinata*) were foung to be hosts for WSSV (Hameed *et al.,* 2001). Rajendran *et al.,* 1999, conducted experimental studies on the southeast coast of India by injecting or feeding white spot virus obtained from infected *P.monodon* to five species of shrimp (*P.monodon, P.indicus, P.semisulcatus*, *Metapenaeus monocerus and M. dobsonii*), 2 species of freshwater prawns (*Macrobrachium rosenbergii and M.idella*), four species of crab (*S. serrata, S.tranquebarica, Metapograpsus sp and Sesarma sp*) and 3 species of lobster (*Panulirus homarus, P.ornatus, and P.polyphagus*). All species examined were susceptible to the virus. Experimental infections in the shrimp had the same clinical signs and histopathological characteristics as in naturally infected *P.monodon.* A cumulative mortality of 100% was observed with in 5 to 7 days in shrimp injected with white spot disease virus and 7 to 9 days in shrimp fed with infected tissue. Two species of mud crab (*S. serrata and S. tranquebarica*) survived the infection for 30 days without any clinical symptoms. All 3 species of lobster survived the infection for 70 days without clinical symptoms. However, bioassay and histological studies revealed that crabs, prawns and

lobsters may act as asymptomatic carriers/ reservoir hosts of white spot disease virus. This is the first report with evidence of the carrier/ reservoir capacity of these hosts through histological and bioassay evidence. Experimental infection with WSSV in the cray fishes *Cherax quandricarinatus and Pacifastacus leniusculus* revealed it as a potential host of WSSV (Shi *et al .*, 2000, Jiravanichpaisal *et al .*, 2001). An investigation to check artemia as a possible vector for WSSV proved that it cannot transmit the disease and so cannot be considered as a vector (Hameed *et al.*, 2002) The tolerance of fresh water prawn *Macrobrachium rosenbergii* to WSSV was also studied (Hameed *et al .*, 2000).

The following species have been reported in peer reviewed literature to be hosts or carriers of the White Spot Syndrome Virus (WSSV). Under the heading "Type of Infection" the source of the animals are noted as either from culturing facilities (Cul), the wild (Wild), nuisance species that are naturally present in culturing facilities (Nat), and experimentally infected animal (EXP) (Lan *et al.*, 1996).

Scientific Name	**Common Name**	**Type of Infection**	**Reference**
Marsupenaeus japonicus	Kuruma shrimp	Cul, Wild, Exp	Inouye *et al .*, 1994; Takahashi *et al .*, 1994; Lo *et al .*,. 1996b
Penaeus monodon	Giant tiger shrimp	Cul, Wild, Exp	Woongteerasupaya *et al .*, 1995; Lo *et al .*, 1996b
Penaeus semisulcatus	Green tiger shrimp	Wild, Nat	Lo *et al .*, 166b; Maeda *et al .*, 1998
Fenneropenaeus penicillatus	Redtail shrimp	Cul, Wild	Chou *et al .*, 1995; Lo *et al .*, 1996b
Fenneropenaeus indicus	-	Cul, Nat	Woongteerasupaya *et al .*, 1996; Lightner, 1996
Fenneropenaeus chinensis	Fleshy shrimp	Cul, Nat	Woongteerasupaya *et al .*, 1996; Lightner, 1996

Fenneropenaeus merguiensis	-	Cul, Nat	Woongteerasupaya *et al* ., 1996; Lightner, 1996
Metapenaeus ensis	Greasyback shrimp	Cul, Wild, Exp	Wang *et al* ., 1997, 1998; Lo *et al* ., 1996b
Litopenaeus setiferus	White shrimp	Nat, Exp	Lightner, 1996
Litopenaeus stylirostris	-	Exp	Lightner, 1996
Litopenaeus vannamei	Pacific white shrimp	Cul, Exp	Woongteerasupaya *et al* .,1996; Lightner, 1996
Farfantepenaeus aztecus	Brown shrimp	Exp	Lightner, 1996
Farfantepenaeus duorarum	Pink shrimp	Exp	Wang *et al* ., 1999
Palaemonidae	Grass shrimp	Nat	Lo *et al* ., 1996b
Alpheus brevicristatus	Snapping shrimp	Nat	Maeda *et al* ., 1998
Alpheus lobidens	Snapping shrimp	Nat	Maeda *et al* ., 1998
Trachypenaeus curvirostris	Southern rough shrimp	Exp	Wang *et al* ., 1998
Macrobrachium rosenbergii	Giant freshwater shrimp	Cul	Lo *et al* ., 1996b
Palaemon serrifer	-	Nat	Maeda *et al* ., 1998

Charybdis japonica	-	Nat	Maeda *et al* .,1998
Helice tridens	-	Nat	Maeda *et al* ., 1998
Hemigrapsus sanguineus	-	Nat	Maeda *et al* ., 1998
Ocypode stimpsoni	-	Nat	Maeda *et al* ., 1998
Petrolisthes japonicus	-	Nat	Maeda *et al* ., 1998
Portunus trituberculatus	-	Nat	Maeda *et al* ., 1998
Portunus pelagicus	Sand Crab	Wild, Exp	Lo *et al* ., 1996b; Supamattaya *et al* ., 1998
Portunus sanguinolentus	-	Wild, Exp	Lo *et al* ., 1996b;Wang *et al* ., 1998
Upogebia major	-	Nat	Maeda *et al* ., 1998
Scylla serrata	Mud crab	Cul, Exp	Lo *et al* ., 1996b; Kanchanaphum *et al* ., 1998; Supamattaya *et al* ., 1998
Acetes sp.	Krill	Exp	Supamattaya *et al* ., 1998
Calappa lophos	Box crab	Exp	Wang *et al* ., 1998
Charybdis feriatus	-	Wild, Exp	Lo *et al* ., 1996b; Wang *et al* ., 1998
Charybdis granulata	-	Exp	Wang *et al* ., 1998
Panulirus ornatus	Ornata spiny lobster	Exp	Wang *et al* ., 1998
Panulirus longipes	Longlegged spiny lobster	Exp	Wang *et al* ., 1998
Panulirus	Painted	Exp	Wang *et al* ., 1998

versicolor	spiny lobster		
Panulirus penicillatus	Pronghorn spiny lobster	Exp	Wang *et al* ., 1998
Uca pugilator	Calico fiddler crab	Exp	Kanchanaphum *et al* ., 1998
Sesarma sp.	-	Exp	Kanchanaphum *et al* ., 1998
Exopalaemon orientalis	-	Exp	Wang *et al* ., 1998
Procambarus clarkii	-	Exp	Huang *et al.*, 2001
Copepod	-	Nat	Lo *et al* ., 1996b
Larvae of Ephydiran insect	-	Nat	Lo *et al* .,1996b

1.10 Secondary pathogens

Viral infections are typically accompanied by secondary bacterial infections (Karunasagar *et al.*, 1997) which may actually be the ultimate cause of death in a shrimp (Lightner, 1996). Vibrio species are found associated with WSSV (Alapide & Dureza 1997, Karunasagar *et al.*, 1997). The bacteriological study undertaken on white spot diseased shrimp revealed the presence of 4 species of bacteria, *V.alginolyticus, V.parahaemolyticus, V.anguillarum, and Pseudomonas aeruginosa and V.alginolyticus* as the most dominant and virulent species (Jayasree *et al.*, 2000).

1.11 Diagnostic methods

a. Histology:

Histological observation revealed that the virus has tissue tropism specifically to tissues and organs of mesodermal and ectodermal origin. (Wongteerasupaya *et al.*, 1995, 1996, Chang *et al* ., 1996, Rajendran *et al* ., 1999, Flegel *et al* ., 1996,). Histopathological

condition includes severe nuclear hypertrophy, cellular degeneration, multifocal necrosis and hemocytic encapsulation in the infected tissues. (Wongteerasupaya *et al.*, 1995, 1996, Chang *et al* ., 1996, Rajendran *et al* ., 1999, Lo *et al* ., 1997, Karunasagar *et al* ., 1997, Flegel *et al* ., 1996(b), Mohan *et al* ., 1998, 1997, Lo *et al*(a) ., 1996, Sudha *et al* ., 1998, Wang *et al* ., 1997(a). Tissues of ectodermal and mesodermal origin such as sub cuticular shell epithelium, gill epithelium, sub cuticular stomach epithelium, connective tissue, haematopoietic tissue, antennal gland and nervous tissue are severely infected by the virus. Development of intercellular hypertrophy observed in cells in the necrotic tissue was different in different stages of the viral infection. Eosinophilic intranuclear inclusions surrounded by marginated basophilic chromatin were found in the early stage. It was followed by enlargement of the eosinophilic intracellular inclusions and finally the swollen nuclei were filled with a prominent pale basophilic inclusion, which occupied most of the cytoplasm of the infected cell (Kasornchandra *et al.*, 1998).

b. Electron microscopy:

Electron microscopic examination revealed the presence of double walled enveloped, non-occluded rod shaped virions. Complete virus is typically characterized by an apical envelope extension. The nucleocapsid displays a superficially segmented appearance. Each segment seems to be formed of sub units, which are arranged in 2 parallel rows. The cylinder representing the nucleocapsid is closed at one extremity by a smaller segment those forms a slightly rounded end while the opposite extremity is squared. Different views on the pattern of morphogenesis exist (Durand *et al.*, 1996, Huang *et al.*, 1995, Huang *et al.*, 2001, Inouye *et al.*, 1994, Takahashi *et al.*, 1994, Wongteerasupaya, 1995).

c. DNA based diagnostics:

DNA hybridization probes for the white spot disease virus have been developed by several laboratories (Chang *et al.*, 1996, Durand *et al.*, 1996). The primers for detection of this virus by PCR technology have also been developed,

(a) F1 5'ACTACTAACTTCAGCCTATCTAG3',

R15'TAATGCGGGTGTAATGTTCTTACG3',

F2 5' GTAACTGCCCCTTCCATCTCC3',

R2 5' TACGGCAGCTGCTGCACCTTGT3'(Lo *et al* ., 1996) where primers F1 and R1 amplify a 1447 bp fragment on the WSSV genome while F2 and R2 amplify a 941 bp fragment internal to the 1447bp fragment. Kasornchandra *et al* .,1998 developed another primer,

(b) F1 5'TCACATCGAGAGACCTCTGTAC3'

R1 5' TCTAGGACGGACGGACTATGGCAA3' Which amplifies a 520bp fragment. Amplified DNA of viral isolates from Thailand, Indonesia, Malaysia, China, Taiwan and Japan.

Yet another primer developed by Thakahashi *et al* ., 1996 is,

(c) F1 5'GACAGAGATATGCAGGCCAA3'

R1 5'ACCAGTGTTTCGTCATGGAG3'

Various other primers have been developed for the detection of WSSV (Wang *et al.,* 1996 (a), Nunan and Lightner 1997, Marielle *et al.,* 2000, Karunasagar *et al.,* (unpublished), Vijayan *et al.,* (unpublished). Two commercial kits are available in India marketed by Mangalore Biotech (P) Ltd, Mangalore and Bangalore Genei (P) Ltd, Bangalore.

Several methods are available for the detection of white spot disease virus, which include PCR (Kim *et al.,* 1998, Nunan *et al.,* 1998, Peng *et al.,* 1998, Hsu *et al.,* 1999, Lo *et al.,* 1996 (b), Kaitpathomchai *et al* ., 2001, Tang & Lightner, 2000, Tan *et al* ., 2001 Otta *et al.,* 1999), *in situ* hybridization (Chang *et al.,* 1996, Durand *et al.,* 1996, Wongteerasupaya *et al.,* 1996, Chang *et al.,* 1998, Chang *et al.,* 1996, Tsai *et al.,* 1999) dot blot hybridization (Wongteerasupaya *et al.,* 1996, Hameed *et al.,* 1998) and ELISA (Hameed *et al* ., 1998). A non-stop, single tube, semi-nested PCR technique for grading the severity of WSSV was also put in use (Kiatpathomchai *et al.,* 2001). Quantification of White spot syndrome virus DNA through a competitive polymerase chain reaction was also done (Tang & Lightner, 2000). Tapay *et al.,* 1999 developed primers for PCR based on the sequence of a cloned fragment of the white spot disease virus genome and used the primers to detect white spot disease virus from both experimentally and naturally infected shrimp. They developed one step and two step PCR protocol as a very sensitive and

specific alternative protocol to Western blot assay for the detection of white spot disease virus. A sensitive immunodot assay for WSSV was developed using the specific rabbit polyclonal antiserum developed from a truncated version of the WSSV 27.5 KDa envelope protein (You *et al.*, 2002, Zhang *et al* ., 2001). A dot blot nitrocellulose enzyme immunoassay has been developed against WSSV (Nadala and Loh, 2000). Westernblot (Nadala *et al* ., 1997 , Bruce *et al.*, 1993), dot blot (Chang *et al* ., 1998 (b), Southern blot hybridization (Wang *et al* ., 2000 (b)) Monoclonal antibodies (Zang *et al* ., 1999, Zhan *et al.*, 1999, Poulos *et al* ., 2001, Shih *et al* ., 2001, Liu *et al* ., 2001,Anil *et al* ., 2002) were also used as diagnostic tools. An immunoassay with recombinant antigen of WSSV was also carried out. Primary shrimp cell culture was also used for the study on WSSV (Kasornchandra & Boonyaratpalin, 1998).

1.12 Management

Prevention and control of WSSV infection:

The major routes of infection are the infected water and carrier shrimp (Flagel *et al.*, 1995).

The best immediate approach to manage this virus is to implement a package of preventive measures. These include pond preparation by disinfection and elimination of potential viral carriers, the use of filters at the inlets to remove potential carriers, the refusal to use fresh feed inputs, disinfection of ponds before discharge, and cessation of water exchange for 4 days after a discharge. Monitoring of brood stock, post larvae and pond reared shrimp using DNA probes. The most effective disinfection agent appears to be chlorine at approximately 30 ppm. However, since the virus does not seem to remain infections for more than a few days when free in seawater a simple process of storage can remove this threat, so long as no carriers are present. Implementing this package will require a good deal of cooperation on the part of the shrimp farmers.

Although no treatment are known that will rescue infected shrimp, work originating at NICA has indicated that some medicinal plant extracts may be effective in preventing YHV infection in aquarium trials (Direkbusarakom & Ruangpan, 1998).

Further tests are underway to confirm these results and to try to determine the mechanism of protection. In addition there are indications that various nutrient supplements (eg. Vitamin- C, HUFA's, Astaxan) may improve chances of escaping from the virus. There are still claims that various bacterial amendments can be used to prevent YHV infections, if they are used continuously. It appears that the most effective disinfectant for WSSV is formalin (Pratanpipat *et al .,* 1996). It is effective at 70 ppm (or even as little as 20 ppm in aquarium tests) preventing transmission through water. This level may not directly harm the plankton bloom and a consequent drop in DO. The situation is such that the treatment may not deal with the carrier status of the virus and application of 70 ppm of formalin at 6 hourly intervals is apparently required to prevent the transmission by cohabitation.

The post larvae are strongly implicated as the possible route of WSSV transmission to grow out systems.However, there are ways to block this route effectively. It is shown that the impact of WSSV & MBV can be substantially reduced or essentially eliminate by simply washing nauplei, with or without disinfectant after they are harvested from spawning tanks (Chen, 1992). The practice of feeding fresh crab to brood stock animals should also be stopped. If these measures are combined with prior brood stock screening with a DNA probe, PL assay with either DNA probe or by way of diagnostic PCR, before stocking, it should be possible to close this route completely.

Mohan and Shankar, 1997 are of the opinion that the endodermal cells are not affected and the infected shrimp may not shed the virus along with faces as it happens in the case of monodon baculovirus (MBV) which is found only in midgut and hepatopancrease. Death and disintegration of a WSSV infected shrimp appears to contribute significantly to the viral load in the water. Removal of dead and moribund shrimps in practical as an important management tool in shrimp farms of Thailand to minimize the viral load in the water.

Karunasagar *et al.,* (1996) reported the use of an immunostimulant developed by them as 'Aquastim' containing yeast glucan and a bacterial product. According to them,

the use of 'Aquastim' is perfectly environment friendly technology unlike the technology of using chlorine and other anti microbial chemicals. However, regular application and good water quality management would be important for successful cultivation. In corporation of vitamin C in the feed can be recommended because this has been shown to enhance the immune response in shrimp.

Rao, 1996 proposed the following management techniques for successful cultivation of prawns, which include Pond preparation as the major task. As a precautionary measure to present the virulence of shrimp virus, an antiviral herbal powder made by fairly pulverizing the sun dried complex plant of *Phyllanthus niruri*, a herb belonging to *Euphorbiaceae* family has been reported. The antiviral herbal powder should be administrated in four doses during the culture period in monthly intervals. The single dose should be 1-2 g/Kg feed for 3 days.

In case of white spot disease infected farms the disease can be cured by adopting following procedures.

Continuous or daily draining of bottom water and pumping in fresh water till the problem is solved. Make sure that there will be no abnormal or sudden change of vital parameters. If change in inevitable, it should be gradual. Step should be taken to ensure proper phytoplankton management. Administer anti-viral powder made from *Phyllanthus ninuri* at the dose of 2 tp 3 g/Kg feed /day for 4 to 5 days. The use of *Phyllanthus spp* and *Clinicanthus mutants* for shrimp viral disease cure was supported by Dr. Boonsirm Withyachumnarnkul, Department of Anatomy, Machidol University, Bangkok, Thailand and the group - C.P. One litre / acre of a standard iodophore to inactivate virus and to oxidise NH_3 has been recommended.

The technique known as SLC-URINUM therapy is carried out by mixing SLC-URINUM with the supplementary pelleted feed to the shrimp which was kept at room temperature for about 2-3 hrs (Chondar, 1996).

Yaligar and Pai, (1996) described the use of *Calotropis gigantea* to manage WSSV in shrimp ponds.

Anti viral activity has been reported in shrimp for lipopolysacchrides (Newman,1999), peptidoglycans from several species of bacteria such as *Bifidobacterium* (Itami *et al* ., 1998) , *Bacillus* (Takahashi *et al.,* 1998) and glucans from *Schizophyllan commune* and *Saccharomyces cereviseae* (Song *et al.,* 1997) all were reported to have a protective effect on shrimp against WSSV. Meanwhile a sulfated polysaccharide fucoidan has been found to be active on enveloped virus (Takahashi *et al.,* 1998).

For the management of WSSV rapid diagnostic technique for the avoidance, determination of carriers, reservoirs of infection, development of specific pathogens free (SPF) or resistant brood stock have to be given top priority. Another management strategy shall be individual spawning of females and separate rearing of the resulting larvae. Further, a set of similar non-destructive quantitative measures of shrimp health, which can be used as part of early warning system, has to be developed. Perhaps studies on crustacean immunity (eg. types of haemocytes, enzyme activities, haemolymph factors etc.) will give some of the needed tools. Immunostimulants, probiotics and vaccines as desired to replace antibiotics along with good management factors can be incorporated in the management regime.

1.13 Conclusion

Since its emergence as the most important single pathogen in shrimp culture systems WSSV has been given utmost importance among researchers. Thanks to their efforts, so much is known now about the pathogen but still inadequate to have a fool-proof management strategy. Considering this requirement, the present work was undertaken on three aspects such as,

1. Demonstration of the WSSV by histological and electron microscopic preparations and unraveling its morphogenesis,
2. Development of hyperimmune polyclonal antiserum and
3. Development of a vaccine.

It is hoped that the achievements in the three fronts shall contribute to develop an appropriate strategy for the management of WSSV.

CHAPTER – 2

HISTOLOGICAL DEMONSTRATION OF WHITE SPOT VIRUS AND THE HISTOPATHOLOGY IN EXPERIMENTALLY INFECTED MORIBUND *PENAEUS INDICUS* H.MILNE EDWARDS IN INDIA

CHAPTER – 2

HISTOLOGICAL DEMONSTRATION OF WHITE SPOT VIRUS AND THE HISTOPATHOLOGY IN EXPERIMENTALLY INFECTED MORIBUND *PENAEUS INDICUS* H.MILNE EDWARDS IN INDIA

2.1 Introduction

White Spot Syndrome Virus (WSSV) continues to be highly pathogenic to penaeid shrimp causing significant mortalities since its advent in 1992. It has adversely affected the shrimp industry considerably in Asia, spreading throughout East and South East Asia, Indonesia, Thailand, The Philippines, Japan, China, India and all other shrimp growing countries in the region (Mohan *et al.,* 1997). The virus has tissue tropism specifically to tissues and organs of mesodermal and ectodermal origin (Wongteerasupaya *et al.,*1995, 1996, Chang *et al.,*1996, Rajendran *et al.,*1999, Flegel *et al.,*1996 ,Wang *et al.*, 1999(a)). Histopathological studies carried out in wild specimens of *Penaeus monodon* have revealed, in general, severe nuclear hypertrophy, cellular disintegration, multi-focal necrosis and hemocytic encapsulation in the infected tissues (Wongteerasupaya *et al.*, 1995, 1996, Chang *et al.,*1996 , Rajendran *et al.,*1999, Lo *et al.,*1997, Karunasagar *et al.*,1997, Mohan *et al.,*1998) . These studies were based mostly on *Penaeus monodon* and comparatively very little work had been done on *Penaeus indicus.*

Our study is the first comprehensive approach describing the histopathology of White Spot Disease (WSD) in *P.indicus* and it includes an investigation in the histomorphology of healthy and white spot syndrome virus infected tissues, and organs such as gill, foregut, stomach, midgut, hindgut, hepatopancreas, nerve, ovary, eye and integument. The suitability of each organ/tissue for easy demonstration of the viral involvement by histological means is illustrated.

2.2 Materials and methods

2.2.1 Source of Virus

A heavily infected brood stock of *P.monodon* with clinical manifestations of the disease such as white spots in the inner surface of carapace, reddening of pleopods and empty intestine was the source of the virus. Matsyafed, Government of Kerala supplied this animal, from a batch of wild spawners brought from Vishakapatnam, Andhra Pradesh, in 1995, for larval production.

2.2.2 Experimental animals

A batch of *P. indicus* post larvae generated from a single brood stock was reared in a hatchery for more than four months in aged (5-6 months, Salinity 30ppt) seawater and on attaining a weight of 5 to 7g was used for the experimental infection and passage of the virus. Before experimental infection, the shrimps were subjected to formalin stress test by maintaining them for one hour in 20-ppt seawater containing 100-ppm formalin with adequate aeration. They were then observed for three days for the manifestation of any disease. The healthy ones which survived the stress test were transferred into experimental tanks for further study.

2.2.3 Pathogen

Presence of white spot syndrome virus (WSSV) in the source tissue and experimental test shrimps was confirmed by Electron microscopy (Fig –1a) as well as by diagnostic PCR(Fig -1b) following Lo *et al.,* (1996(b). The primers were synthesized by M/S Bangalore Genei, Bangalore, India.

2.2.4 Preparation of virus inoculum

A sample of about 500 mg gill tissue was dissected from the donor *Penaeus monodon*, and macerated in cold 10 ml PBS (NaCl - 8g, KCl- 0.2g, Na_2HPO_4-1.15g, KH_2PO_4- 0.2g, double distilled water –1000 ml) with glass wool to a homogeneous mass using mortar and pestle in an ice bath. The homogenate was centrifuged at 8200-x g in a refrigerated centrifuge (REMI C.24) at 4°C, and the supernatant fluid was filter-sterilized by passing it through a 0.22μ pore size membrane filter. The preparation was streaked on ZoBell's agar plates and incubated at 28 ± 2°C for 72 hours to determine the presence or absence of bacteria.

2.2.5 Experimental infection

An aliquot of 0.01ml filtrate was inoculated at the dorsal side of the abdomen of *P.indicus* in the space between the telson spine and the last abdominal segment using a 1ml-tuberculin syringe. Five shrimps were kept in 40 x 25 x 10 cm fibre-glass tanks containing 20L filtered and aged sea water (20ppt) at a temperature of 28 ± 2°C, with continuous aeration. The shrimps were fed *ad libitum* with pelleted Higashi Maru feed containing 40% protein. One third of water was replaced daily with fresh filtered and aged seawater (20 ppt). The animals were observed for cessation of feeding, lethargy and mortality. The virus was re-isolated following the above procedure and inoculated into the next batch of animals. This was repeated in five batches of shrimps and the moribund shrimps of the fifth passage were fixed for histopathological studies.

A set of healthy shrimps, subjected to stress test, was maintained as control and used for histological preparations.

2.2.6 Histopathology

Moribund animals as well as control animals were fixed by injecting 1-3 ml (depending on size) Davidson's fluid at the 6th segment at a dorsal point, in-between the telson spine and the segment. Immediately after the injection, the cuticle was split sagittally using a surgical scalpel blade and immersed in Davidson's fluid (95% Ethyl alcohol-330ml: Formalin-220ml; Glacial acetic acid- 115ml made up to 1 L using distilled water) for 24 hours. Subsequently, the animals were dissected and the gills, heart, nerve cord, stomach, foregut, midgut, hindgut, hepatopancreas, eye, integument and ovary were transferred to 70% ethyl alcohol, processed for histopathological studies as shown in the Table-1 and later, blocked, sectioned and double stained with haematoxylin and eosin (Bell and Lightner, 1988) and examined using a light microscope. (Nikon Type 104).

2.2.7 Electron Microscopy

For Electron Microscopy, gill tissue from experimentally infected *Penaeus indicus* was removed and fixed in 2.5% glutaraldehyde (Electron Microscopy Sciences, USA) in PBS (1M), at pH 7.4 for 24 hours at 4°C and post fixed in 2% Osmium tetroxide (Electron Microscopy Sciences, USA) in PBS (1M) at pH 7.4 for 2 hours at 4°C. After dehydration through an ascending series of acetone the tissue pieces were embedded in epoxy resin (Nonenyl Succine Anhydride 7.8ml; Vinyl cyclohexane dioxide - 3ml; DER resin- 1.8ml; 2- dimethylamino ethanol- 0.12 ml) as shown in the Table-2, Ultra thin (0.5µ) sections were, stained with uranyl acetate and lead citrate (Electron Microscopy Sciences, USA) and examined under CM-10, Philips Electron Microscope.

2.3 Results and discussion

2.3.1 Foregut

The foregut is an elongated sac-like region of the alimentary canal consisting of mouth, oesophagus, and stomach and associated glands. In infected P. *indicus* the entire foregut showed marked degeneration and disintegration especially in the ventral median channel (VMC), ventro lateral folds (VLF) (Fig 2 & 3) and in the dorsal grooves (DG), dorsal median folds (DMF) and intra lateral cardiac plate (ILCP) (Fig 4 & 5). Rajendran *et al.* (1999) on investigating white spot virus in shrimps crabs and lobsters demonstrated acute degeneration, marked hypertrophy of nuclei with chromatin margination and karyorexis. However, this was a generalized observation unlike the specific pathological changes, which could be demonstrated in the foregut epithelial lining in our study. As Sudha *et al.*, (1998) pointed out, the foregut cuticular epithelium was one of the target tissues in which the viral infection could be easily demonstrated as hypertrophied nuclei, at higher magnification (100X) in deeply stained columnar cells of the inner epithelial lining (Fig 6). The underlying muscular layer of the foregut showed multifocal necrosis, an observation hitherto not reported.

2.3.2 Stomach

The stomach is the posterior region of foregut embedded in hepatopancreas (Fig 7). In infected specimens the stomach wall (SW) was found to have disintegrated and detached from the outer cuticular lining (OCL) coupled with lysis and disintegration of the inner epithelial layer (IEL). The detached stomach wall had been transformed into a syncytium (SY) (Fig 8). Hypertrophied nuclei were not seen in infected stage as there was total lysis and sloughing off of IEL unlike the observations made by Karunasagar *et al.*, (1997) in *Penaeus monodon* who reported hypertrophied nuclei with eosinophilic to basophilic inclusions. Strikingly, during the moribund stage, the OCL adhered to the hepatopancreatic tubules. Lo *et al.*, (1997) observed infected nuclei of the cuticular epidermis of the stomach dramatically hypertrophied and 2 to 3 times larger than those of

normal nuclei in *P.monodon*. Karunasagar *et al.*, (1997) also stated that in some of the infected cells the nuclei had completely disintegrated leaving vacant areas. A similar observation had already been made by Wongteerasupaya *et al.*, (1995) in gill tissue of *P.monodon* where, in the later stages of infection the nuclei also disintegrated leaving vacant spaces. The statement of Chang *et al.*, (1996) that the white spot syndrome virus caused severe damage to the stomach leading to cell lysis was essentially true as had been demonstrated in this study with the total sloughing off of the inner epithelium. This observation could be correlated with the cessation of feed intake by the infected animals, which was generally highlighted as one of the manifestations of the onset of the disease. Based on the evidence obtained from our histopathological study, no movement of food materials was possible in the intestine of an infected animal. However, more information is required on the histopathology of stomach during the early stages of infection.

2.3.3 Hepatopancreas

The hepatopancreas forms a large, paired glandular mass of tubules occupying much of the cephalothoracic cavity. The tubules were ensheathed by thin connective tissue (Fig 9). The hepatopancreas of experimentally infected shrimp exhibited vacuolization of the entire tissue as reported by Wang *et al.,* (1999)(a). The four different cells- embryonic cells (E- cells), vacuolated storage /absorptive cells (R- cells), the large vacuolated excretory cells (B-cells), and the fibrillar cells (F-cells) (Fig 9) which line the hepatopancreatic tubules were not distinguishable in the moribund animals suggesting dysfunction of the organ (Fig 10). However, the tubules were intact, the lumen in the tubules discernible and hemocytes were not seen. (Fig 11). Earlier studies revealed that the white spot virus grows in the myoepithelial cells of the hepatopancreatic sheath and the fibroblast of the connective tissue in *P.monodon* (Chang *et al.*, 1996) and not in the hepatopancreatic tubular epithelial cells. But during this study, even though the hypertrophied nuclei could not be demonstrated in the tubular epithelial lining, most of the secretory cells in the tubules had lost their chromatic character and had become acidophilic (Fig 10). Chang *et al.*, (1996) reported lysis of some parts of hepatopancreas in the later stages of infection without detecting any virus due to the unhealthy state of

the infected shrimp and also due to the autolysis of the infected tissue. More than autolysis, loss of integrity of the four types of cells and extensive vacuolization were the characteristic features of the disease as observed in this study. This poses a question as to how such an extensive vacuolization of the tubular epithelial lining takes place without the direct involvement of the virus. Wang *et al.,* (1997)(a) and Wang *et al.,* (1999)(a) reported the presence of virus infecting hemocytes between the hepatopancreatic lumens. The connective tissue also did not demonstrate prominent hypertrophied nuclei as seen in foregut and in other tissues. Lo *et al.*, (1997) also observed infected nuclei of hepatopancreas close to the normal size. Unlike the observation made by Woongteerasupaya *et al.*, (1995), hemocytic infiltration was not seen in the infected hepatopancreatic tissue.

2.3.4 Midgut

In the infected animals the inner surface of the midgut, with thin columnar epithelial cells forming the internal epithelial lining (IEL) with small oval nuclei (Fig 12) had under gone extensive atrophy and was found to have sloughed off from the wall into the lumen (Fig 13). The circular and longitudinal muscle layers, which were situated beneath the epithelial layer, showed extensive multi focal necrosis (MFN). No hypertrophied nucleus was found in the entire epithelial layer or in any of the underlying tissue. This observation matched with that of Lo *et al.*, (1997) who stated that the nuclei were not so obviously hypertrophied and remained close to normal size. According to Chang *et al.*, (1996), the white spot virus infected the connective tissue of the midgut, subsequent to the infection in the connective tissue of stomach wall. The midgut comprised the bulk of the alimentary tract and the extensive damage caused by the virus in this region might be proposed as the reason, for the cessation of food intake along with the manifestation of the disease.

2.3.5 Hindgut

The hindgut is a narrow, comparatively thick walled tube extending from the sixth abdominal segment and opens to the exterior through the anus. The internal epithelial lining of the hindgut which was raised into a series of longitudinal ridges called rectal pads (RP) with the lumen in between (Fig 14 and 15) showed nuclear hypertrophy, degeneration and necrosis (NC) of the underlying connective tissue (CT) in the infected animals. This is contrary to the observation made by Chang *et al.,* (1996 who reported that the hind gut was mildly infected in *Penaeus monodon* subsequent to infection of the stomach. On examining stomach, midgut and the hindgut of moribund *P.indicus* in this study, significant variations in the level of infection, in terms of pathological changes, could not be observed between them.

2.3.6 Nerve

The inner epithelial lining of the blood sinus (BS) (Fig 16) inside the nerve sheath was found to have lost its integrity (Fig 17). The neurosecretory cell nuclei (NCN) (Fig 18) could be seen hypertrophied with eosinophilic inclusions (Fig 19) and in some cases with hemocytic encapsulation and subsequent atrophy. The hemocytes were found to be deeply stained and dense in appearance (Fig 20). The eosinophilic stage of hypertrophied nucleus was also seen sporadically (Fig 21). Karunasagar *et al.*, (1997) Sudha *et al.*, (1998) and Wang *et al.,* (1999) (a) have reported connective tissue of the nerve cord as the target tissue of virus multiplication. But in our observations, the neurosecretory cells were found to have hypertrophied nuclei along with disintegration of connective tissue. The report of Chang *et al.*, (1996) that the nerve cord in *P.monodon* was slightly infected was not found to be true in the light of the evidence presented here in *P.indicus*. Similarly, the observation of Lo *et al.,* (1997), in *P.monodon* that the infected nuclei of nervous tissue were not obviously hypertrophied was also contrary to our findings. However, it has to be examined whether this is an incidence of difference in response of the two species to the same situation.

2.3.7 Eye

Histology of the infected eye showed tissue damage in the crystalline tract region (CTR) and the nuclei of the crystalline tract cells were hypertrophied with complete necrosis of the tissue forming undifferentiated mass (Fig 23) followed by spontaneous infiltration of oesinophilic granules (OG) from the base of the crystalline cone (Fig 24 & 25). These granules are not identified and more investigation is required for their elucidation. Even though no hypertrophy could be detected in the lamina ganglionaris (LG) region, necrosis could be seen in it. The retinular cell nuclei (RCN) layer was lysed and had lost its integrity compared with the healthy shrimp eye (Fig 22). Many of the earlier studies were on eyestalk tissue rather than on the compound eye. According to Chang *et al.,* (1996), the eyestalk was the severely infected region, involving nerve cells and cells of the ganglia. According to Lo *et al.*, (1997) the cuticular epidermis of the eyestalk was dramatically hypertrophied. These observations coupled with ours suggest the feasibility of using eyestalks and the eyes for demonstrating white spot virus.

2.3.8 Heart

The heart is a sac-like contractile structure, which lies immediately dorso-posterior to the hepatopancreas (Fig 26). In the infected animals hypertrophied nuclei characteristic of White Spot Virus could not be demonstrated in heart tissues. However, a generalized vacuolization (V) of the tissue as a whole and constriction of muscle bundles along with increased infiltration of blood cells (BC) (Fig27) in between the muscle bundles and oedema in the heart wall were seen (Fig 28). Regarding nuclear hypertrophy as a pathological change our observation matched with those of Lo *et al.,* (1997) as the nuclei remained close to normal size.

2.3.9 Gills

The dendrobranchiate gill consisting of a median gill axis (MGA) and the gill filament (F) (Fig29) showed massive disintegration and vacuolization in infected

specimens (Fig 30). The median blood vessel (MBV) which traverses longitudinally along the septa of the main axis had dilated and laterally ruptured in the infected shrimp (Fig30). The gill filaments in apparently healthy shrimps contained numerous lacunae (GL) occluded by hemocytes (H). During infection they showed massive disintegration and generalized vacuolization (V) and the nuclei of the epithelial cells were hypertrophied (Fig30 & 31). But the two major pathological changes such as, 1.vacuolization, disintegration and rupturing of median gill axis, gill filaments, gill lacunae and 2. dilation of the median blood vessel as observed here have not been reported by the earlier workers. (Karunasagar *et al.*, 1997, Sudha *et al.*, 1998, Chang *et al.*, 1996, Woongterasupaya *et al.*, 1995, Rajendran *et al.*, 1999).

2.3.10 Ovary

The ovarian wall consisting of three layers, outer epithelial layer (EP), a comparatively thicker connective tissue (C), and the innermost germinal layer (G) (Fig 32) showed necrosis (Fig 33). Hypertrophied nuclei were not seen in the developing oocytes as reported by the earlier workers (Wongterasupaya *et al.,* 1995, Rajendran *et al.,* 1999, Chang *et al.,* 1996) but the nucleus of the connective tissue was found to be enlarged. Sudha *et al.*, (1998) observed that supportive connective tissue of gonad is a target tissue and the oocytes when infected became atrophied. Lo *et al.*, (1997) has also stated that the infected oocytes do not complete maturation and consequently disintegrate. Under these circumstances a true vertical transmission is doubtful. Probably, as Chang *et al.*, (1996) suggested, virus might be getting released from the infected connective tissue during spawning and may be infecting either fertilized eggs or embryonic stages. This is an area for further investigation, having many practical implications.

2.3.11 Integument

The outer exocuticle (EX) and the underlying procuticular (PRO) layers of the integument (Fig 34) were discernable in the infected state. The underlying epidermis (EP) which secretes the integument showed massive necrosis and nuclear hypertrophy

(H) (Fig 35). In several epizootics of white spot viral infection, moderate softening of the exoskeleton has also been reported. It appears that the changes in the structural integrity of the exo and pro cuticle might be the reason for this syndrome. Cuticular epithelial cells have been reported as the target of the white spot syndrome virus as demonstrated by Karunasagar *et al.*, 1997. They have uniformly demonstrated hypertrophied basophilic nuclei in the cuticular epidermis.

Of the various tissues examined the foregut and gill was found to be specifically suitable for the postmortem demonstration of the virus because of the severity of infection and ease of tissue processing.

Several of the histopathological observations in this study were found contradicting the previous ones and this could be, to some extent, due to the difference in the species of penaeids dealt with as all previous work were centered on *P. monodon.*

Table-1 Protocol followed for histology

Sl:No	**Reagent**	**Time**
1	Ethyl Acohol - 80%	1/2 hour
2	Ethyl Acohol -90%	1/2 hour
3	Ethyl Acohol -90%	15 minutes
4	Ethyl Acohol -96%	15 minutes
5	Ethyl Acohol -100%	15 minutes
6	Ethyl Acohol -100%	2 minutes
7	Acetone	Dip
8	Xylene-1	10 minutes
9	Xylene-2	10 minutes
10	Xylene : Wax (3:1)	15 minutes
11	Xylene : Wax (2:2)	15 minutes
12	Xylene : Wax (1:3)	15 minutes
13	Pure wax- 1	15 minutes
14	Pure wax- 2	15 minutes
15	Pure wax- 3	15 minutes

Table- 2 Protocol followed for electron microscopy

Sl:No	Reagent	Time	Temperature
1	2.5% Glutaraldehyde	24 hours	4°C
2	1st wash	10 minutes	4°C
3	2nd wash	20 minutes	4°C
4	2% Osmium Tetroxide	2 hours	4°C
5	1st wash	15 minutes	4°C
6	2nd wash	15 minutes	4°C
7	3rd wash	30 minutes	4°C
Dehydration			
8	30% acetone	10 minutes	4°C
9	30% acetone	20 minutes	4°C
10	50% acetone	30 minutes	4°C
11	70%acetone	1 hour till further process	4°C
12	80%	30 minutes	4°C
13	95%	30 minutes	4°C
14	Absolute Acetone	15 minutes	37°C
15	Absolute Acetone	45 minutes	37°C
Clearing			
16	Toluene	1 hour	37°C
17	Toluene :embedding media(3:1)	2 hours	37°C
18	Toluene :embedding media(2:2)	2 hours	37°C
19	Toluene :embedding media(1:3)	2 hours	37°C
Infiltration			
20	Absolute embedding medium	3 hours	37°C

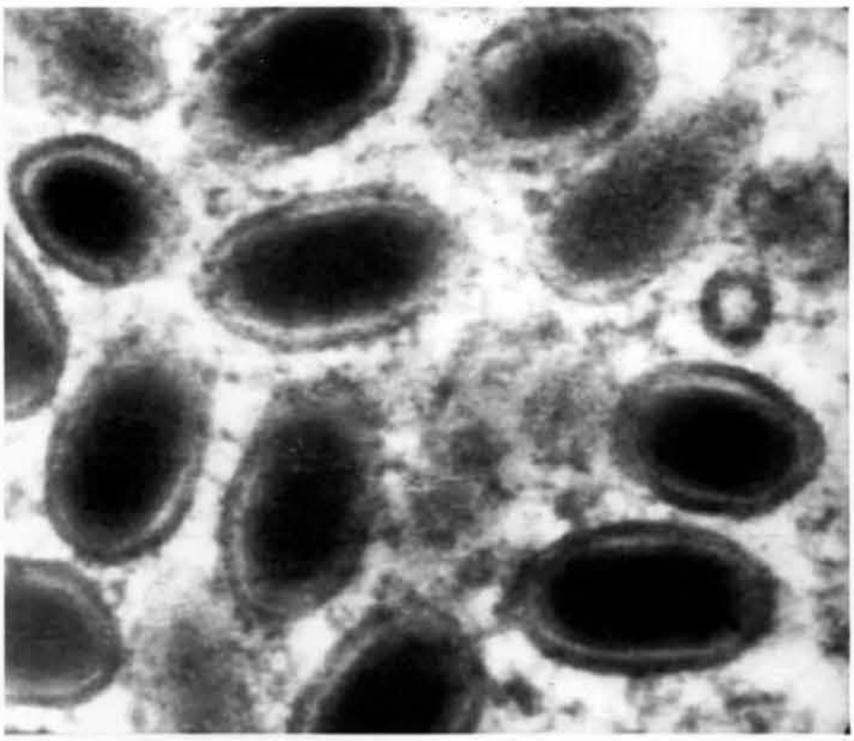


Fig: 1 (a) Electron micrograph showing enveloped white spot syndrome virus particles in the virogenic stroma of infected cell nucleus.

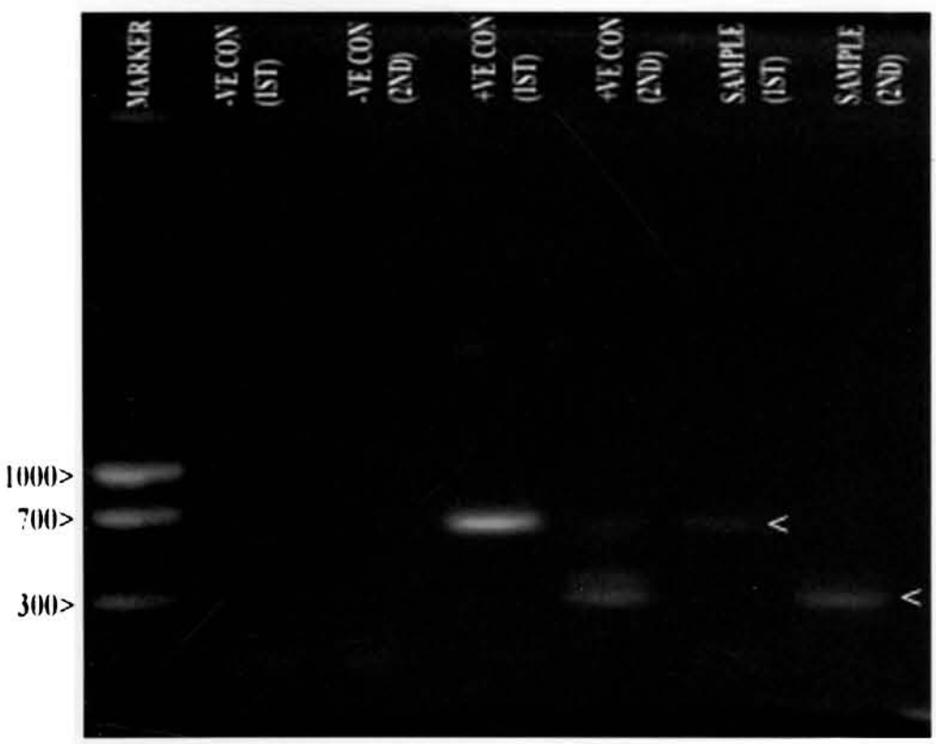
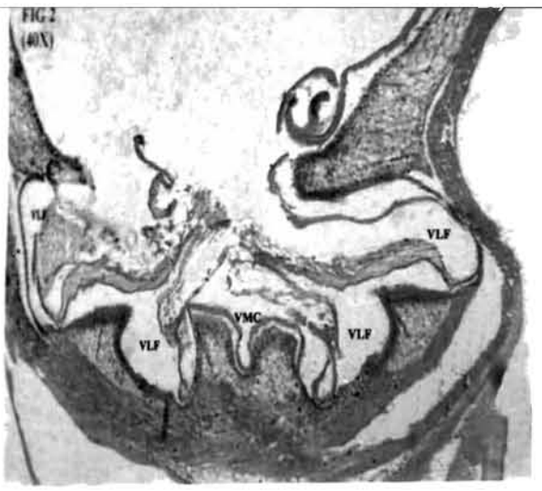
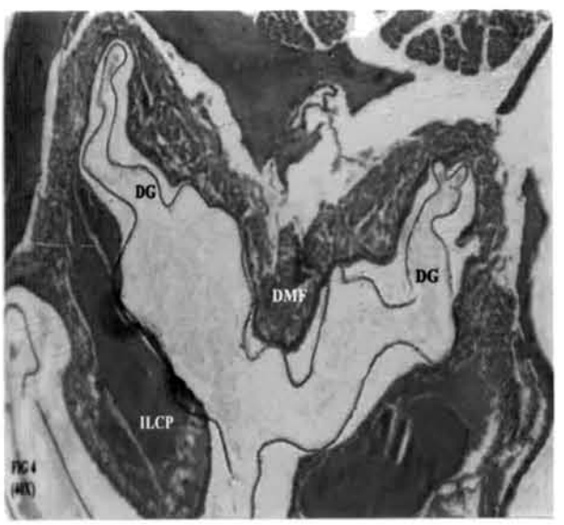
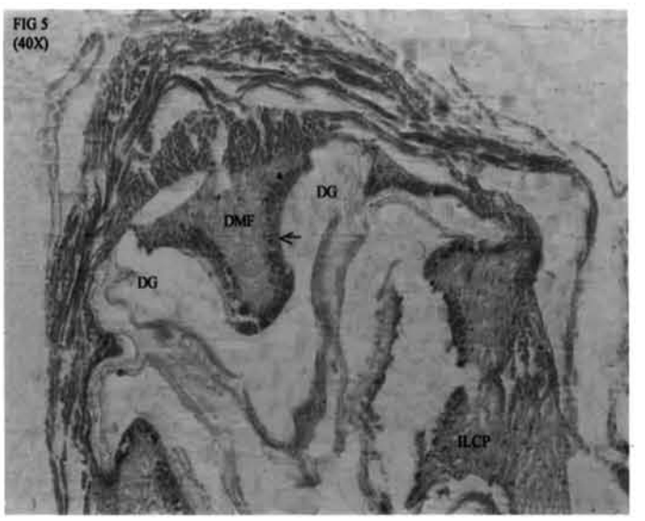


Fig: 1 (b) Diagnostic first step and nested PCR of WSSV







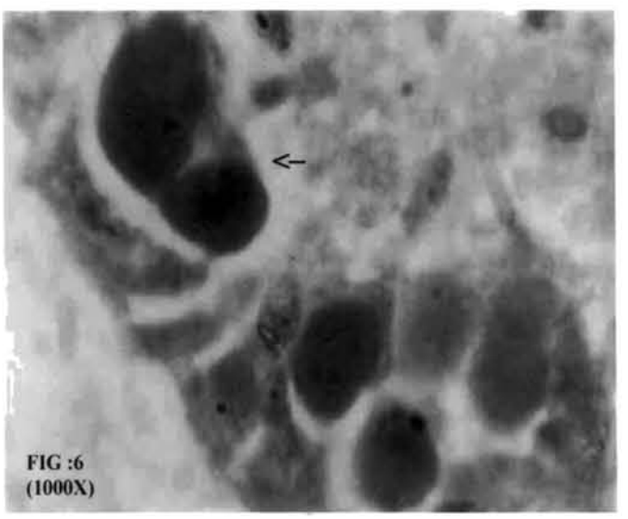
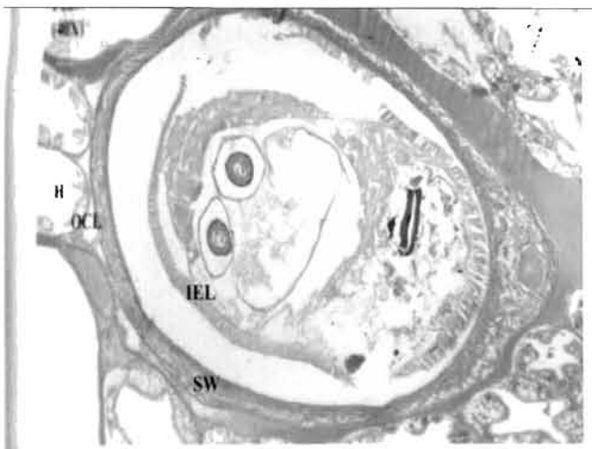
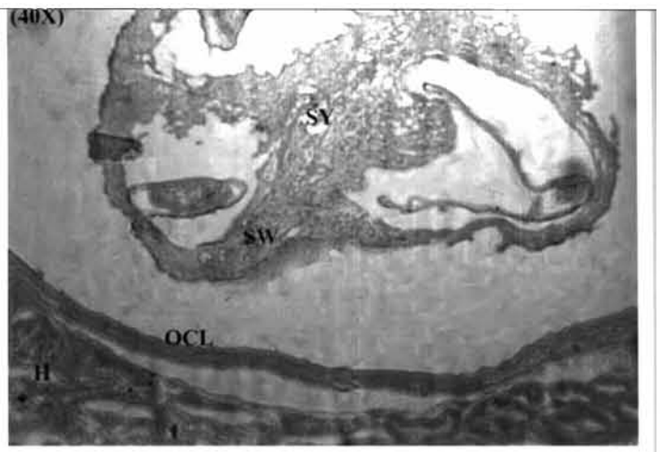
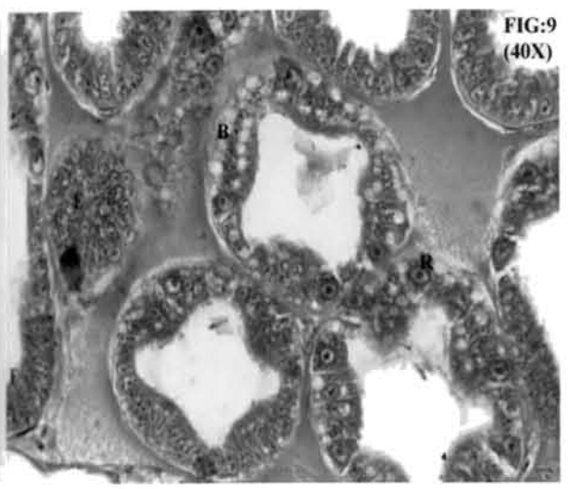
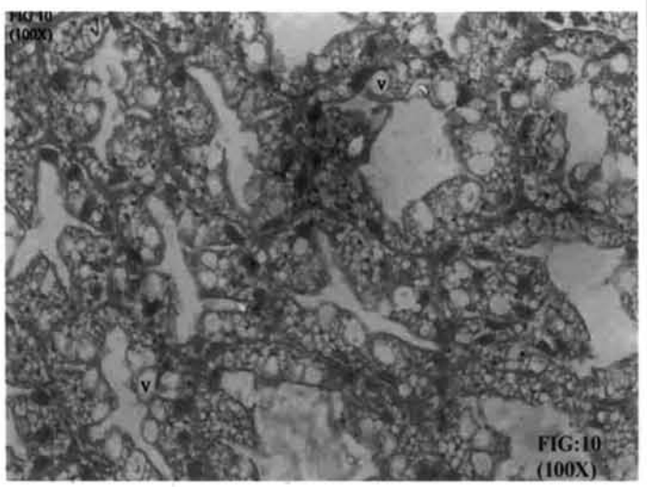


Fig - 2 Healthy prawn foregut tissue showing the ventral folds, Fig - 3 Infected prawn foregut tissue showing ventral folds, Fig - 4 Healthy prawn foregut tissue showing dorsal folds, Fig - 5 Infected prawn foregut tissue showing dorsal folds, Fig - 6 Hypertrophied nuclei. Arrows in Fig -5 & Fig -6 shows hypertrophied nuclei. DG -Dorsal Groove, DMF - Dorsal Median Fold , ILCP - Intra Lateral Cardiac Plate, VLF - Ventral Lateral Fold, VMC - Ventral Median Channel.









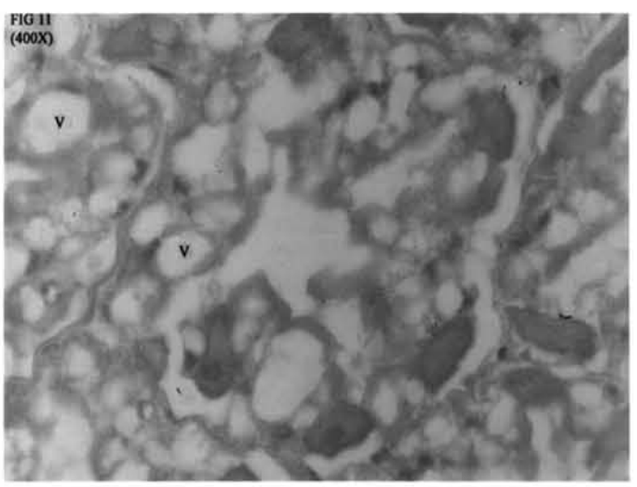


Fig - 7 Healthy prawn stomach tissue, Fig - 8 Infected prawn stomach tissue, Fig - 9 Healthy prawn hepatopancreatic tissue, Fig- 10 Infected prawn hepatopancreatic tissue, Fig - 11 Infected prawn hepatopancreatic tissue.B - Excretory Cell, E - Embryonic Cell, F - Fibrillar cell H - Hepatopancreatic tubules, IEL - Internal Epithelial Lining, OCL - Outer Cuticular Lining, R - Absorptive/Storage Cell, SW - Stomach Wall, SY - Syncytium, V - Vacuolization.

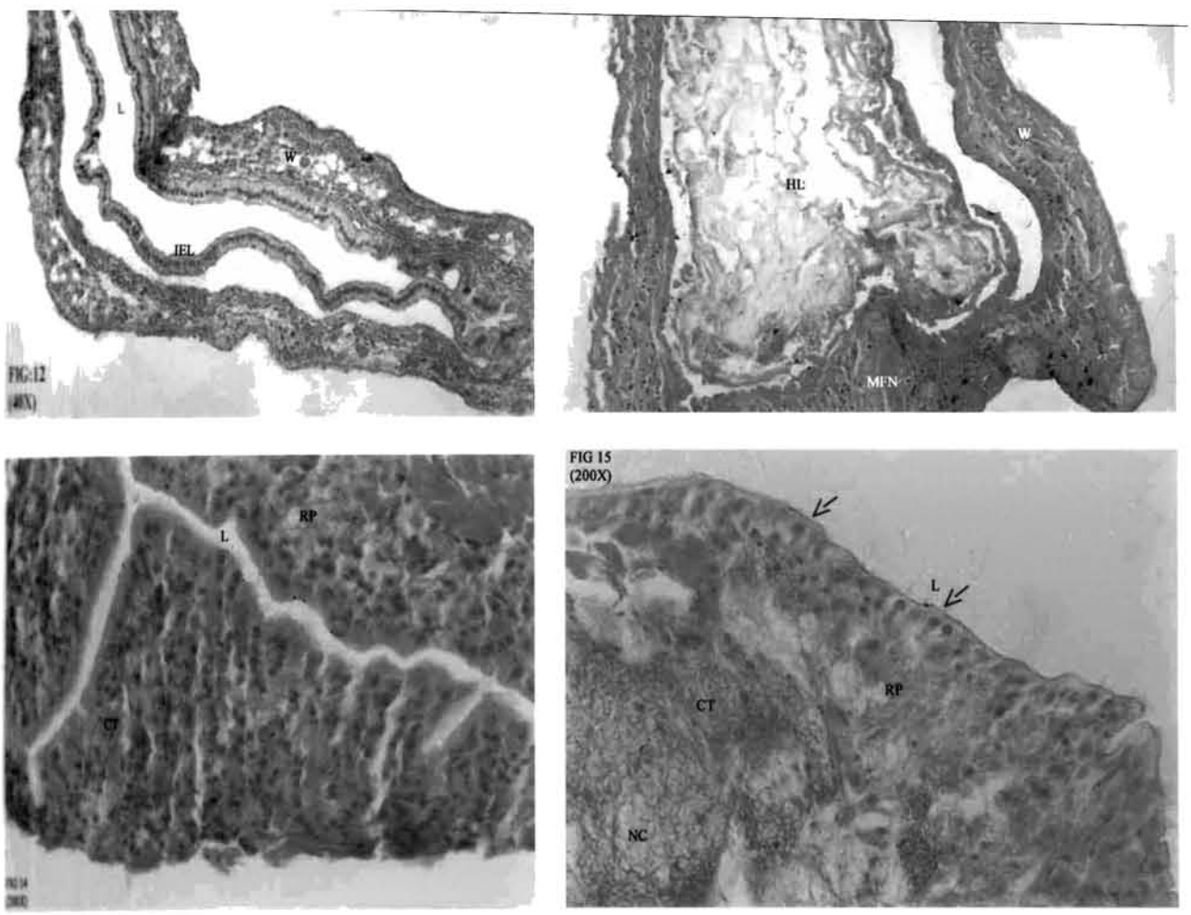
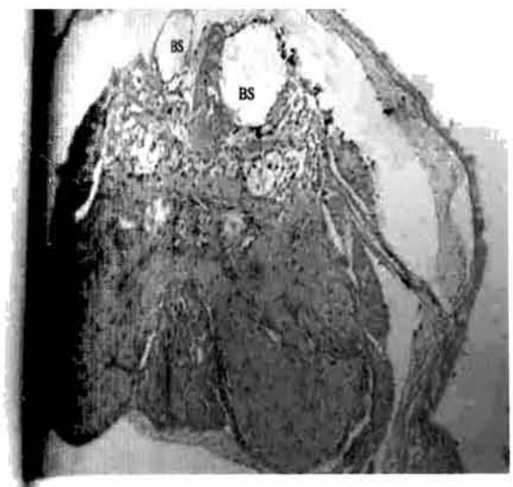
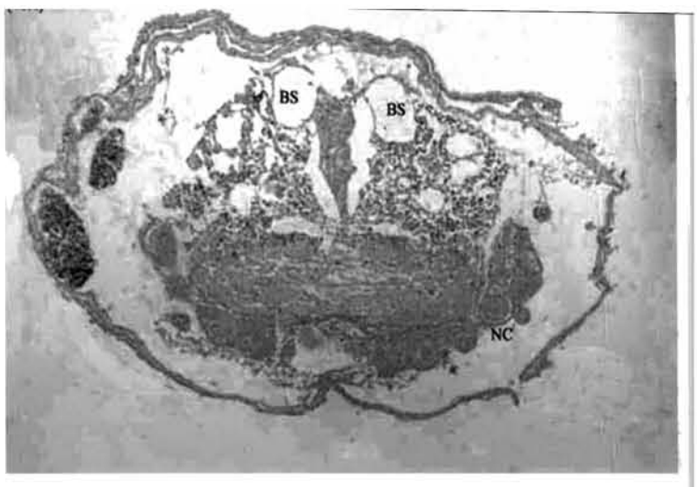
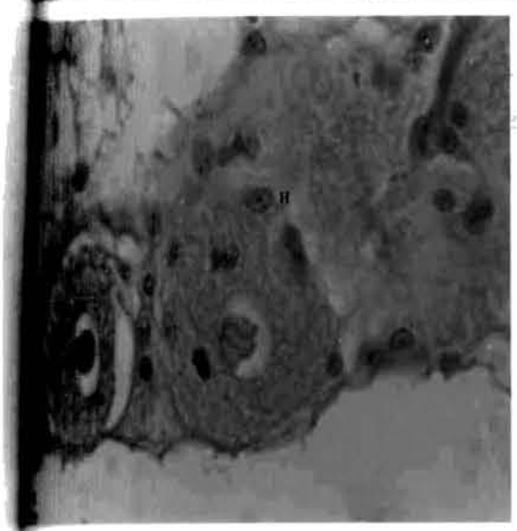
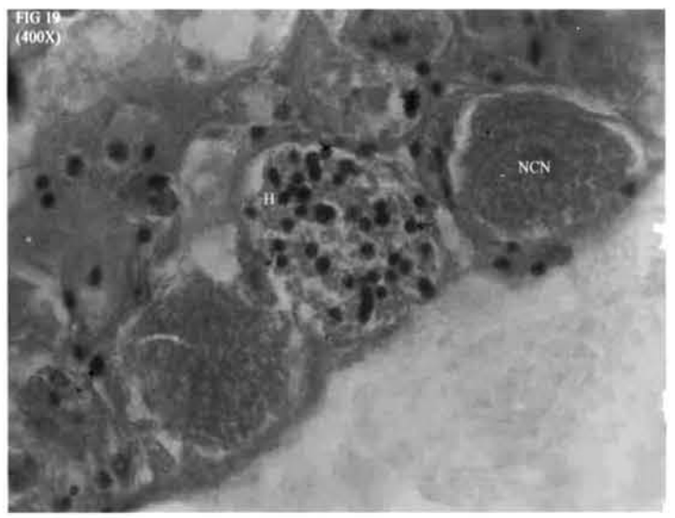


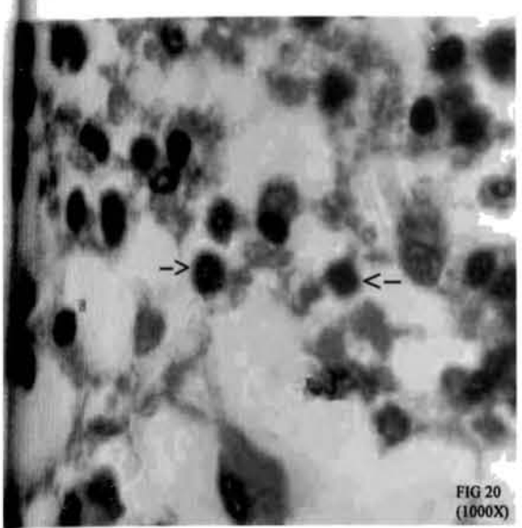
Fig - 12 Healthy prawn midgut tissue, Fig - 13 Infected prawn midgut tissue, Fig - 14 Healthy prawn hindgut tissue, Fig - 15 Infected prawn hindgut tissue. CT - Connective Tissue, HL - Hindgut Lumen, IEL - Internal Epithelial Lining, L - Lumen, MFN - Multi Focal Necrosis, NC - Necrosis, RP - Rectal Pads of Hindgut, W - Wall of midgut. Arrows in Fig - 15 shows hypertrophied nuclei.











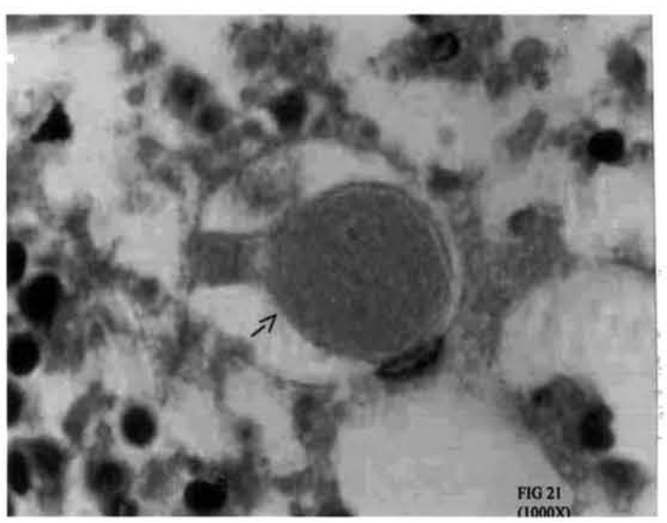


Fig - 16 Healthy nerve tissue, Fig - 17 Infected nerve tissue, Fig - 18 Healthy neurosecretory cells, Fig - 19 Infected neurosecretory cells, Fig - 20 Darkly staining hemocytes in the nerve tissue (Arrows), Fig - 21 Eosinophilic state of hypertrophied nucleus with basophilic chromatin. BS - Blood Sinus, H - Hemocytes, NC - Neurosecretory Cell , NCN - Neurosecretory Cell Nucleus.

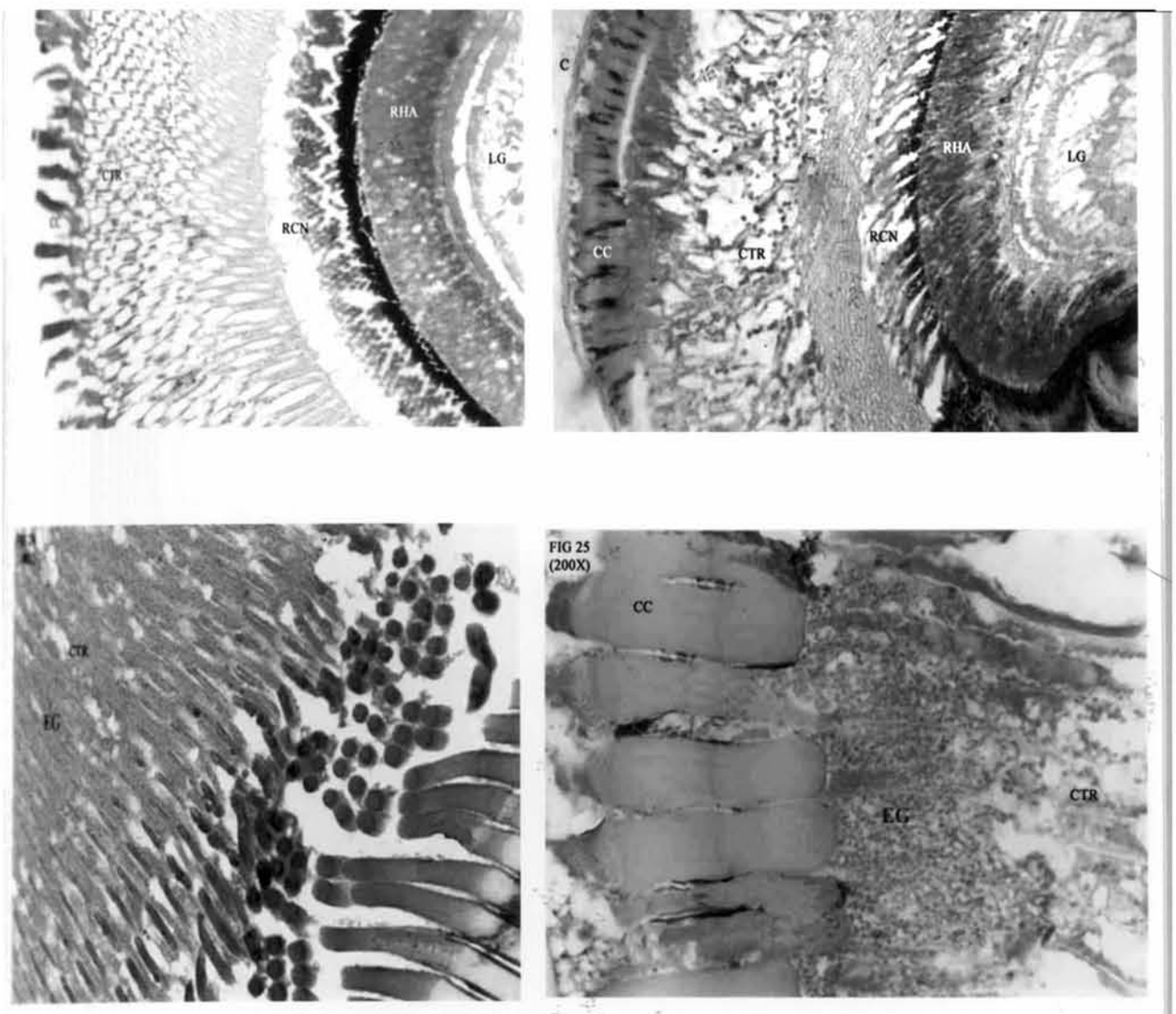
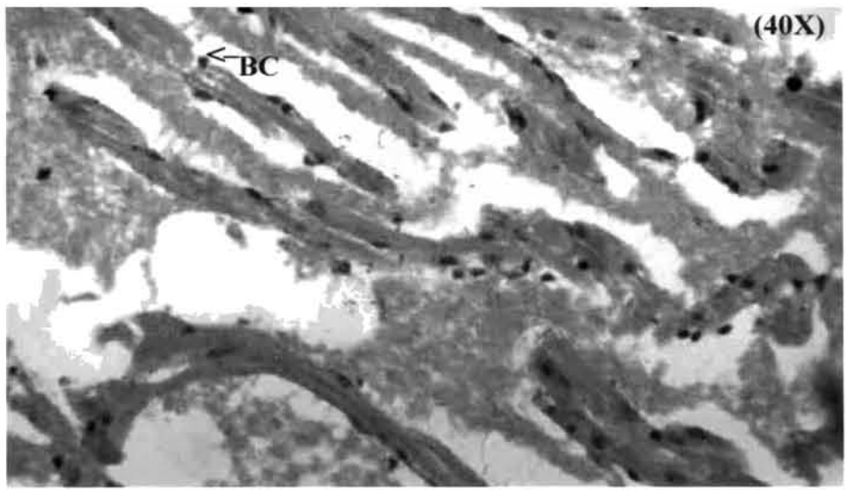
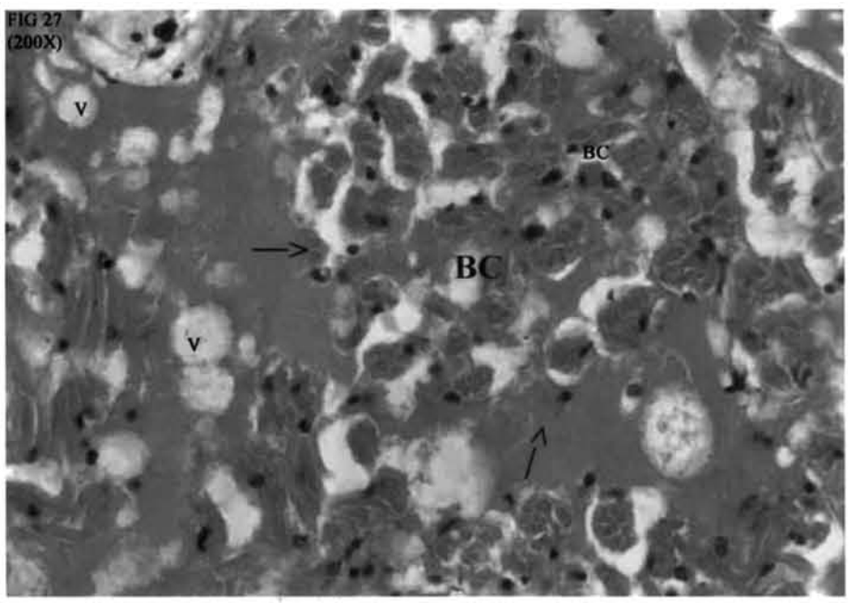


Fig - 22 Healthy prawn eye ball tissue, Fig - 23 Infected prawn eye ball tissue, Fig - 24 & Fig - 25 Infected eye tissue. C - Cuticle, CC - Crystalline Cone, CTR - Crystalline Tract Region, LG - Lamina Ganglionaris, EG - Eosinophilic Granules, RCN - Rectinular Cell Nuclei, RHA - Rhabdom





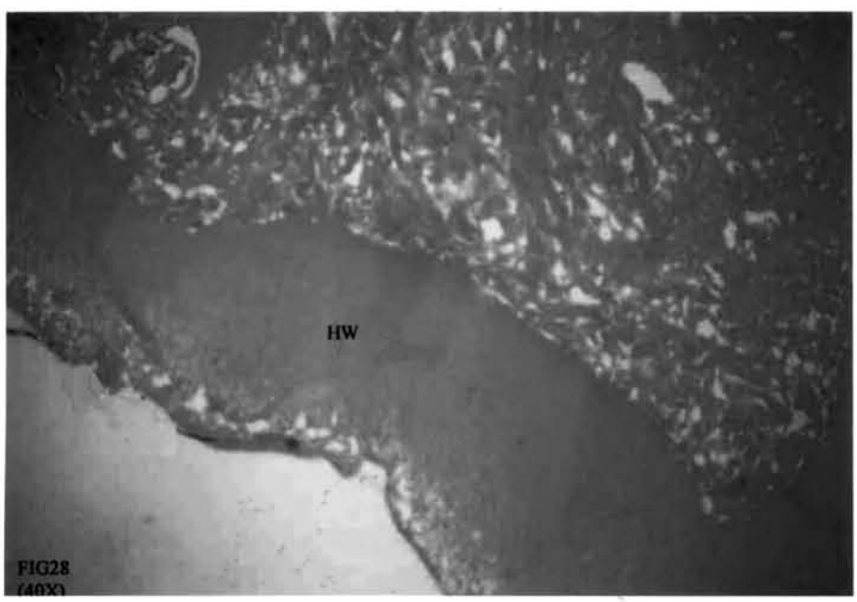
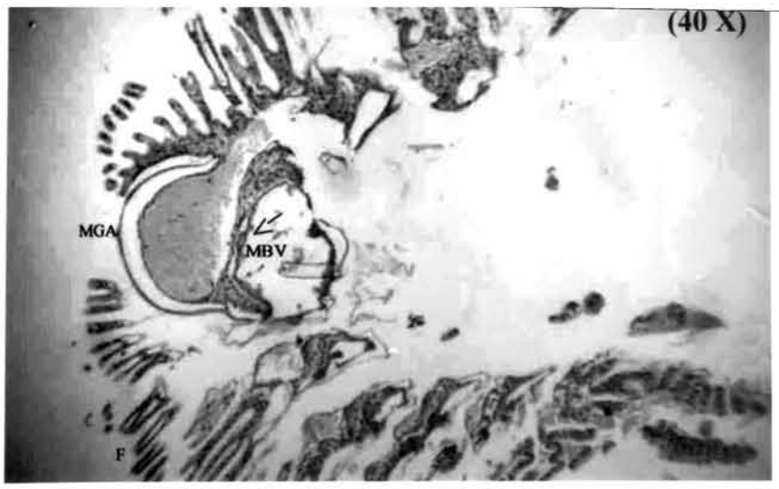
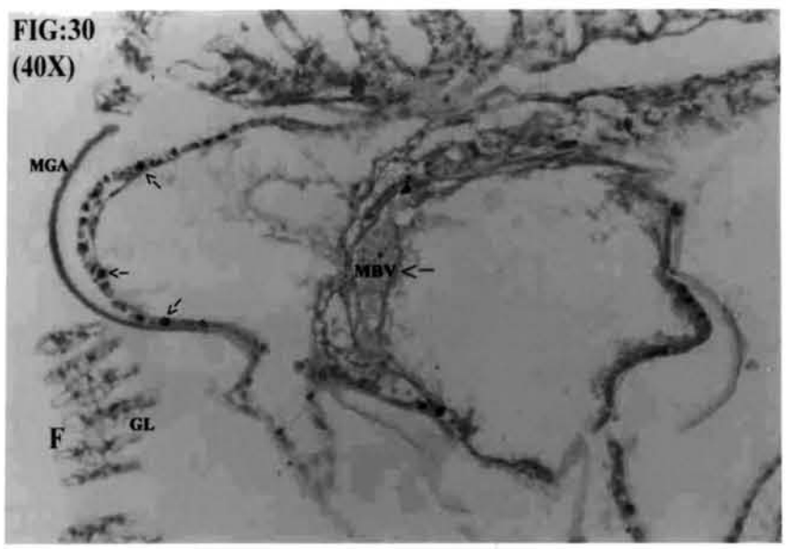


Fig - 26 Healthy heart tissue, arrows showing hemocytes, Fig - 27 Infected heart tissue, arrows showing hemocytic infiltration, Fig - 28 Infected heart tissue showing edema. BC - Blood Cells, HW - Heart Wall, V - Vacuolization.





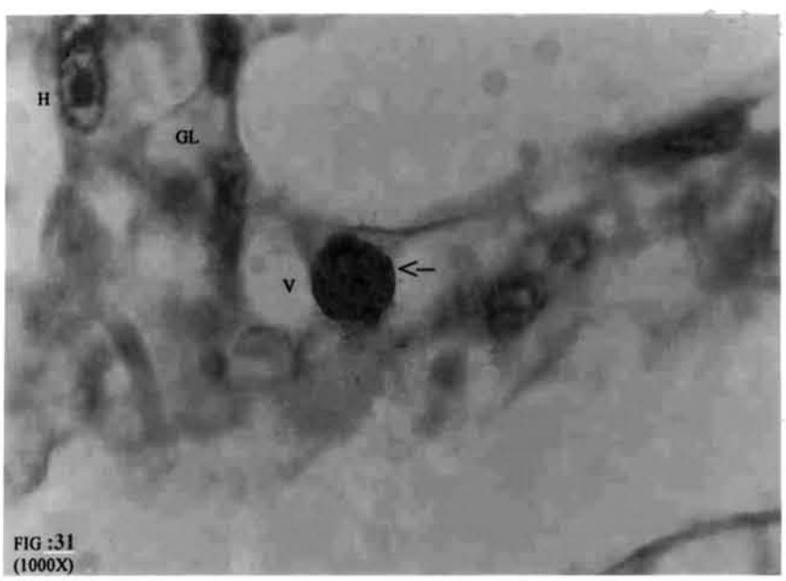
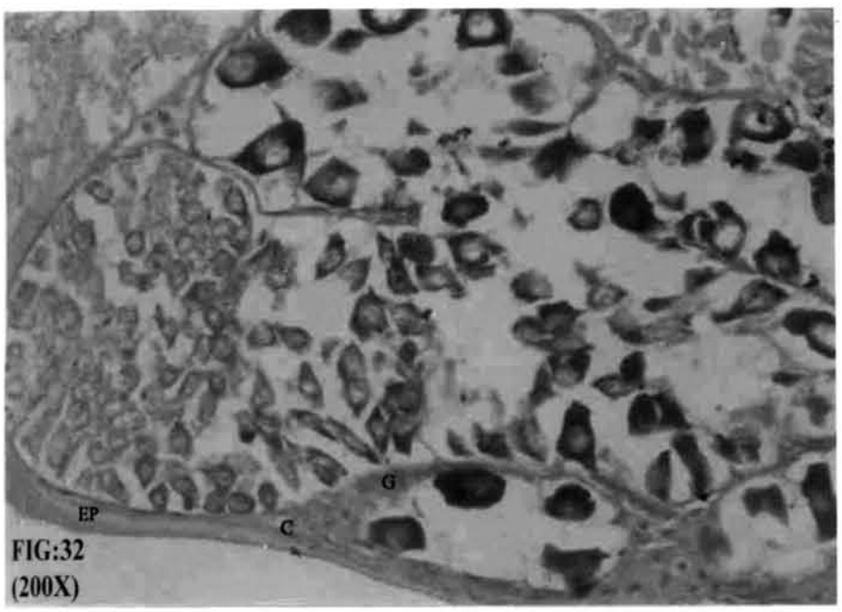


Fig - 29 Healthy gill tissue, Fig -30 Infected gill tissue, Fig - 31 Infected gill tissue, arrow showing hypertrophied nucleus. F- Gill Filaments, GL - Gill Lacunae, H - Hemocyte, MBV - Median Blood Vessel, MGA - Median Gill Axis, V - Vacuolization.



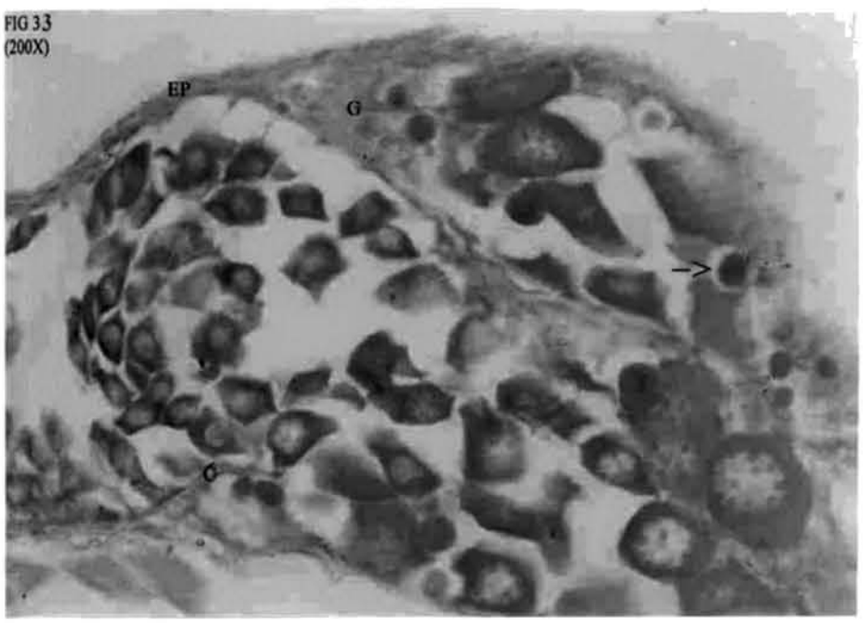


Fig - 32 Healthy prawn ovarian issue, Fig - 33 Infected ovarian tissue. EP - Epidermis G - Germinal Epithelium, C- Connective tissue
Arrow in Fig - 33 show hypertrophied nucleus.

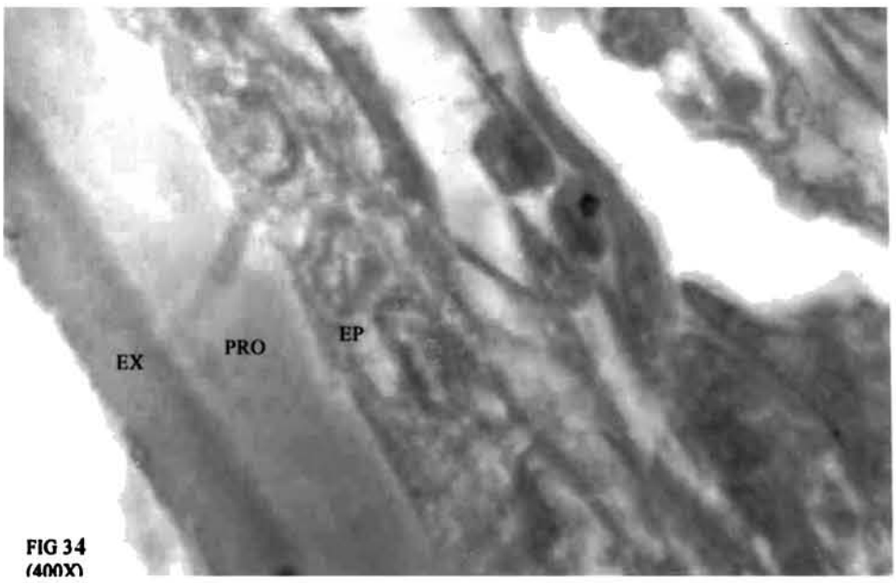


FIG 34
(400X)

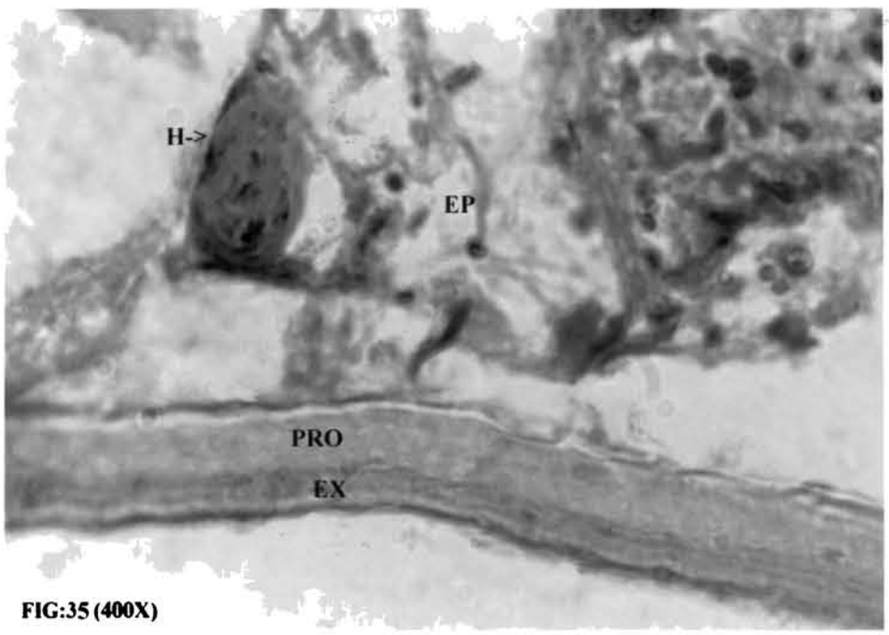


FIG:35 (400X)

Fig - 34 Healthy integument and under lying epidermis,
Fig - 35 Infected integument and underlying epidermis. EP - Epidermis, EX - Exocuticle, H - Hypertrophied nucleus, PRO - Procuticle.
Arrow in Fig 35 shows Hypertrophied nucleus.

CHAPTER - 3

TRANSMISSION ELECTRON MICROSCOPIC EVIDENCE AND ULTRASTRUCTURE OF WHITE SPOT SYNDROME VIRUS IN VARIOUS TISSUES OF *PENAEUS INDICUS* AND VIRAL MORPHOGENESIS

CHAPTER - 3

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3.1 Introduction

Several investigators have looked in to the ultra structure of WSSV and attempted to unravel the viral morphogenesis.

3.1.1 Ultrastructure

Takahashi *et al.,* 1994, subjected the lymphoid organ of WSSV infected *P.Japonicus* for electron microscopy and demonstrated rod shaped virions in the nucleus of cells. The virions possessed an envelope around a central nucleocapsid. The envelope was approximately 10nm in thickness and the space between the envelope and the nucleocapsid was about 5nm. Average size of the complete virions was 83nm in diameter and 275nm in length; the size of nucleocapsid being 54nm in diameter and 216nm in length. They did not observe any occlusion body in TEM. They named it as non - occluded bacilliform virus and with the present understanding of the virus if one look back it can be easily recognized as the white spot syndrome virus and this can be considered as the first electronmicroscopic demonstration of WSSV.

In 1997, Wang *et al.,* reported rod shaped, enveloped non-occluded virions with the nucleocapsid composed of rings of sub units in a stacked series.

One year later, in 1995, Wongteerasupaya *et al.,* demonstrated non-occluded systemic baculovirus that occured in cells of ectodermal and mesodermal origin and that

ised high mortality in P. *monodon*. They named it as Systemic Ectodermal Mesodermal Baculovirus (SEMBV) as it measured 121± 9nm in width at the widest point and 276 ± 26 nm in length and the envelop was clearly trilaminar, consisting of 2 electron opaque layers divided by an electron transparent layer characteristic of the baculovirus. In cross sections, the envelopes for complete virions varied from round to pentagonal or hexagonal in shape. They observed that the epithelial cells of the hepatopancreatic tubules were normal but with signs of abnormal cytology in the connective tissue such as hypertrophied nuclei.

Durand *et al.,* 1997 made an extensive study on the ultrastructure of the virus and confirmed that the virus was typically characterized by an apical envelope extension sometimes observed in one side of the particle. The nucleocapsid displayed a superficially segmented appearance each segment forming 8nm-diameter sub units arranged in two parallel rows. Nucleocapsid of the 250 x100nm long rod shaped virion is closed at one extremity by a small segment that formed a slightly rounded end while the opposite extremity was squared. The envelop was 6 to 7 nm thick and had the structure of a trilaminar unit membrane. The area between the nucleo capsid and the envelop varied from about 2 to 7.5nm. The nucleocapid was cylindrical about 200 X 65 nm long with a 8nm thick external wall. The core of the nucleocapsid was highly electron dense. This study confirmed the observation made by Wang *et al.,* 1995 that nucleocapsids were formed by a series of ringed structures.

Wang *et al.,* 1997(a) made TEM examinations of lymphoid organ, gill and stomach and revealed rod shaped, enveloped virions in hypertrophied nuclei. The mean size of the complete virion was 298±21 X 107 ± 8 in *P.monodon* and 248 ± 12X 104 ± 8nm in *P.japonicus*. The WSD virus particles consisted of two structural units such as the nucleo capsid and envelope. The virion envelops were clearly trilaminar, consisting of two electron opaque layers separated by an electron transparent layer, a regular characteristic of baculoviruses. In addition, some virion showed a nipple like extension at one end.

Hameed *et al.,* 1998 studied a virus strain from India and observed that the nucleocapsids measured 420 ± 18nm in length and 68 ± 5nm in width. The nucleocapsids showed a pattern of electron opaque striations (17nm) altering with electron – transparent striations (5.5nm) arranged perpendicular to the long axis of the nucleocapsid. Sen *et al.,* 1999, demonstrated WSSV in the gill tissue of artificially infected *P.indicus* from India as oblong shaped, rounded at the ends and measuring approximately 224± 21 nm X 100 ±13nm. Infected cells exhibited paracrystalline arrays of virus as reported by Wongteerasupaya *et al.,* 1995, Wang *et al.,* 1995.

Cesar *et al.,* 1998 isolated a non-occluded baculovirus like agent from moribund *P.japonicus* obtained from China and the virus was named Chinese baculovirus (CBV). Under electron microscope, negatively stained virus particles were rod shaped, enveloped, and measured 322 to 378 nm in length and 130 to 159nm in diameter. The nucleoprotein core exhibited a unique striated structure and measured 316 to 350nm in length and 65 to 66nm in diameter. These striations appeared to be the result of the stacking of ring like structures. These rings consisted of two rows of 12 to 14 globular sub units, each measuring approximately 10 nm in diameter. Cesar *et al.,* 1998 made a comparative study of these different isolates of white spot virus purified from three different penaeid shrimp species from different countries by characterizing morphologically, biochemically and genomically using various techniques. Under the electron microscope, the three isolates were in distinguishable and their nucleoprotein core exhibited the unique striated structure characteristic of the baculovirus like agent associated with White spot syndrome. The dimensions of the nucleoprotein cores were also identical for all these isolates.

3.1.2 Morphogenesis

Inouye *et al.,* 1994 on studying mass moralities of cultured *P.japonicus* in 1993 observed that during morphogenesis the capsid was formed from a 'capsid originator' and the envelop was formed *de novo* in the nucleoplasm. The presumptive indications of the virus assembly in the hypertrophied nucleus has come from Wang *et al.,* 1997 (a) who

observed in some hypertrophied nuclei empty capsids, circular envelops and capsid originators. Meanwhile presence of stacked elongated structures during the initial stage of viral morphogenesis have been explained by Durand *et al.,* 1997 as precursor of capsid and Wang *et al.,* 1999 explained it as the precursor of nuclear material. Undoubtedly, viral multiplication occurs inside the nucleus of the infected cell; morphogenesis initiated by the margination of chromatin material resulting in the formation of an electron dense peripheral zone known as 'Ring Zone ' and a clear electron lucent central region called the 'virogenic stroma' (Durand *et al.,* 1997, Wang *et al.,* 1999, Wang *et al.,* 2000). Viral replication takes place inside the central virogenic stoma of the infected nucleus.

Different views persist for viral morphogenesis. Primarily the virogenic stroma gets filled with many empty capsid shells most of which surrounded loosely with an envelop. Both shell and envelop are open at one end and later electron dense threads extend directly through the open end (Wang *et al., 2000).* Presence of naked nucleocapsid suggested that, envelope was formed later after the densification of the empty capsid with the nuclear material. (Wang *et al.,* 2000).

Meanwhile certain multi vesicular bodies could be observed in the cytoplasm, which formed connections with the nuclear membrane during viral morphogenesis but later disappeared. The role of such vesicular structures in viral morphogenesis is yet to be resolved (Wang *et al.,* 2000).

Yet, another version of viral morphogenesis is from Wang *et al.,* 1999 (a). According to them, it is initiated by the formation of certain fibrillar fragments in the nucleoplasm of the infected cell. These fibrillar structures give rise to envelop material as vesicles. Parallel to this a long rod shaped structure with numerous stacked repeating sub units designated as 'viral nucleosome' could be observed. This structure was considered to be the nucleo-protein precursor of the viral nuclear material. Meanwhile, the vesicular, circular, membranous structures found in the virogenic stroma are filled with nuclear material. Along with this naked capsids appear which grow over the vesicles containing the nuclear material. Later the envelop grows around the capsid covering it and during

time, the capsid acquires a stacked appearance. Formation of a tail is observed after formation of the envelop. An alternate pathway as suggested by Wang *et al.,* 1999 (a) explained the formation of nucleo capsid which later got surrounded by the envelop.

On reviewing the investigations carried out on the ultrastructure of the virus and morphogenesis, very little attempt has been found to be made precisely from India in demonstration of the ultrastructure of the strain of WSSV seen in Indian waters. Moreover, it was speculated that there existed variations in the morphology and morphometry of the virus in various tissues and organs although they were all in the same animal. As explained above morphogenesis of WSSV is an important component still not fully resolved. These lacunae in information prompted to undertake this investigation.

2 Materials and methods

2.1 Source of Virus

A heavily infected brood stock of *P.monodon* with clinical manifestations of the disease such as white spots in the inner surface of carapace, reddening of pleopods and empty intestine was the source of the virus. Matsyafed, Government of Kerala supplied the animal, from a batch of wild spawners brought from Vishakapatnam, Andhra Pradesh, in 1995, for larval production.

2.2 Experimental animals

A batch of *P. indicus* post larvae generated from a single brood stock was reared in a hatchery for more than four months in aged (5-6 months, Salinity 30ppt) seawater and the animals on attaining a weight of 5 to 7g were used for the experimental infection and passage of the virus. Before the experimental infection, shrimps were subjected to formalin stress test by exposing them for one hour in to 100-ppm formalin in 20ppt seawater, with adequate aeration. They were then observed for three days for manifestation of any disease.

12.3 Pathogen

Presence of white spot syndrome virus (WSSV) in the source tissue and experimental test shrimps was confirmed by Electron microscopy as well as by diagnostic PCR following Lo *et al.,* (1996) (b). The primers were synthesized by M/S Bangalore Genei, Bangalore, India.(Refer chapter -1)

12.4 Preparation of virus inoculum

A sample of about 500 mg gill tissue was dissected from the donor *Penaeus monodon*, and macerated in cold 10 ml PBS (NaCl - 8g, KCl- 0.2g, Na_2HPO_4-1.15g, KH_2PO_4- 0.2g, double distilled water –1000 ml) with glass wool to a homogeneous mass using mortar and pestle in an ice bath. The homogenate was centrifuged at 8200-x g in a refrigerated centrifuge (REMI C.24) at 4°C, and the supernatant was filter-sterilized by passing it through a 0.22µ pore size membrane filter. The preparation was streaked on McBell's agar plates and incubated at 28 ± 2°C for 72 hours to determine the presence of bacteria.

12.5 Experimental infection

An aliquot of 0.01ml filtrate was inoculated at the dorsal side of the abdomen of *indicus* in the space between the telson spine and the last abdominal segment using a ml-tuberculin syringe. Five shrimps were kept in 40 x 25 x 10 cm fibre-glass tanks containing 20L filtered and aged sea water (20ppt) at a temperature of 28 ± 2°C, with continuous aeration. The shrimps were fed *ad libitum* with pelleted Higashi Maru feed containing 40% protein. One third of water was replaced daily with fresh filtered and aged seawater (20 ppt). The animals were observed for cessation of feeding, lethargy and mortality. The virus was re-isolated following the above procedure and inoculated into the next batch of animals. This was repeated in five batches of shrimps and the moribund shrimps of the fifth passage were fixed for electron microscopic studies.

A set of healthy shrimps, subjected to stress test, was maintained as control.

12.6 Electron Microscopy

Moribund animals as well as control animals were collected, various tissues such as gill, foregut, heart, hepatopancreas, hindgut, nerve, dorsal aorta, and midgut were dissected out into small pieces of 1mm size and fixed in 2.5% glutaraldehyde (Electron Microscopy Sciences, USA) prepared in PBS (1M), at pH 7.4 for 24 hours at 4°C and post fixed in 2% Osmium tetroxide (Electron Microscopy Sciences, USA) in PBS (1M) at pH 7.4 for 2 hours at 4°C. After dehydration through an ascending series of acetone the tissue pieces were embedded in epoxy resin, (Electron Microscopy Sciences, USA) Ultra thin (0.5μ) sections were stained with uranyl acetate and lead citrate (Electron Microscopy Sciences, USA) and examined under CM-10, Philips Electron Microscope.

13 Results

WSSV could be demonstrated in all tissues subjected for electron microscopy. This included nuclei of gills, foregut, heart, hepatopancreatic connective tissue, hindgut, nerve and dorsal aorta. In gill tissue (Fig.1) bunches of fully formed virions were seen in paracrystalline array towards the margin of the hypertrophied nucleus. The virions ranged from 267 to 317 nm long and 105 to 158 nm wide. In the hypertrophied nucleus of foregut (Fig.2) also bunches of fully assembled virions were seen mostly towards the margin of the nucleus, showing a high level of infection. The virions measured 289 to 317 nm long and 144 to 217 nm wide. Margination of chromatin and less electron dense inner nuclear core as the virus stroma could be demonstrated in both gill and foregut as the characteristic features of higher level of viral multiplication and severity of infection. In hypertrophied nucleus of heart tissue, virus particles were seen in the inner virogenic stroma. The broader ring zone and a small stromal middle region without a definite arrangement of the virions which ranged in size from 260 to 347 nm in length and 144 to 173 nm in width suggested the initial stage of viral replication (Fig.3). The virus could also be demonstrated in the hepatopancreocytes, instead could be seen in the connective

ssue (Fig.4). Alike afore mentioned tissues, in hindgut the hypertrophied nuclei had lly formed virus aggregations in the virogenic stroma with little chromatin margination ggesting the tissue's higher susceptibility for viral multiplication when compared to ert tissue. Size of the virions ranged from 347nm long and 173nm wide (Fig.5). In rve tissue different stages of viral morphogenesis could be demonstrated (Fig.6). This cluded the envelop (E), empty capsid (EC), partially filled capsids (PC), probable point f entry of nuclear material to capsid (N) and fully formed virions (V). Notably, the ucleocapsid ranges from 318 to 463 nm in length and 46 to 86 nm in width. Meanwhile e fully formed viral particles recorded a length of 240 to 318 nm length and 115 to 144 m width. In dorsal aorta, aggregates of virions were found to migrate towards the rginal chromatin leaving the virogenic stroma with a few virions (Fig 7). The virus rticles ranged in size from 260-318 nm in length and 115-144 nm wide. In midgut ucleus, the virus could not be demonstrated, rather the nuclei were normal without any nlargement and were with nucleolus (Fig 8).

Precisely among the tissues examined gill, foregut and hindgut exhibited mparatively severe and greater degree of virus multiplication and margination of romatin.

Morphogenesis of WSSV could not be made clear so far due to the compulsion on e investigators to study the whole process in animal model and not in cell lines. In this dy also prawn animal model had to be used and the figures 9-14 represented different ages/components in the WSSV morphogenesis. In all ultrathin sections observed, rgination of chromatin and formation of middle electronlucent central virogenic oma could be observed as the prelude of viral multiplication. To delineate the viral rphogenesis, different stages in the viral multiplication cycle as evidenced in the ctron microgarph were used. A prominent structure was the elongated empty capsid th a trilaminar outer covering. Some of the capsids were closed at one end while the ers have both their ends open (Fig. 9). There were empty vesicles seen in the virogenic oma (Fig.10) suggesting the formation of envelop as evidenced principally due to its minar nature. The capsid got progressively densified (Fig 11, 12 & 13) with an

ectron dense nuclear material. Fig.14 depicted, an enlarged view of the empty capsid, ith trilaminar outer covering forming the envelop with an electron dense nuclear aterial at one end borne in a trilaminar vesicle resembling the trilaminar envelop. Fig 15 presented a completely formed WSSV with a trilaminar outer covering and electron nse inner core inside the virogenic stroma of the infected nucleus.

4 Discussion

On comparing the ultrastructure of WSSV infected nuclei with histopathological anges in the corresponding tissues, greater degree of correlation between the two odes of investigations in depicting the severity of infection was noticed. In the previous apter it has been demonstrated that gill, foregut and hindgut were the most severely fected tissues in a moribund animal with extensive pathological changes. In infected l tissue two major pathological changes such as 1. Vacuolization, disintegration and pturing of median gill axis, gill filaments and gill lacunae and 2. Dilation of the median ood vessels were observed. The bunches of fully formed virions seen in the ypertrophied nuclei, as demonstrated here by ultrastructural study, reason out the higher egree of pathological changes occurred in gill tissue. Normally when the animals get fected they come to the surface of water and move towards the periphery of the pond pparently to get more oxygen, a behavioral trait considered as an outward manifestation f the impairment of the respiratory system.

Foregut cuticular epithelium was found to be a prominent target tissue in which e viral infection could be easily demonstrated (Sudha *et al.,* 1998) with multifocal ecrosis of the underlying muscle layer and disintegration of the inner epithelial lining as escribed in the previous chapter. This observation is greatly supported by the astructure of the infected nuclei with bunches of fully assembled virions towards the argin of the nucleus. The histopathological and electronmicroscopic evidences of the verity of the viral infection in the foregut answer to the question why the animals do t feed once the disease gets manifested.

Histopathological examination of hindgut, as presented in the previous chapter, showed nuclear hypertrophy, degeneration and necrosis of the underlying connective tissue in the infected animal. Supporting that acute viral infection ultrastructure of nuclei were showed signs of acute viral involvement with bunches of fully formed virions.

In midgut, histologically, there were profound degenerative changes in the thin columnar epithelial lining, but without any nuclear hypertrophy (Refer chapter-1). Ultrastructurally the nucleus exhibited very narrow electron lucent stroma with surrounding chromatin. However, certain vesicular structures seen in the less electron dense zone was intriguing. Even though viral multiplication could not be demonstrated in the nuclei pathological changes such as virogenic stroma, membranous vesicles etc, were well evident.

In heart tissue nucleus, the viral multiplication was poor in its magnitude as characterized by broader ring zone, smaller virogenic stroma and disorganized and fewer virions. This is in agreement with the histopathology of heart tissue of moribund animals, where hypertrophied nuclei could not be demonstrated, instead generalized vacuolization, constriction of muscle bundles along with increased infiltration of blood cells and edema (Chapter- 1) were seen.

Hepatopancreocyte nuclei were characteristically normal without any virions. However, viral multiplication could be seen in the connective tissue nuclei. May be because of this, as a secondary response to the collapse of the supportive connective tissue, profound pathological changes were able to be demonstrated in the hepatopancreatic tubules even though they themselves were not susceptible to virus invasion (Chang *et al.,* 1996).

Alike heart tissue, nerve tissue was also slow in responding to the virus as seen in the moribund stage the virus was in different stages of morphogenesis without attaining full assembly. In agreement to this the histopathological investigations had revealed that

In the nerve tissue it were the neuro secretory cells, found to have hypertrophied nuclei along with disintegration of connective tissue.

No histological studies on dorsal aorta of infected shrimp have been made previously to make any comparison. However, electron microscopically dorsal aorta was also found to be greater susceptible to virus multiplication with bunches of virions migrating to the periphery of nucleus.

Precisely very high order of correlation could be obtained between the histopathology and electron microscopy of various tissues in moribund *P.indicus* which illustrated variations in the response and susceptibility to WSSV infection. Another observation is the extensive pleomorphism as evidenced by variations in the morphometric measurements of the virions seen in the nuclei of various tissues.

Viral morphogenesis is initiated by the margination of chromatin resulting in the formation of an electron dense peripheral ring zone and clear electron lucent virogenic stroma. (Durand *et al.,* 1997, Wang *et al.,* 1999 (a) & 2000). Viral multiplication takes place in the virogenic stroma in locci where they are arranged in paracrystalline array during assembly.

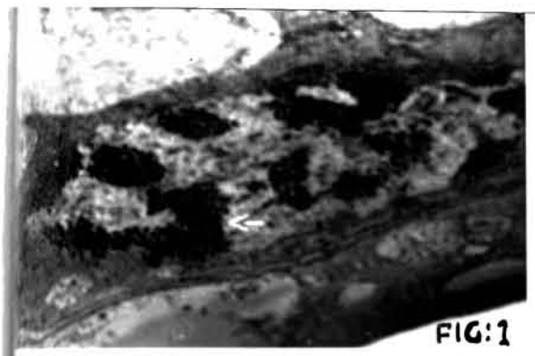
The unique feature of morphogenesis of WSV is the synthesis of all the three major components of the virus such as the nuclear material, capsid and envelop within the nucleus *de novo*. There is no evidence of the trilaminar envelop getting derived from the nuclear membrane. Instead it appears that the lipid envelop is synthesized within the nucleus by transporting the precursors from cytoplasm across the nuclear membrane. Durand *et al.,* 1997 reported the formation of certain circular membranous structures *de novo* within the nucleus which later gave rise to the envelop. As per the available evidence the envelop formed readily covers the capsid by sliding over the latter which when formed remains one end fused and the other end open.

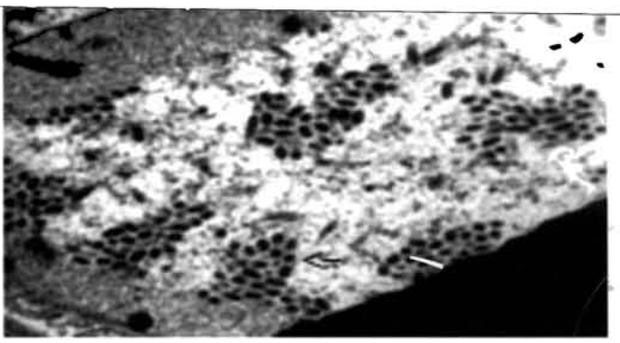
As suggested by Durand, 1997 the empty capsid formed gets enveloped before getting it filled with the nuclear material. This densification of capsid with the nuclear material is a dynamic process, the true mechanism of which is poorly understood. The nuclear material was reported to have filamentous appearance (Durand *et al.,* 1997) which entered the capsid through the open end. Contrary to that, in the present investigation the nuclear material was found spherical borne by trilaminar vesicular structures having the same composition of the envelop. Similar vesicular structures were observed by Wang *et al.,* 1999 (a) which were filled with the nuclear material from viral nucleosome as long rod shaped structures with numerous repeating sub units, as considered to be the precursor of the viral nuclear material. Contrary to in the vesicle is delivered to the capsid through the open end driven by an unknown mechanism. The capsid get fused and since the vesicle which carried the nuclear material and the envelop surrounding the capsid have the same structure and probably the same chemical composition both of them fuse together leaving occasionally a tail like extension. This is contrary to Wang *et al.,* 1999 (a) who suggested that the capsid grew over to the nuclear material. Wang *et al.,* 2000 was of the opinion that the virogenic stroma first gets filled with many empty capsid shells, most of which are surrounded loosely with an envelop. Both the shells and envelop were open at one end and later the electron dense threads extends directly through the open end. However, based on the evidence generated in the present study we are not able to agree with Wang *et al.,* 2000 who states that the envelop is formed later after densification of the empty capsid with the nuclear material. Similar disagreement is there to the observation of Durand *et al.,* 1997 who reported that tubular capsid precursors are formed which later get segmented to form smaller capsids.

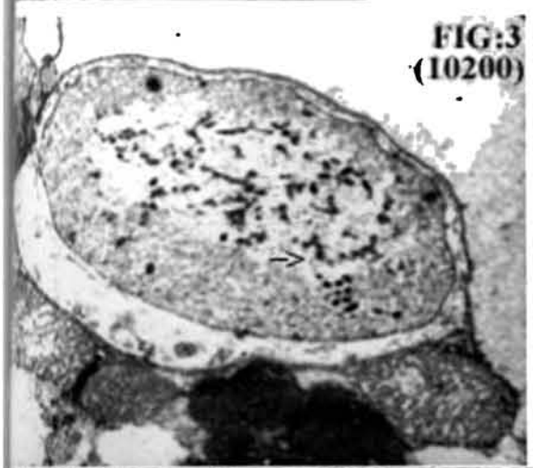
Based on the evidence obtained in the present investigation and the supporting literature the following sequence in the viral morphogenesis has been revolved.

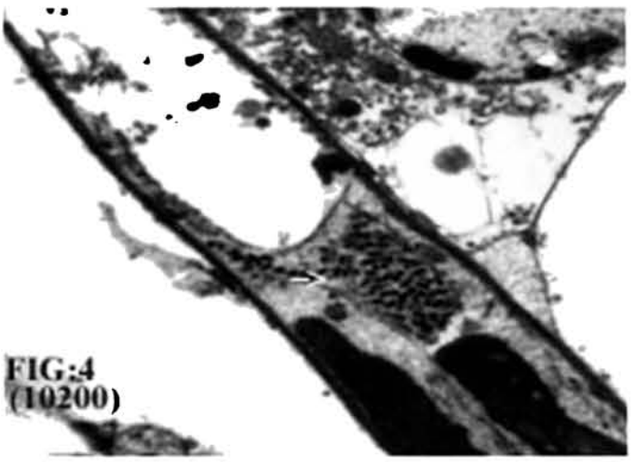
Virus nuclear material, capsid and envelop are synthesized within the virogenic stroma of the nucleus; 2. They migrate to various loci for virus assembly; 3. The trilaminar envelop with one open-end slide over to the single open-ended capsid; 4. The virus nuclear materials get surrounded by a trilaminar vesicle and are carried to the open

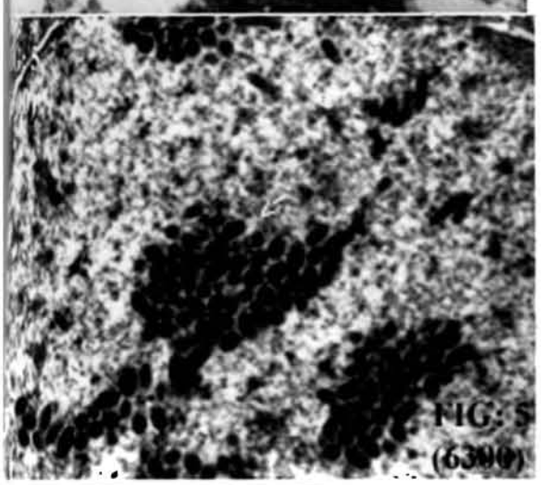
ed of the enveloped capsid; 5. By an unknown process the spherical nuclear material is rushed to the core of the capsid; 6. Capsid gets closed, the vesicular structure fuses with the envelop to form fully assembled virions; 7. While the virus assembly takes place they assume a paracrystalline structure which later gets loosened facilitating migration of the virions towards the base of the nuclear membrane within the marginated chromatin; 8. The nuclear membrane lyse releasing the virions to cytoplasm from where the virus will be getting released when the animal dies and cells are decayed.

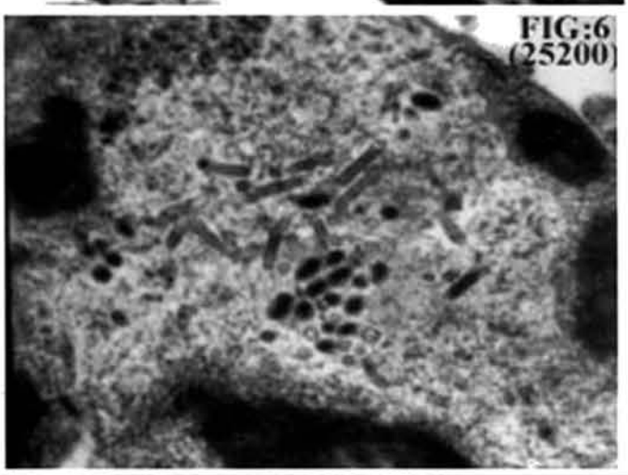


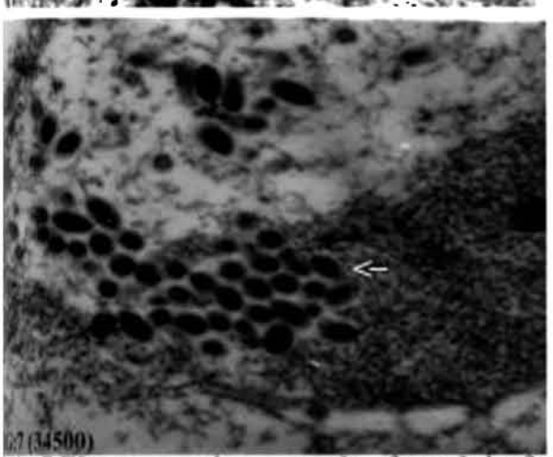


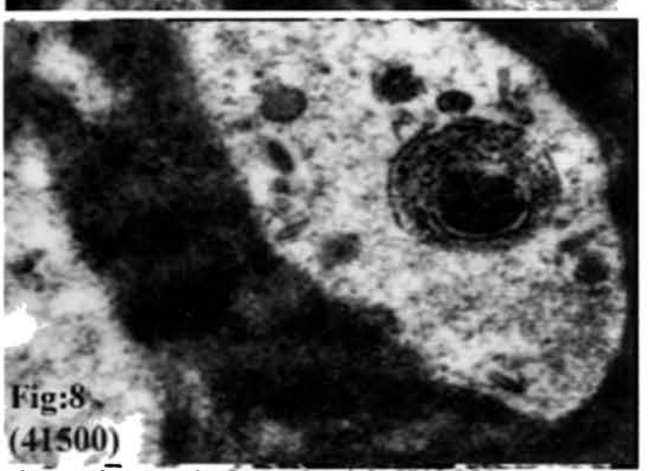




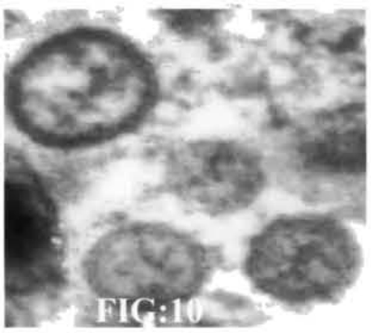


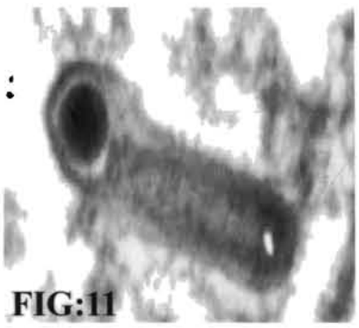


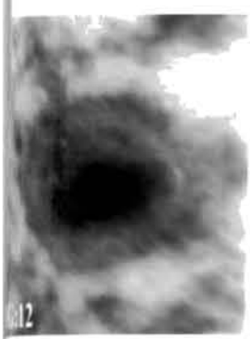


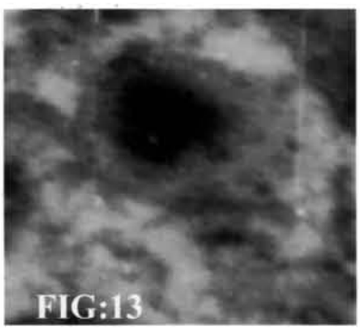


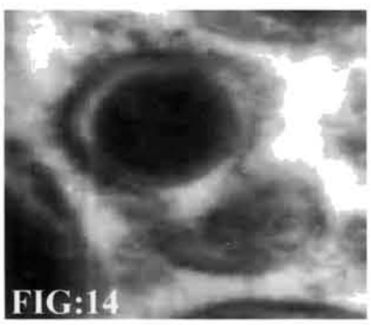
g 1 - 7 Electron micrograph of nuclei of various tissues infected with WSSV. g 1 Gill tissue with paracrystalline array of the virions, Fig 2 Foregut tissue th bunches of virions, Fig 3 Heart tissue with dispersed virions, Fig 4 Connective ssue of hepatopancreas packed with virions, Fig 5 Hindgut with bunches of virions. g 6 Nerve tissue with different stages of viral morphogenesis, Fig 7 Dorsal aorta th virions migrating to the periphery of the nucleus. Arrow indicates virions. Fig 8











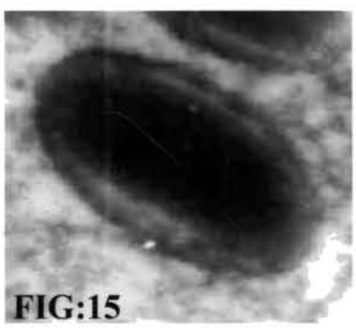


Fig 9 : Elongated empty capsid with trilaminar outer envelop

Fig 10: Cross section of tubular structures with trilaminar wall characteristic of the viral envelop

Fig 11: Fusion of nuclear material born by a trilaminar vesicle with the capsid just before its delivery into the capsid core

Fig 12,13 &14: Different stages in the progressive densification of the enveloped capsid

Fig 15: Fully assembled WSSV

CHAPTER - 4

DEVELOPMENT OF POLYCLONAL ANTISERA TO SHRIMP CELL CULTURED WHITE SPOT SYNDROME VIRUS

CHAPTER - 4

DEVELOPMENT OF POLYCLONAL ANTISERA TO SHRIMP CELL CULTURED WHITE SPOT SYNDROME VIRUS

4.1 Introduction

Among shrimp viruses, White Spot Syndrome Virus is of major concern in all shrimp growing countries. The virus was originally identified in Thailand in 1990s and subsequently in several other Asian countries (Nakano *et al.,* 1994, Chen 1995, Chou *et al.,* 1995, Wang *et al.,* 1995, Durand *et al.,* 1996(a), Lo *et al.,* 1996, Lu *et al.,* 1997(a), Nagbanua *et al .,* 2000). In India this virus virtually paralyzed the shrimp industry (Anon 1994, Ninawe 1997, Mohan *et al.,* 1997, Karunasagar *et al.,* 1997, Otta *et al.,* 1998, Hameed *et al.,* 1998, Shankar & Mohan 1998, Sudha *et al.,* 1998, Sen *et al.,* 1999, Rajendran *et al.,* 1999).

As part of the managerial measures, early, accurate and quick detection of the virus was given top priority. Thanks to these efforts, DNA based diagnostics such as diagnostic PCR based on several primers (Lo *et al.,* 1996(b), Nunan *et al.,* 1997, Kim *et al.,* 1998, Tapay *et al .,* 1999, Peng *et al .,* 1998), DNA probes for *in situ* hybridization (Chang *et al .,* 1996, Durand *et al .,* 1996, Wongteerasupaya *et al.,* 1996, Chang *et al .,* 1998) and dot blot hydridization (Wongteerasupaya *et al.,* 1996 , Hameed *et al.,* 1998) with varying degree of sensitivity and levels of application have been developed. PCR based diagnostic being most sensitive (5pg virus), is routinely employed for screening post larvae for stocking grow out systems. *In situ* hybridization shall be useful at a slightly advanced stage of infection and requires sophisticated laboratory facility. Dot blot hybridization although could be performed at field level, sensitivity is still lower, and the procedure is not so simple. A major drawback of all these techniques, apart from the cost involved, is the higher chances of false positive and false negative reactions and

possible block in DNA amplification, which specifically happens in the case of PCR methods.

However, for surveillance of the disease and routine monitoring of the culture systems, less expensive, simple to perform but fairly sensitive methods are required. Aiming at this requirement several investigators have reported the production of polyclonal (PAbs) and monoclonal antibodies (MAbs) to the virus. However such serological methods have not been put into routine use by diagnostic laboratories for the detection of WSSV in clinical specimens, in part because the antibodies are not commercially available (Hameed *et al.,* 1998, Zhan *et al.,* 1999, Nadala and Loh 2000, Van Hulten *et al .,* 2000(a).

One of the impediments in commercializing the antibody-based diagnostics is the labour intensive process involved in virus purification from the host tissue. In addition, even the exhaustive purification does not completely remove trace amounts of shrimp proteins which may be immunogenic (You *et al.,* 2002). As the WSSV is a very effective immunogen (Nadala *et al.*, 1997) prospects for generating PAbs are comparatively higher if sufficient purity of the antigen is attained, as it often recognize multiple epitopes making them more tolerable to small changes in the nature of antigen, can detect denatured proteins, can be generated in a variety of species and the process economically viable than Mabs (Poulose *et al.,* 2001, Anil *et al.,* 2002). Where in the case of Mabs, the cost of production and maintenance of a clone is very high and are more vulnerable to the loss of epitope through chemical treatment of the antigen, which further demands the pooling of the clones (Harlow & David 1988 and Lehninger *et al.,* 1993). As reported earlier (You *et al.,* 2002) purification of WSSV from infected tissues of prawns was not successful for us too to yield sufficiently larger virus titre to be used as the antigen to yield higher antibody titre. One of the important reasons for this impediment was the enveloped nature of the virus itself, which made it difficult to get physically separated from the tissue mass. Consequently the virus titre declined to a very low level during the purification process, not sufficient enough to be used as the antigen. This difficulty in purifying antigen led You *et al.,* 2002 to go for the expression of a truncated version of

ip 27.5 KDa WSSV envelop protein in *E.Coli* and to use it for the generation of hyper mmune serum in rabbit. While efforts were on to attain pure virus with sufficiently large rus titre here, it happened so that a couple of primary cell cultures and diploid cell lines ould be developed from *Penaeus indicus* (Indian white prawn) in this laboratory, thanks the project 'Development of cell culture systems from penaeids for the isolation of hite spot syndrome virus' sponsored by Department of Biotechnology, Govt of India. onsequently, the WSSV could be isolated in the diploid cell lines generated from estalk (Kumar *et al.,* 2001) and hepatopancreas (unpublished) as well. These hievements prompted us to use the virus generated from cell cultures as the antigen for olyclonal antibody production. Even though the diploid cell line from eyestalk and patopancreas could support the virus rather uniformly, the one generated from the ploid cell line of hepatopancreas (*Pi* HPT-1) was used for the purpose, as the cell line as showing positive signs of establishment.

2 Materials and methods

2.1 Generation of WSSV antigen from prawn hepatopancreas cell culture (*Pi* HPT-1)

Several bottles of *P.indicus* hepatopancreas cell culture (*Pi*HPT-1) were prepared d inoculated with 0.1 ml aliquots of virus suspension which was prepared by cerating (1.5 g) infected prawn tissue in cold 10 ml PBS (NaCl - 8g, KCl- 0.2g, Na_2HPO_4-1.15g, KH_2PO_4- 0.2g, double distilled water –1000 ml) with glass wool to a mogeneous mass using mortar and pestle in an ice bath. The homogenate was ntrifuged at 8200-X g in a refrigerated centrifuge (REMI C.24) at 4°C, and the pernatant fluid was passed through a 0.22µ pore size membrane filter (Sartorius India Ltd). Prior to inoculating the bottles the preparation was streaked on ZoBell's agar ates and incubated at 28 ± 2°C for 72 hours to examine the presence of bacteria. After culating with the virus suspension the bottles were incubated for 4 to 7 days at 25°C osed system) till more than 90% cells dislodged and lysed (visual observation). To ease the virus from the infected cells the bottles were frozen (-20°C) and thawed

peatedly for 3 times and the cell debris removed by centrifugation at 2500g for 30 inutes at 4°C. The supernatant was saved and centrifuged at 11000g for 30 minutes at °C. The supernatant was again saved and subjected to ultra centrifugation at 1 lakh g for hours. The pellet was resuspended in minimum quantity PBS (NaCl - 8g, KCl- 0.2g, Na_2HPO_4-1.15g, KH_2PO_4- 0.2g, double distilled water –1000 ml) and distributed in liquots of 1ml each and maintained at -35°C. Protein content of the suspension was stimated (Bradford, 1976).

As control, uninoculated cell culture in bottles were subjected to the above reatment and the pellet obtained after ultra centrifugation was saved, resuspended in PBS nd maintained at -35°C after estimating the total protein content. Identity of the virus tock was confirmed by diagnostic PCR for WSSV employing the PCR kit supplied by Bangalore Genei (P) Ltd, Bangalore and by employing immunoblot using the MAbs upplied by Dr. K. M. Shankar (Anil *et al.*, 2002).

4.2.2 Preparation of antigen- adjuvant emulsion for immunization

The concentrated virus suspension (ultracentrifuged) stored at -35°C in aliquots as used as the antigen. An aliquot of 0.5ml of this suspension containing a minimum of 50µg protein was mixed with equal quantities of Freund's adjuvant (complete for the first injection & incomplete for the remaining injections) (Bangalore Genei (P) Ltd, Bangalore) and emulsified thoroughly using a 5ml glass syringe with 21 gauge needle attached.

4.2.3 Immunization

The quantity of antigen mixture used and the schedule of injection are given in Table-1. Three months old New Zealand white male rabbit maintained under laboratory conditions was used for immunization. Five injections of 0.5ml antigen + 0.5ml adjuvant were given at multiple sites intradermally using insulin syringe (29 gauge) as per the schedule given in Table -1. After 14 days of the first set of immunization, a test bleeding

as done to determine the antibody titre by drawing 3ml blood from the marginal ear ein of the test rabbit. The blood was aseptically collected in screw capped tubes and lowed to clot at room temperature for half an hour. The clot formed was detached from ass wall using sterile glass rod and kept at 4°C overnight. The serum formed was ansferred into Eppendorff's tubes and centrifuged at 1000g for 10 minutes. The pernatant was transferred to fresh tubes in small aliquots and stored at 4°C. The tibody titre was determined by microwell plate precipitation method and the cross activity was checked by single gel diffusion technique, radial Immunodiffusion chnique and Ouchterlony technique.

2.4 Microwell plate precipitation test

The antibody titre was determined by titrating equal quantities of antigen to a paration of serially diluted antiserum as summarized in Table 2 and 3. The highest lution of the antiserum where a clear precipitation could be obtained was counted as the tibody titre.

2.5 Simple agarose gel immunodiffusion

This was done according to Mancini *et al.,* (1965) using 1.5% agarose gel. On a an dry microscope slide, 1mm thick 1.5% molten agarose prepared in PBS was poured d solidified. Using a template and gel puncture, 2 wells of 3mm diameter at a distance 3mm were punched. Bottom of both the wells was sealed with 5µl of 1% molten arose prepared in distilled water. One of the wells was loaded with 10 µl antiserum and the other with the same quantity of antigen. A negative control slide was run with one of the wells with the control preparation of cell culture and the other with the antigen. The ides were observed for the precipitation arc after incubating them overnight at room mperature (28± 1°C).

2.6 Single radial immuno diffusion technique

In this technique (Ingild, 1983) 60µl antigen was mixed with 3ml of 1.5% molten agarose solution prepared in PBS and a gel layer of 1mm thickness was made on micro slide. As control 60µl back ground protein (cell culture extract pelleted) was mixed with 3ml of 1.5% molten agarose solution as described above and converted into slides. These were allowed to gel for 10 min and two wells of about 3mm diameter each were punched in each slide using a gel puncture and template. The bottom of the wells was sealed using 1µl of 0.5% molten agarose solution. Subsequently one set of wells was filled with 10µl antiserum and the other set with PBS as the negative control. The preparation was incubated overnight at room temperature in petridishes with wet cotton to reduce evaporation and drying of the gel. The preparation was observed for precipitation around the margin of the well.

2.7 Ouchterlony technique

1.5% agarose solution was prepared and poured into a glass Petridish. After solidification, depots were punched using a gel cutter in circular pattern in the gel. The central depots were filled with a positive control (Shrimp cell cultured WSSV), a negative control (Shrimp cell culture extract- pelleted), suspected samples from wild as well as PCR negative sample. The antibody was placed in the central well (Fig 3). The Petridish was then incubated at 28°C ± 1°C overnight and observed for precipitation arc between the central well and peripheral wells.(Ouchterlony & Nilsson 1978).

3 Results and Discussions

The antibody titre obtained by microwell plate precipitation as presented in Table 1 indicated a titre value of $1:10^8$ after the 3rd booster. The results of the simple agarose gel immuno diffusion assay are presented in Fig 1. A clear precipitation arc could be seen between the wells containing the antiserum and the antigen and no precipitation arc

tween those of the antiserum and the control. In the single radial immuno diffusion ssay a ring of precipitate could be seen around the well in the gel plate with the ntiserum. However, no precipitate could be noticed in the control slide which was repared by incorporating the background protein (Fig 2). In Ouchterlony technique, ear precipitation arcs could be observed in the case of viral antigen and the wild virus spected samples, while it was absent in the PCR negative samples. Very faint recipitation could be seen between the control well filled with the cell culture antigen nd the central well with antiserum (Fig 3).

Thus in this work the procedure for generating PAbs could be very much mplified by employing the cell cultured WSSV as the viral antigen. Earlier investigators ho developed PAbs to WSSV were following the cumbersome procedure of virus rification by differential ultracentrifugation, sucrose and Cesium chloride gradient ntrifugation (Cesar *et al.,* 1997, 1998 (a), Cesar & Loh, 1998 (b), Wang *et al.,* 2000(a)). he antibody raised in this work by the simplified procedure could recognize its own ntigen and also the WSSV in suspected wild samples. A series of diagnostic PCR ositive and negative sample for white spot virus could be checked for the recognition of e viral antigen as presented in Table 3. The data as a whole suggest the feasibility of mploying the cell cultured virus antigen for raising PAbs. A very faint band formed tween the antiserum and the cell culture background protein suggest the presence of mall quantity of immunogenic background molecules in the virus preparation used for mmunizing rabbit. But this reaction was not detected with any of the wild samples sted, which were diagnostic PCR negative for WSSV which suggests that in the ractical sense the small fraction of cross reacting antibodies might not lead to any false sitive reactions. However, this small fraction of IgG formed shall be absorbed on to etone dried shrimp cell culture fractions which would remove antibodies that cross act with normal shrimp antigen.

Precisely, the process of generating PAbs to WSSV could be made very simple d less expensive, opening up the way for commercializing the use of

mmunodiagnostics in WSSV detection. The reagent produced here turns out to be an xcellent material for developing immunodiagnostic kits of varying nature.

Table:1 Preparation of antigen-adjuvant mixture and the schedule of injection performed.

DAY	QUANTITY OF ANTIGEN (ML)	ADJUVANT
0	0.5 (50μg)	0.5ml antigen +0.5ml Freund's complete adjuvant
4	0.5 (75μg)	0.5ml antigen + 0.5ml Freund's incomplete adjuvant
8	0.5 (100μg)	0.5ml antigen + 0.5ml Freund's incomplete adjuvant
12	0.5 (125μg)	0.5ml antigen + 0.5ml Freund's incomplete adjuvant
14	TEST BLEEDING	
22	0.5 (125μg)	0.5ml antigen +0.5ml Freund's incomplete adjuvant

Table:2

Antibody titre by microwell precipitation on reacting equal quantities of antigen with varying quantities of antiserum progressively diluted with buffer

WELL NO	1	2	3	4	5	6	7	8	9	10
ANTIBODY(μl)	100	100	100	100	100	100	100	100	100	100
BUFFER(μl)	900	900	900	900	900	900	900	900	900	900
ANTIGEN(μl	100	100	100	100	100	100	100	100	100	100
1 HOUR	+	+	+	+	+	+	+	+	-	-
4 HOURS LATER	+	+	+	+	+	+	+	+ *	-	-

*Antibody titre

Table 3

Comparison of the efficacy of the polyclonal antiserum developed against WSSV using diagnostic PCR and Ouchterlony technique.

No	Sample	Diagnostic PCR	Ouchterlony technique
	LRT-10 (24-1-03)	- -	No precipitation
	MFM Narakkal (27-1-03)	+ +	Precipitation
	Tissue culture control	- -	Very faint Precipitation
	Virus inoculated cell culture supernatant	+ +	Precipitation arc
	Prawn muscle extract	- -	No precipitation
	WSSV infected prawn generated in the lab	+ +	Precipitation

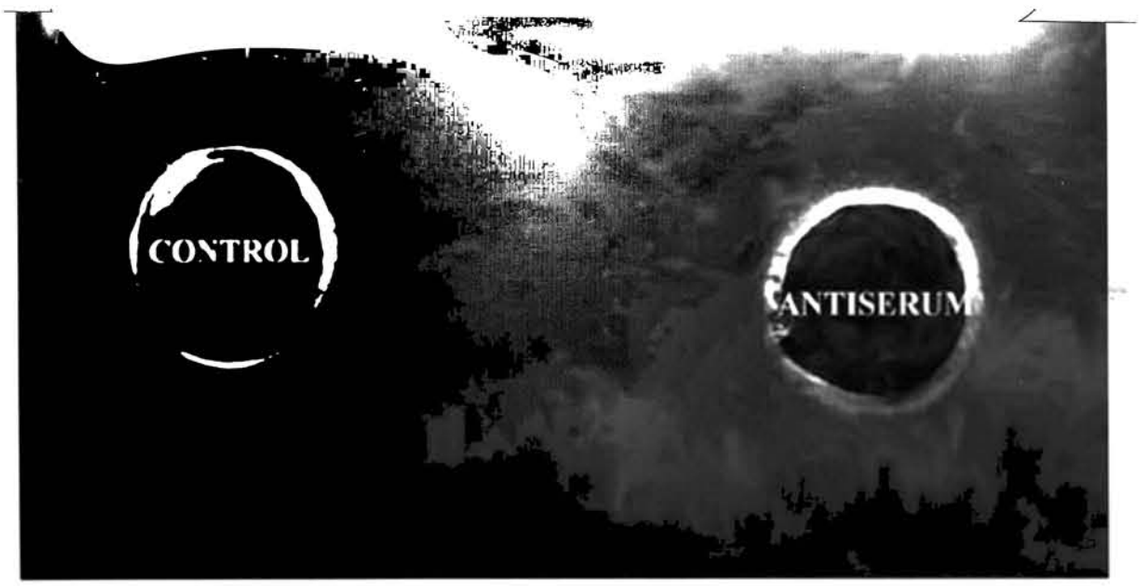


FIG:1(a)

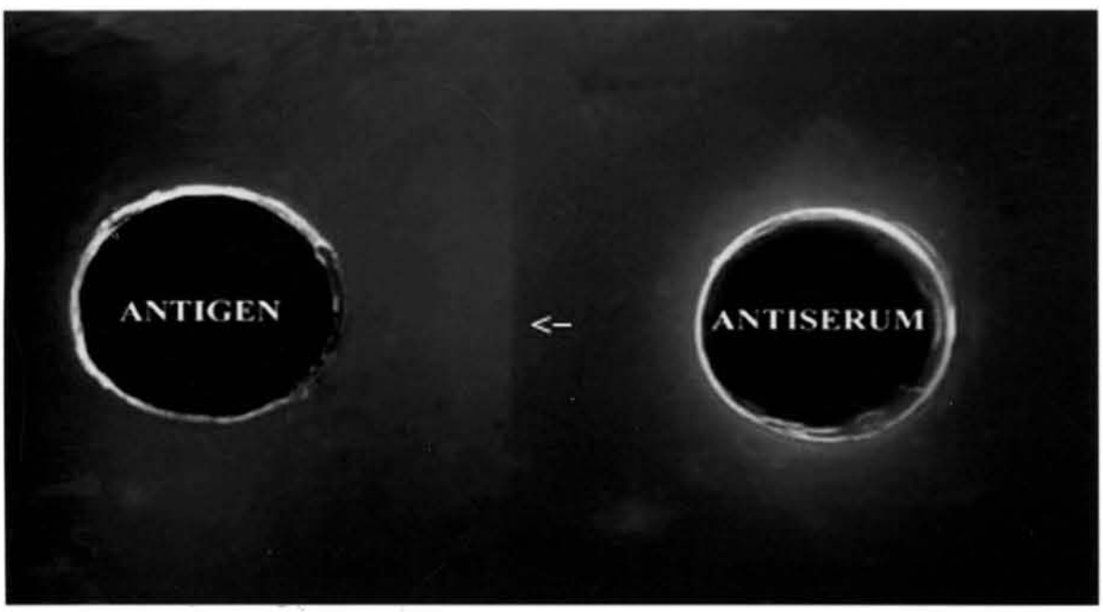


FIG: 1(b)

Fig 1 Simple agarose gel precipitation.

a. Antiserum versus control (T/C control)

b. Antiserum verses antigen (WSSV).

Arrow indicate the precipitation

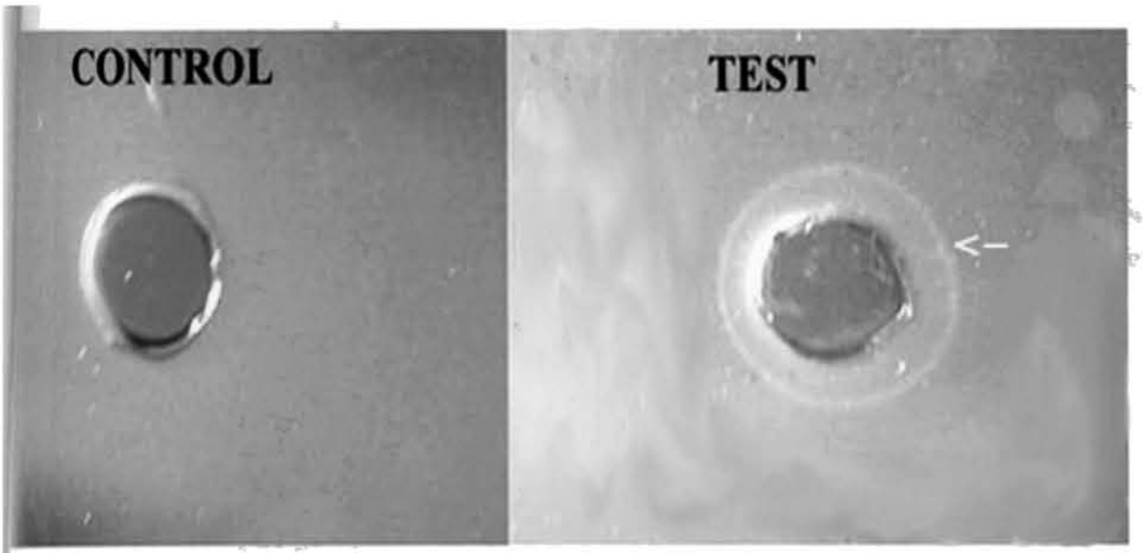


FIG: 2
Single radial immuno diffusion assay. Control: Agarose mixed with background protein; Test: Agarose mixed with WSSV antigen. Arrow indicate antigen-antibody precipitation

FIG: 3
Demonstration of antigen antibody reaction by the Ouchterlony technique. Arrows indicate precipitation arc. 1:LRT-10, 2: MFM Narakkal, 3: Tissue culture control, 4: Virus inoculated cell culture supernatant, 5: Prawn muscle extract, 6: WSSV infected prawn generated in the lab.

CHAPTER - 5

DEVELOPMENT OF VACCINE AGAINST WHITE SPOT SYNDROME VIRUS

CHAPTER - 5

DEVELOPMENT OF VACCINE AGAINST WHITE SPOT SYNDROME VIRUS

Introduction

Development of appropriate measures to manage white spot syndrome virus (WSSV) has been of great concern globally ever since its emergence as the single most important pathogen in shrimps in all shrimp growing countries. Despite the implementation of several managerial measures such as avoidance, optimization of environmental conditions of grow-out systems, application of immunostimulants, probiotics and vitamins, the disease continues to cause havoc. The existing belief that invertebrates lack specific immunoglobulins has hindered the deliberate attempt of vaccinating shrimps against WSSV (Faye, 1990). True that so far no antibody molecules identical to those in vertebrates have been detected in invertebrates. However, several molecules belonging to the immunoglobulin superfamily (IgSF) have been documented (Mendoza & Faye, 1996). They include the adhesion molecules (Mendoza & Faye, 1996) such as Catherins, Ig-like proteins, extracellular matrix proteins, tiggrin (Johansson 1991), peroxinectin (Johansson & Soderhall 1988, 1989, Johansson *et al.,* 1995) in *P. monodon* hemocytes, haemolin (Sun *et al.,* 1990, Ladenhorff & Kanost, 1991, Theopold & Faulcourt *et al.,* 1997), limulus agglutination-aggregation factor (LAF), hemocytin, integrin protein, croquemort, plasmocyte spreading peptide (PSPI) and Drosophila Toll, Cactus/Dorsal (TCD) (Arala-chaves & Sequeira (2000) which are involved in the effective secondary memory immune response. Nevertheless, it is believed that shrimps including all other crustaceans do not possess adaptive immunity, instead have an innate system which include a diverse array of humoral and cellular factors such as phagocytosis (Rabin, 1970, Ratcliff *et al.,* 1985, Armstrong 1991, Soderhall & Cerenius 1992, Martin *et al.,* 1993, Vargas 1995, Noga *et al.,* 1996, Soderhall & Thornqvisit 1997, Kondo *et al.*, 1998(a)), melanization by activation of the prophenol oxidase activating system (Soderhall *et al.,* 1979, Soderhall 1982, Johansson & Soderhall, 1985

89, Soderhall & Cerenius 1992 & 1998, Vargas *et al.,* 1993, Soderhall *et al.,* 1996, ndez- Lopez *et al.*. 1996 , Soderhall & Thornqvisit 1997, Perazzolo & Barracco Soderhall & Cerenius 1998, Sung *et al.,* 1998, Gollas-Galvan *et al.,* 1999), clotting ss (Omori *et al.,* 1989, Kopaeek *et al.,* 1993, Iwanaga *et al.,* 1998), encapsulation of gn material, (Ratcliffe *et al.,* 1985, Soderhall & Cerenius, 1992, Soderhall & nqvist, 1997) antimicrobial action and cell agglutination (Amirante & Mazzalai, Hetru *et al.,* 1994), non- self recognition factors which include lectins, roteins, antimicrobial peptides and reactive oxygen intermediates (ROI) (Amirante Ratanapo & Chulavatnatol, 1990, Bell & Smith 1993 , Song & Hsieh 1994, adakis & Stratakis, 1995, Anderson 1996, Yoshida *et al.,* 1996, Destoumieux *et* 997, Kondo 1998 *et al.,* (b), Suzuki *et al.,* 2002), cytotoxicity and cell to cell unication (Tyson and Jenkin 1974, Ratcliffe *et al.,*. 1985 , Soderhall *et al.,* 1985, co *et al.,* 1991, Soderhall & Cerenius, 1992). As the quest for understanding more the non-specific defense mechanism of crustaceans was dominating the minds of searchers, the prospects of developing vaccines to protect shrimps from specific V-like pathogens were not seriously pursued. Nevertheless, effective vaccination of us *monodon* and *Penaeus japonicus* against vibriosis with formalin killed *Vibrio* d been in progress (Kou *et al.,* 1989, Itami *et al.,* 1989 and Itami *et al.,* 1992). It en proved that vaccination against vibriosis is effective up to even 50 days post nation, and that the vaccinated group evidently had higher survival rate than the n treated group (Teunissen *et al.,* 1998), which they explained as the partial icity of the vaccine induced bactericidins. This prophylactic immunopotentiation is panied by immune cell activation. However, it remains to be clarified if these s can be explained by the existence of adaptive secondary immune response in brates homologous to that observed in vertebrates, or to a distinct type of noprotective pathway (Arala- chaves & Sequeira 2000). Well before that the ce of bactericidins in the hemolymph of *P. monodon* after exposure to heat-killed *molyticus* had been reported (Adams 1991). Undoubtedly this would only hold true e existed some memory for different pathogens in the shrimp immune system ssen *et al.,* 1998). Dealing directly with WSSV, an active accommodation of the resulting from an initial binding step by the host was proposed (Flegel &

sarawipas (1998) and this was as a result of specific memory, such that simultaneous subsequent viral binding for infection (by different receptors) did not trigger cellular ptosis that lead to host death. Exposure of young larval stages to inactivated viral ticles or sub unit viral proteins ("tolerines" as opposed to vaccine) followed by sequent challenge with active virus, would result in innocuous infections rather than tality (Flegel & Pasharawipas,1998). A similar phenomenon was observed in a mp farm in Hiroshima, Japan against experimental PRDV (WSSV) challenge negas *et al.*, 2000). Moreover, by artificial infection they could produce immune mp where resistance was confirmed by a viral re- challenge and the phenomenon they tained as the enhancement of quasi- immune response. Similar incidents have also n observed in different shrimp farms of Kerala, India where a culture could be mpleted successfully with good harvest where an earlier viral attack had been reported.

These previous investigations, observations and our own experience prompted us ok into the possibilities of immunizing shrimp against WSSV using a formalin- tivated virus preparation.

Materials and Methods

Virus strain

One strain of white spot syndrome virus (CFDDM-MCC 102) from the microbial re collection of the Centre for Fish Disease Diagnosis and Management, Cochin versity of Science and Technology, Kochi was used for the experiment. This virus originally isolated in 1995 from a spawner *P.monodon* into a quarantined batch of SV free *P.indicus*, passaged continuously through several batches of such animals solated further in to a newly developed prawn cell line *Pi*HPT-1 (unpublished). The is stocked at -35°C in animal tissue as well as in the cell line. The virus was firmed as WSSV by histopathology (Fig: 1), electron microcopy (Fig: 2) and gnostic nested PCR (Fig: 3).

To generate sufficient quantity of virus for vaccine preparation, the frozen shrimp se with the virus was thawed and fed to a batch of quarantined and formalin stressed *dicus* maintained in a rearing tank fitted with biofilter system for nitrification. The ted animals on entering moribund stage were examined for the characteristic white s on the inner side of carapace and stocked at -35°C till used for the vaccine paration.

Inactivation of the virus

Gill and head soft tissues were dissected out from infected prawn stock and tained at -35°C. From this stock as per the requirement, tissues were homogenized in pt sterile seawater as the diluent using a tissue homogenizer. Volume of the paration was made up to 100ml for 15g tissue used, with the same diluent. The paration was passed through a sieve of 100 μ mesh to make sure that the particles in preparation were all uniformly smaller. The ratio, 15g tissue: 100 ml sea water, was ed at by trial and error method to facilitate easy passage of the homogenate through mesh. The preparation was subjected to continuous freezing and thawing twice to se virus from the tissue pieces. To make sure that the preparation contained virus, an ot of 0.01ml suspension was injected into 10g size healthy shrimps (*P. indicus*) ediately after preparation, in duplicate at the last body segment and observed for nfestation of the disease. Inactivation of the preparation was achieved by adding aldehyde solution (Qualigens, India) to a final concentration of 0.2%(v/v) and taining at room temperature (28 ± 1°C) for 48 hours. Subsequently, the preparation s maintained at 4°C till used. As the control gill and head soft tissues of apparently thy animals tested diagnostic PCR negative were subjected for the same treatment maintained at 4°C and used.

Maintenance of experimental animals

Juvenile samples of *Penaeus indicus* of average body weight of 3gms were ght to the laboratory, quarantined and subjected to stress test by exposing to 100ppm

malin for 1 hour and rearing subsequently under normal conditions. The animals, ch survived the test, were transferred to Fiber Reinforced Plastic (FRP) rectangular s of 30L capacity for carrying out the experiment. Water quality was monitored daily ave the parameters within the specific range (pH: 7.5 to 8.5; Salinity:20-22ppt; 0.01 – 0.02ppm; NO_2:0.1-0.2ppm and dissolved oxygen: 6-7 mg oxygen L^{-1}). enever the above values deviated, water exchange was given partially. Aeration was vided from a 1HP compressor through air spargers.

The experimental animals were fed *ad libitum* twice daily at 10am and 5pm with appropriate feed type (vaccine/control/normal).

Determination of the time required for inactivation

To determine the time required for attaining inactivation 10 ml aliquots of the paration was absorbed onto 5 gms pelleted feed (Higashimaru , Kochi), air dried and to fresh batches of juveniles of *P.indicus* (in triplicate) continuously for 9 days at an val of 24 hours from the point of addition of formalin as mentioned above. The mals were observed for manifestation of the disease if any and mortality for 7 days. As itive control freshly prepared virus suspension in the form of tissue homogenate was rbed on to pelleted feed as mentioned above and fed to animals. Inactivated cessed) tissue homogenate from healthy animals devoid of the virus administrated in same pattern served as one of negative controls. A set of un-inoculated shrimps ntained on normal diet under the same conditions served as the second negative trol. To examine the extent of inactivation of bacteria and fungi (which might rally present in the preparation) a loopfull of the preparation was streaked on to ell's and Sabouraud Dextrose agar plates (prepared in seawater) and incubated for 7 . This preparation henceforth is designated as 'vaccine' in this text.

2.5 Efficacy of the vaccine preparation to immunize shrimps against WSSV

The inactivated virus preparation designated as 'WSSV vaccine' and the control ssue homogenate which is devoid of the virus were lyophilized (FTS Systems, Inc, SA) and a yield of 35.6 ± 0.5gm dry mass was obtained from 500ml tissue suspension. he lyophilized material was coated on to pelleted (Grower) shrimp feed (Higashimaru eeds (P) Ltd, Kochi) at a ratio of 1:10 (w/w) using a commercial binder, Bindex. Matrix vet Pharma (P) Ltd, Hyderabad, India) at the ratio of 0.1:10 g feed on v/w basis nd dried in vacuum desicator for 2 days. After drying, the vaccine-coated feed was aintained aseptically at 4°C in closed containers.

Of 12 sets of experimental tanks set apart, four were used to administer vaccine oated diet, and four were maintained on feed coated with the control tissue preparation nd another set was maintained on normal diet. The feeding was *ad libitum* and later the ed consumption was worked out to be at the rate of 0.035gm feed/gm body weight /day. he feeding was continued for 7 days.

Challenge of the above sets of animals was done subsequently on the 1st, 5th, 10th nd 15th day sequentially after the completion of the 7-day vaccination schedule. WSSV fected shrimp meat maintained at -35°C was used for the challenge at the rate of pproximately 1gm infected tissue for three animals. This was done after removing all eaten feed and fecal matter and after partial water exchange. Subsequently the animals ere examined for manifestation of the disease and mortality.

2.6 Minimum vaccine required

The quantity of vaccine incorporated feed administrated per kg body weight of rimp was found to be 35g. Since this value was the one derived from the quantity of ed consumed per animal on feeding *ad libitum*, it was felt to determine the minimum quired quantity of vaccine without compromising with the effectiveness of mmunization considering the commercial viability. The animals were fed on the

perimental diet coated with 0.25 g vaccine preparation /10g feed, the second two sets th 0.5g vaccine/10g feed the third two sets with 0.75 g vaccine/10g feed and the fourth e with 1.0g vaccine/10g feed. Corresponding control sets of animals were maintained normal diet. The overall duration of the vaccine administration was 7 days. The mals were challenged in two consecutive steps, half the number on fifth day after the mpletion of vaccination programme, and the second half on the 10^{th} day.

2.7 Repeated challenges

The test animals which survived the 5^{th} day and 10^{th} day challenges were jected to repeated challenges, with WSSV infected prawn meat, periodically at an rval of 15 days for four consecutive times as described above.

2.8 Examination of animals which survived challenge with WSSV for the presence the virus

i) **By histological preparation.** Gill tissue of the animals, which survived the challenge th WSSV, were fixed in Davidson's fixative and processed for normal histology (Bell Lightner 1988). The sections were stained with haematoxylin and eosin, examined der bright field microscope (Nikon, Japan) for hypertrophied basophilic nucleii and for integration of the tissue.

ii) **By diagnostic PCR.** Samples of gill tissue of the animals, which survived the allenge with WSSV, were pooled as batches and were used for the examination of SSV by diagnostic PCR employing the WSD detection Kit (Bangalore Genei Pvt. Ltd, ngalore). The Kit uses a 2- step nested PCR protocol for the detection of WSSV in mples, wherein, in the first step a 650bp segment on the viral genome is amplified and the nested step a 300bp fragment internal to the first step product is amplified. All ps of the assay and thermal cycling (MJ Research, PTC-150) were performed as per kit instructions. The products were then analyzed on 2% agarose gels using TAE (IX)

.unning buffer (Tris- HCl 0.04M, EDTA 0.001M, Glacial Acetic acid 5.71%) stained ·ethidium bromide and viewed on a UV transilluminator (Hoefer, Macro Vue UV-20).

3 Results

White spot syndrome virus could be inactivated within duration of 48 hours in the ·sence of 0.2% formalin (v/v final concentration) at room temperature (28 ± 1°C) as ·idenced by RPS of 100 in the experimental groups of animals. Maintenance of the ·us at room temperature without any additives also resulted in inactivation by 72 hours, ·the recipient animals did not exhibit any sign of disease and mortality (Table-1). No ·terial and fungal growth was observed in any of the inactivated virus suspensions ·ile streaking on media plates.

On oral administration of the vaccine for 7 days, the shrimps gained protection 5 ·ys post completion of the schedule of administration and it lasted till 10^{th} day as ·idenced by 100 per cent relative survival during this period against challenge with the ·SSV (Table -2).

On examining the efficacy of minimum quantity of the vaccine preparation to ·it a response, the lowest quantity of 0.25g preparation used (dry weight) per 10g feed ·s sufficient to protect the animals during 5^{th} and 10^{th} day post administration. (Table-

Under light microscope, the haematoxylin - eosin stained gill sections of shrimps ·ich survived the challenge 5^{th} day post termination of the vaccination schedule ·eared identical histologically to that of healthy ones (Fig 4 & 5), without any ·aracteristic hypertrophied nuclei and tissue disintegration. Meanwhile the gill sections ·the animals challenged 10^{th} day post vaccination looked largely normal to the control ·d the 5^{th} day challenged one (Fig 6). However, the nucleii of epithelial lining of ·nchial arch was slightly and irregularly enlarged but were eosinophilic. Other than ·s no gross pathological changes could be observed (Fig 7).

On first step PCR of the gill tissue from the shrimps challenged 5th day post vaccination, no amplification of the viral DNA could be seen (Fig 8). Meanwhile in the gill tissue of 10th day challenged post vaccination, amplification of both 650bp fragment (first step) and 300bp fragment (nested) (Fig 8) could be observed. The results precisely suggested the absence of the viral genome in the 5th day challenged group and its apparent presence in the 10th day challenged group.

4 Discussion

Formalin was used as the inactivating agent considering its wide acceptance in vaccine preparation. Basically formaldehyde cross links proteins and stabilizes antigenic epitopes (Wesslen *et al.,* 1957, Bottiger *et al.,* 1958, Barteleng & Woortmeyer, 1984). Even otherwise the virus gets inactivated normally when outside the cell within 4 to 7 days (Fegan *et al.,* 1991) and on exposing to 0.2 % formalin (v/v) the period required for inactivation gets shortened to 48 hours. This treatment makes the preparation quite safe for application by killing bacteria and fungi also, which are normally present in the tissue macerate. The animals were fed on the diet coated with the inactivated virus preparation for 7 days and they were found to have acquired resistance to virus invasion 5th day post completion of the vaccination schedule. The resistance acquired was found to have extended for 5 more days. However, the periods before and after were found vulnerable to the virus invasion and death. Since the challenges with WSSV were made only on 1st, 5th, 10th and 15th day post completion of the vaccination schedule the above timings are quite approximate not specific to the days of challenge. Precisely, oral administration of small doses of inactivated virus over a period of time elicited a positive response in shrimps, which protected the animals from WSSV infection between 5th and 10th day post completion of the schedule of administration.

The schedule of 7 days administration of the vaccine preparation was adopted based on the experience of workers like Itami *et al.,* 1992who found a 7 day period of oral administration of immunostimulants most appropriate to elicit maximum immunological response. Requirement of a couple of days, from the point of termination

the vaccine administration schedule, for the expression of the immunological response as been noticed by many (Sung *et al.,* 1994, Teunissen *et al.,* 1998).

On combining the information gathered through histological observation of nimals that survived the 5th day challenge post vaccination with the results of PCR, ubstantial evidence of a refractory phase in the immunological cycle of the animals, as a nsequence of vaccination, could be observed. During this period, strikingly, virus could ot be detected in the animals, which survived the challenges as evidenced by the normal stology and negative nested PCR. It has to be remembered that these surviving animals ere the ones fixed and processed after experiencing a total mortality of the control roup. This refractory phase almost looks like that of the immune system of vertebrates pecifically mediated by immunoglobulin molecules under the IgSF which react with pecific antigens facilitating their elimination by subsequent phagocytosis. On exposing *monodon* to heat-killed *V.alginolyticus* Adams, 1991 had found bactericidins, a specific roup of molecules, which can react specifically with the corresponding antigen, in the emolymph. Teunissen *et al.,* 1998 maintained that this would hold well only if there isted some memory for different pathogens in the shrimp immune system. In the light f these previous observations and the strong evidence of the elimination/ rejection of SSV from the body of *P.indicus* during the initial phase of 5^{th} day post vaccination hedule we propose the formation and interplay of 'Viricidins', a group of molecules hich can specifically bind with virulent WSSV and facilitate their elimination by ubsequent phagocytosis. The cascade of events which lead to the elimination of the virus re sufficient enough to even digest the viral genome as evidenced by the negative nested CR.

However, a different picture was obtained on examining the gill tissue of those nimals, which survived the 10^{th} day challenge post vaccination. Even though stologically the gill tissue looked largely normal to that of healthy ones, slightly nlarged eosiniphilic nuclei and the first step and nested positive PCR of the same, point the presence and multiplication of the virus in the host cells in this phase of post ccination immunological response. It is reminiscent of the hypothesis of Flegel and

sharawipas, 1998 who proposed an active accommodation of the virus subsequent to an tial binding step by the host cells. This resulted in specific memory, such that multaneous and subsequent viral binding for infection (by different receptors) did not gger cellular apoptosis and host cell death. Deviating slightly from their proposed pothesis, entry of the virus in to a multiplication phase, as evidenced by slightly larged nuclei and first step positive PCR, could be demonstrated here. But that nation did not lead to apoptosis and mortality, rather the animals seemed to commodate the virus without causing tissue damage and death. It is not known how uld the animals achieve this situation of not permitting the virus to damage the tissue, spite of its multiplication. It can be reasonably postulated that a specific kind of hesion molecules or a new class of molecules belonging to IgSF released during this se of the immune cycle might be taking a leading role in protecting the animals from ther destructive multiplication and death.

Flegel and Pasharawipas, (1998) named the inactivated viral particles or subunit al proteins as 'tolerines' as opposed to 'vaccine' probably due to their observation of the erance of host cells to the virus and its accommodation which prevented apoptosis. But could move a step forward in recording four discrete time depended phases in the mune cycle of penaeids post vaccination. The first phase, starting from the 1st day ccination to the 5th day post completion of vaccination schedule, as the immune tivation phase, 5th day post vaccination, as the refractory phase and 10th day post ccination as the accommodative phase and 15th day post vaccination as the immune clining phase. The molecules involved during the refractory phase appear to be ifferent from those involved in the accommodative phase. Since the inactivated virus rticles could elicit a response similar to that of vertebrate system during the refractory se we propose the term vaccine, to 'tolerine'.

The concept of vaccine and vaccination gain added support from the situation of rvival of the 5th day and 10th day post vaccinated groups against repeated four time allenges with WSSV. This implies that subsequent challenges served as vaccinations milar to what happened in vertebrates where the specific memory system recognized the

tigens and led to rapid production of immunoglobulins. The shrimp immune system is Grey area awaiting research with advanced molecular tools. Nevertheless, with the ailable information it could be stated that once vaccinated and protected from WSSV, e situation can be prolonged by repeatedly vaccinating once in 10 days by oral inistration of the inactivated virus.

The situation is quite different from the immunostimulatory response of the non-ecific immune system of crustaceae as demonstrated against WSSV by several vestigators (Sung *et al.,* 1994, Teunissen *et al.,* 1998, Itami *et al.,* 1992). In all such stances only a part of the population could be protected and that too for a shorter riod, and the onset of mortality could be only extended for a few more days but not vented. Contrary to that a relative survival of 100% could be obtained uniformly in all perimental trials provided the challenge was made on the 5^{th} and 10^{th} day post ccination.

The process of vaccination shall be made economically viable as evidenced by the all quantity (0.25g vaccine/ 10g feed w/w) of vaccine preparation, which would be fficient enough to elicits a response in the recipient animals. Investigations are derway to workout the smallest quantity of vaccine required for the above level of otection and to demonstrate and validate it under field conditions. The exact virus titer ailable in such preparations could not be quantified due to lack of standardized WSSV ation protocols. Establishment of a cell line by this Centre from shrimps (*Pi*HPT-1, ublished and patent pending) susceptible to WSSV brightens the prospects of veloping commercial vaccine preparations with an appropriate delivery system for field eld application.

Table- 1. Demonstration of the duration required for inactivating WSSV in terms of its loss of viability and virulence on administering in shrimp. (*P.indicus*) n = 24

Preparations	Relative per cent survival(RPS) of *P.indicus* on administering with inactivated WSSV at specific hour of inactivation									
	0	24	48	72	96	120	144	168	192	216
Inactivated virus	0	0	100	100	100	100	100	100	100	100
IHSTS (-ve control)	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
Uninactivated (+ve control)	M=100%	M=100%	M=100%	M=0%	M=0%	M=0%	M=0%	M=0%	M=0%	M=0%

-ve control: IHSTS (Inactivated healthy shrimp tissue suspension)
+ve control: uninactivated virus
M: Mortality

Table-2. Demonstration of efficacy of inactivated WSSV preparation to protect shrimps (*Penaeus indicus*) from WSSV.

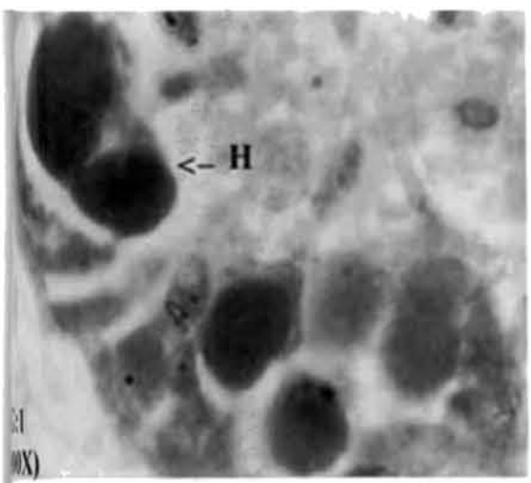
Experimental group of animals fed on	Relative per cent survival on challenging with WSSV post vaccination			
	1st day	5th day	10th day	15th day
1.Lyophilized vaccine coated feed	0 (n= 24)	100 (n= 24)	100 (n= 24)	0 (n= 24)
2.Lyophilized normal tissue coated feed	0 (n= 24)	0 (n= 24)	0 (n= 24)	0 (n= 24)
3. Normal diet	M= 100% (n=12)	M= 100% (n=12)	M= 100% (n=12)	M= 100% (n=12)

M: Mortality

Table-3. Determination of the minimum quantity of inactivated WSSV preparation required for eliciting protective response in *P.indicus*

Experimental groups fed on the diet coated with	Relative per cent survival on challenge with WSSV 5th day post vaccination(n=12)	Relative percent survival on challenge with WSSV 10th day post vaccination(n=12)
1. 0.25g vaccine/10g feed	100	100
2. 0.50g vaccine/10g feed	100	100
3. 0.75g vaccine/10g feed	100	100
4. 1.0 g vaccine/10g feed	100	100
5. Fed on normal diet	(n=6) M=100%	(n=6) M=100%

M: Mortality



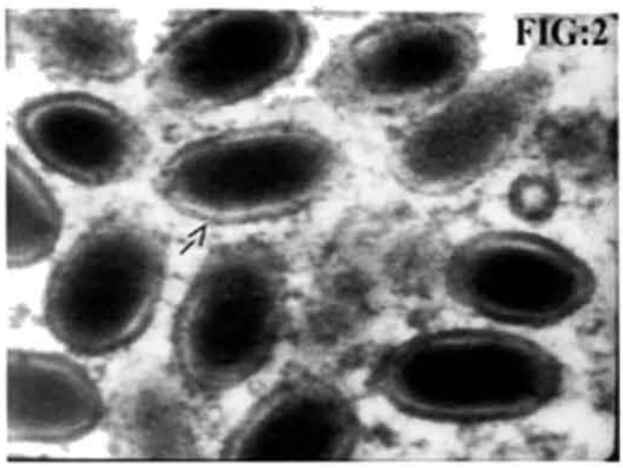


FIG:2

FIG: 3

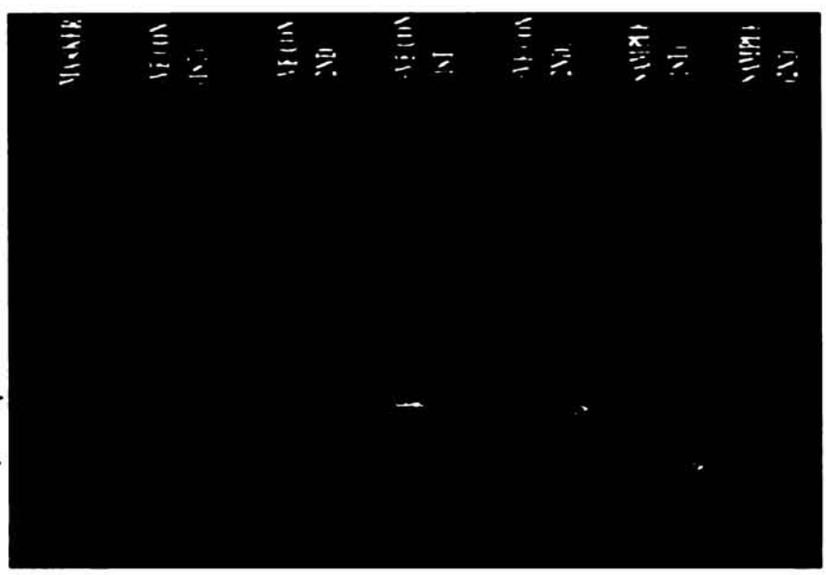
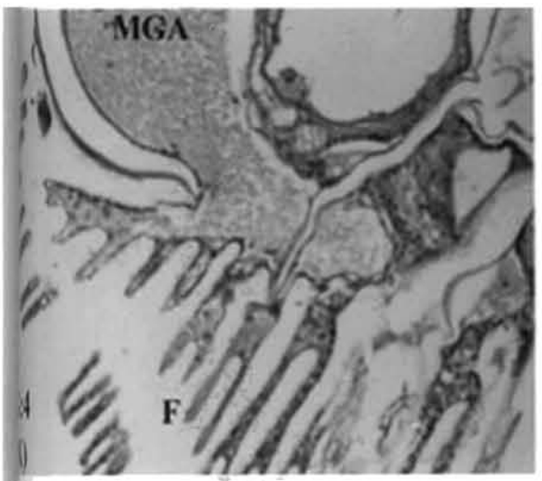
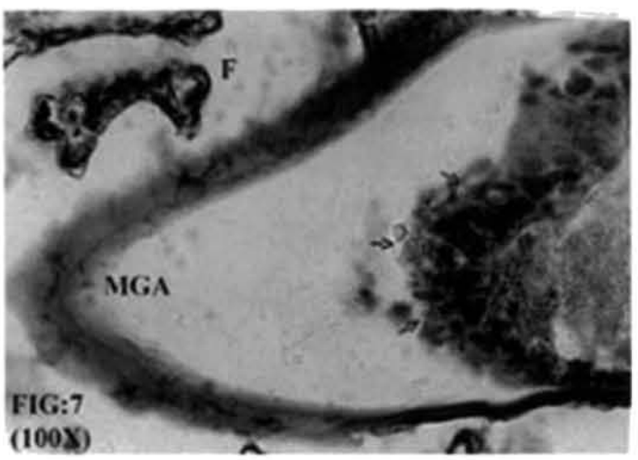
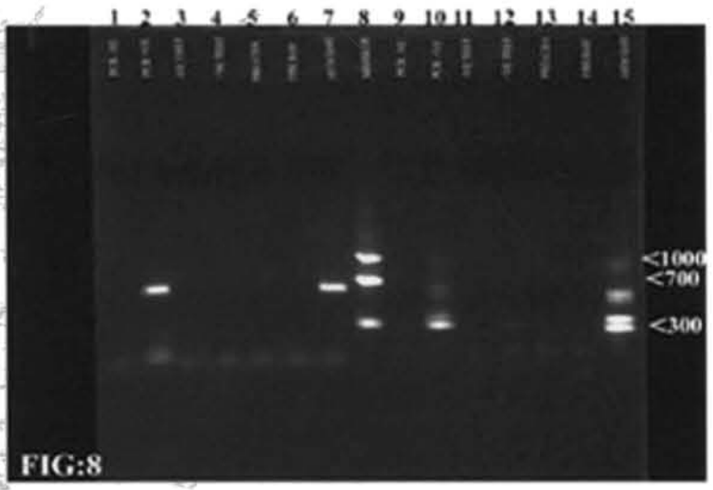


Fig 1 Haematoxylin - eosin stained histological preparation of foregut tissue showing characteristic hypertrophied nucleus caused by the WSSV strain (CFDDM-MCC 102) used in the vaccine preparation Arrow indicate hypertrophied nucleus. Fig 2 Electron photomicrograph of hypertrophied gill tissue nucleus with rod shaped enveloped WSSV strain (CFDDM-MCC 102) used in the vaccine preparation. Arrow indicate the characteristic trilaminar envelope of WSSV. Fig 3 Agarose gel with 650bp 1st step and 300bp nested PCR products of DNA of the WSSV strain (CFDDM-MCC 102) used in Vaccine preparation. H - Hypertrophied nucleus







4 Haematoxylin - eosin stained histological preparation of normal gill tissue
n apparently healthy *P.indicus*. Fig 5 Haematoxylin- eosin stained histological preparation
ill tissue drawn from *P.indicus* challenged with WSSV (CFDDM- MCC 102) on 5th day post
pletion of the vaccination schedule. Arrow indicate epithelial cells of gill arc with normal nuclei.
6 & 7. Haematoxylin - eosin stained histological preparation of gill tissue drawn from *P.indicus*
allenged with WSSV (CFDDM-MCC 102) on 10th day post completion of vaccination schedule.
row indicates eosinophilic partially hypertrophied nuclei. Fig 8 PCR detection of WSSV DNA in
imps vaccinated but not challenged (Lane 5), vaccinated & challenged with WSSV (CFDDM-
C 102) on the 5th day (Lane 6) and on the 10th day (Lane 7) post completion of the vaccination
hedule. F - Gill filament. MGA - Median gill arc.

CHAPTER - 6

DEVELOPMENT AND DEMONSTRATION OF SHRIMPVAC –I' FOR THE MANAGEMENT OF WHITE SPOT SYNDROME VIRUS AND VIBRIOS IN SHRIMP GROW-OUT SYSTEM

CHAPTER - 6

DEVELOPMENT AND DEMONSTRATION OF 'SHRIMPVAC-I' FOR THE MANAGEMENT OF WHITE SPOT SYNDROME VIRUS AND VIBRIOS IN SHRIMP GROW-OUT SYSTEM

6.1 Introduction

As part of prophylactic measure to protect shrimps from White Spot Syndrome Virus (WSSV), vaccination was shown to be effective under laboratory conditions (Refer chapter - 5) and had been described as a viable option under field conditions too in addition to the already accepted managerial measures such as avoidance of the virus in a region, optimization of the environmental conditions of grow-out systems, application of immunostimulants, probiotics and vitamins (Scholz *et al.,* 1999, Tournut 1989 and, Prabhu *et al.,* 1999). While immunizing *Penaeus indicus* orally with formalin inactivated prawn tissue derived WSSV, four discrete time dependent phases in the immune cycle of shrimp post vaccination could be observed. The first phase, designated as immune activation phase, extends from 1st day to the 5^{th} day post completion of a 7 day long vaccination schedule (12 days in aggregate). The second phase, entitled as the refractory phase is on the 5^{th} day post vaccination, the third phase, the virus accommodation phase, is on the 10^{th} day post vaccination, and the 15^{th} day onwards it enters in to a declining phase. The animals, which survived the 5^{th} and 10^{th} day post vaccination schedule, could be repeatedly challenged where in each challenge turned out to be vaccination. The molecules which protected the animals during the refractory phase was named as 'viricidins' and the ones which played the role of virus accommodation as a specific kind of adhesion molecules or a new class of molecules altogether, both belonging to IgSF of invertebrates. Existence of four phases in the immune cycle of shrimp during immunization, and the fact that every repeated challenge with the WSSV being turned out to be vaccination, brightens the possibility of protecting shrimp from WSSV during an entire culture period by repeatedly vaccinating them once in 10 days.

Second to WSSV in shrimp grow-out systems are the pathogenic strains of *Vibrio* which cause devastating conditions during several occasions. To combat this menace Kou *et al.,* 1989 and Itami *et al.,* 1989, 1992 had proved vaccination, with formalin killed *Vibrio* sp. as an option. Adams, 1991 reported even the presence of bactericidins in the hemolymph of *P.monodon* after the exposure to heat killed *V.alginolyticus.*

The viral and bacterial antigens elicit a response in shrimp immune system leading to the production of specific molecules, probably within the IgSF, which are endowed with specificity and short memory (Refer chapter-5). However, immunostimulants have a great role to play in stimulating the non-specific defense mechanisms which is the innate system that includes a diverse array of humoral and cellular factors such as phagocytosis, phenol oxidase activating system, clotting process, encapsulation of foreign materials, anti microbial action and cell- agglutination, non-self recognition factors, cytotoxicity, and cell to cell communication, as summarized by (Soderhall and Cerenius, 1992). More over immunostimulants can serve as adjuvants also for specific antigens in a vaccination schedule (Itami *et al.,* 1994). Therefore any preparation containing inactivated virus and vibrios and an immunostimulant could reasonably be considered as a viable option to equip the animal to fight against both WSSV and *Vibrio* and several other potential and opportunistic pathogens of the aquatic system by way of interplay of the stimulated adaptive secondary immune system and the innate defense mechanism over a period of 120 days by repeatedly administering it in small doses. Accordingly, a cocktail vaccine named 'Shrimpvac-1' was designed, prepared and tested its efficacy under laboratory conditions and subsequently subjected to a field level demonstration as an effective prophylactic preparation to WSSV and *Vibrio* in a semi intensive grow out phase as described in this paper.

6.2 Materials and Methods

6.2.1 Preparation of Shrimpvac-1

Shrimpvac-1 contains shrimp tissue derived inactivated WSSV aqueous suspension (Refer chapter - 5) supplemented with bacterins derived from pathogenic strains of *Vibrio* and an immunostimulant, both in powder form (Patent pending). Based on the previous work 351ml WSSV vaccine was found required for coating 1000g feed of which 35g would be sufficient for administering in 1000g shrimp biomass.

6.2.2 Efficacy of the preparation and its components to immunize shrimp against WSSV

a) Maintenance of experimental animals.

The protocol followed in the maintenance of experimental animals is the same as the one described in chapter -1. In short, quarantined juveniles (3g) of *P. indicus* were exposed to 100 ppm formalin for one hour and those survived for 24 hours were quarantined for 3 days and used for the experiment. The animals were maintained in 30L capacity FRP rectangular tanks under strict monitoring and control of water quality with in a range (pH: 7.5 to 8; salinity: 20 to 22 ppt; NH_3: 0.01 to 0.02 ppm; NO_2: 0.1 to 0.2 ppm; Dissolved Oxygen 6 to 7 mg/L). Whenever deviation of parameters was noticed from the above range water exchange was resorted to. The animals were fed twice daily at 10am and 5pm *ad libitum* with the appropriate feed type (vaccine/ cotrol/ normal).

6.2.3 Efficacy of Shrimpvac-1 and its component to immunize shrimps against WSSV

The experimental design consisted of administration of Shrimpvac-1 and its components such as the inactivated virus, bacterins, and immunostimulants as test groups along with 2 controls such as the groups of animals fed on formalin treated shrimp muscle extract and normal feed was used for delivering the vaccine. As described in the

previous chapter lyophilized inactivated WSSV (25g) equivalent to 351ml infected tissue suspension was used for coating 1Kg pelleted feed. Incorporation of 351-ml shrimpvac-1 in 1-kg feed was practically and economically not feasible. Therefore, the experiment was designed with the incorporation of Shrimpvac-1 100 times lesser in quantity per Kg feed. Accordingly, 0.35ml Shrimpvac-1 was coated on to 100g pelleted feed. While doing so, the quantity of the bacterins, immunostimulants, inactivated normal tissue suspension and inactivated virus suspension (vaccine) was maintained proportionately to the quantity of the same as contained in 351ml shrimpvac-1 when applied to 1 kg feed. Each of the above components was administrated into 4 tanks, holding 3 animals each in duplicate and the process was repeated 3 times. The feeding was done *ad libitum* twice a day and the rate of consumption was 0.035g feed/ gm body wt / day. Administration of the feeds coated with the above preparation was continued for 7 days and, 5 and 10 day after completion of schedule, the animals were challenge with WSSV in the form of infected meat maintained at -35°C at the rate of approximately 75mg for 3 animals. This was done after removing all uneaten feed and faecal matter and after partial water exchange. Subsequently the animals were examined for manifestation of the disease and mortality (Table-1).

6.2.4 Vaccination of the shrimp *P. monodon* against WSSV and *Vibrio* using Shrimpvac-from post larvae to sub adults

The vaccination programme employing 'Shrimpvac-1' was commenced from post larvae extending to sub adults in grow-out system. Considering the loss, which would be met with during delivery of the vaccine under field conditions, the quantity of Shrimpvac-1 was increased by 10 times limiting to 4ml /100gm feed. A nested PCR (for WSSV) negative larval stock was identified at Matsyafed Shrimp Hatchery, Quilon, Kerala by performing 3 consecutive PCR analysis one at naupli, second at Zoea –3 and the third at PL-10 stages using a diagnostic PCR kit (Bangalore Genei, Bangalore). As part of the managerial measures the 10 million capacity larval rearing system was maintained on indigenous probiotics such as 'Detrodigest' and 'Enterotrophotic' (I.S. Bright Singh -personnel communication) without the application of antibiotics. A strict

ater quality regime was maintained as presented in Table-2 in the identified larval earing tank.

Vaccination of post larvae commenced at PL-10 and continued for 5 days. Post arval feed (Higashimaru (P) Ltd, Cochin) was coated with Shrimpvac-1 at a rate of 4.0 l/100gm feed using the commercial binder 'Bindex' (Matrix vet Pharma pvt Ltd , yderabad) at a ratio of 1ml bindex to 100g feed. The coated feed was dried at room mperature (30 ± 1°C) for 48 hours and packed and stored at 4°C. Feeding larvae with e coated feed was at a rate of 62.5g/million larvae /day splitting the ration into three. his quantity of feed required, was arrived at on the basis of the total biomass of PL-10 lmillion) calculated based on the weight of the larvae (0.004g) and the quantity of accine (1.25ml) required per Kg shrimp biomass (Table 4). Five days after the rminations of the vaccination schedule the larvae were lifted for stocking.

2.5 Pond preparation

A 0.80-hectare shrimp pond of Matsyafed Shrimp Farm at Narakkal, Cochin, erala, India was chosen for the field level demonstration of 'Shrimpvac -1'. The pond ith earthen bunds of 2 m height, fitted with a sluice gate of 2 meter dimension was ubjected to eradication of weed fishes by applying tea seed cake powder, having 6% aponin content, to 10 cm water column to attain a final saponin concentration of 5ppm. he shrimps and bivalves which survived the treatment were destroyed by adding lime - mmonium sulphate mixture (5:1) to attain NH_3-N > 10ppm at the time of application. fter confirming destruction of weed fin fish and shellfishes water was allowed to get nto the pond through sluice gate filled with a nylon mesh of 100μ size during high tide. n attainting 70-cm water column the sluice gate was closed and subjected for analysis f water and sediment quality by sampling from five spots fixed at equal distance. hysico-chemical parameters such as salinity, pH, alkalinity, hardness, NH_3, NO_2, hosphate (for water) and pH and E*h* for sediment were analyzed. As part of the ioremediation programme 'Detrodigest' was brewed (150ml in 100 L pond water) and pplied through out the pond following which 'Nutrimix', a mixture of macro and micro

rients, dissolved in an aqueous extract of 100kg dry cowdung was applied to enhance yto and zooplankton production. The pond was fitted with five paddle wheel aerators. e pond preparation was done in such a way that while the vaccination of the post rvae had been completed the pond was made ready for stocking. Maturation and nditioning of the pond for stocking was determined based on the extent of phyto and o plankton production (1 X 10^6 and 1 x 10^3 /ml respectively), and from the quantity of monia NH_3: < 0.01ppm; pH : 7.5 to 8.5 and alkalinity 75 to 100 ppm, in the water lumn.

2.6 Packing, transportation and stocking vaccinated post larvae

Fifty thousand vaccinated larvae were packed in polythene bags having 2000 rvae per bag with 5 liters fresh chlorinated- de chlorinated seawater diluted to suit the linity of the conditioned grow-out system (15 to 25 ‰). While doing so, a bag of 200 rvae was diverted to this laboratory to examine the efficacy of vaccination on the rvae. Stocking was done during the early morning hours and the first 20 days feeding as done in the area nearer to the bunds and subsequently when the juveniles started igrating towards the interior of the pond the required quantity and type of feed was oadcast to the entire pond. The physical, chemical and biological factors monitored, medial measures taken are summarized in Table-5.

2.7 Test of efficacy of vaccination at PL level

The batch of larvae brought to the lab were transferred to the bioassay system in oups of 100 each per 40 x 25 x 10 cm size FRP tanks having 10 litre water and with 1/3 ater exchange everyday. The larvae were challenged on 5^{th} and 10^{th} day post mpletion of vaccination schedule by inoculating 3.5 ml tissue suspension per tank rived from 1.5g infected tissue macerated in 10ml sea water (20ppt). The larvae were served for mortality with clinical signs (Table-3).

6.2.8 Repeated vaccinations under grow - out system

From 10th day post stocking the animals in grow out were vaccinated regularly once in 10 days for three consecutive days for a period of 90 days. Precisely the Shrimpvac-1 was coated on the required quantity of pelleted feed (4ml/100g) using the commercial 'Bindex' at the ratio of 1ml bindex to 100g feed. The coated feed was shade dried and broadcast as summarized in Table -6.

6.2.9 Health assessment of shrimp

Periodic health assessment of shrimp was carried out once in a week for which five to ten animals were brought to laboratory in thermo-cool boxes. Besides weekly increment of weight and general health status were also assessed and remedial measures were under taken accordingly.

6.2.10 Bio-remediation programme implemented

Functionally the system was maintained semi-closed with restricted water exchange. Whenever water level went down due to evaporation loss and seepage the required quantity was pumped in during high tide. Detritus management was given top priority, for which 'Detrodigest' was applied once in 10 days on regular basis as described above.

6.2.11 Completion of the culture, harvest and challenge with WSSV

The culture was completed on attaining 30g average weight and the harvest was performed. During this time the animals weighing 30 to 40 gm were brought to the laboratory and maintained in the bioassay system individually in FRP tanks of 40 x 25 x 10 cm size in 20 ppt sea water. Soon after reaching the lab, five uniform sized animals were used for drawing haemolymph from the rostral sinus for examining the hematological parameters and, the gill tissue was used for diagnostic PCR for WSSV

mploying the PCR kit supplied by Bangalore Genei, Bangalore. After 3 days of climation the animals were challenged with WSSV infected tissue stored at -35°C at a te of 25mg/ animal only once after removing the un-eaten pelleted feed, fecal matter d subsequent to partial exchange of water. The challenged group and the control set ere maintained on normal diet henceforth and examined for clinical manifestation of the sease and mortality. Altogether four repeated challenges were made in the same way ce in fifteen days. After the fourth challenge, all challenged prawns and the control oup were sacrificed and haemolymph collected individually, and the gill tissue was ed for histopathology and diagnostic PCR. (Table- 8).

2.12 Haematology

Collection of haemolymph was with a specially designed capillary tube rinsed ith an anticoagulant (Tris HCl- 0.01M, Sucrose 0.25M, Trisodium citrate 0.1M). The llected haemolymph was transferred into Eppendorff tubes of 1ml volume rinsed with e above anticoagulant and subjected for total haemocyte count (using Neubaeur's emocytometer), prophenol oxidase (Smith & Soderhall, 1983) and NBT reduction. heng *et al.,* 2000). Subsequently the protein profile of the haemolymph samples was alyzed using 12 % SDS-PAGE (Laemmli 1970).

2.13 Cost benefit analysis

Based on the harvested biomass, cost of Shrimpvac-1, other inputs such as obiotics, micro and macro nutrient for productivity, feed etc, the cost benefit analysis of e culture was performed.

6.3 Results

6.3.1 Comparative efficacy of Shrimpvac-1 and its components to protect shrimp from WSSV

On examining the efficacy of Shrimpvac-1 and its ingredients such as the inactivated virus, bacterins and the immunostimulant independently and the inactivated tissue suspension from healthy WSSV as the control to elicite an immune response against WSSV, the shrimpvac-1 and inactivated virus were found to have performed identically and uniformly resulting in a relative per cent survival of 100 in all trials. But in all experimental trials with the other ingredients total cumulative mortality was observed. But the only difference observed in between them was in the pattern of mortality, as, on challenging shrimps post administered orally with inactivated tissue suspension, bacterins and immunostimulants there was a corresponding progressive increase in the number of days required for attaining total mortality (Table 1) with respect to the components in the respective order.

6.3.2 Vaccination of *Penaeus monodon* post larvae in hatchery

A 10 tonne capacity larval rearing system with nested PCR negative larvae was identified for the administration of shrimpvac-1. The water quality of the system was closely monitored and maintained with in a range (Table-2) by partial water exchange and the application of probiotics. The post larvae subsequent to vaccination at PL-10 to PL-15 stages were challenged with WSSV in the bioassay system (Table-3) by maintaining a non- vaccinated similar batch as the control. Significantly, 100 % survival of post larvae vaccinated with Shrimpvac-1 was obtained while resulting in a cumulative mortality of 100 % in the control set over a period of 15 days. Relationship between the rate of feed consumption by the animal and the quantity of vaccine delivered per day per Kg body weight of animal under laboratory conditions and in the hatchery are summarized in Table 4.

Subsequent to stocking the pond with vaccinated post larvae the quality of water and sediment was strictly monitored and maintained with in a range (Table-5) by regular application of probiotics and required water exchange. All measurable parameters were with in the acceptable range during the culture operation.

During the grow-out phase the animals were vaccinated once in 10 days, each vaccination schedule continuing for three days (Table-6). During the culture period major unhealthy signs noticed were uropod setae breakage, infestation with *Zoothamnium sp.*, and occasional fungal associations. They were managed by preparing the animals for successful molting by administering Vitamin C (stabilized) Matrix Vet Pharma (P) Ltd @ 2g/kg feed and by the regular application of the gut probiotics 'Enterotrophotic'. Towards the end of culture period partial water exchange was resorted to.

The culture could be completed within 90 days with an overall survival of 56.5% as determined at the time of harvest. A total biomass of 687.7 kg could be harvested which fetched a price of Rs. 1,93,434.00 (US $ 4030.0 @ Rs. 48.00 per USD) (Table- 7). On challenging with WSSV the vaccinated group of animals consecutively for four times by maintaining wild caught *P.monodon* as control, it was observed that the vaccinated lot could survive all the four consecutive challenges performed at an interval of 15 days in between. Meanwhile the wild caught unvaccinated lot died showing clinical signs of the disease at the first challenge itself (Table 8). Histopathologically, the animals vaccinated in the field and maintained unchallenged and those, which were vaccinated and challenged, were with eosinophilic granulated hypertrophied nuclei without any other pathological changes (Fig - 1 to 9). The analysis of hematological parameters demonstrated significant variations in the haemocyte counts as well as in the reactive oxygen intermediates. The normal healthy shrimps (CUC), WSSV infected shrimps (CC) and vaccinated unchallenged (VUC) shrimps showed increased number of hemocytes when compared to the vaccinated challenged (VC) set of shrimps. It was found to be 107.6%, 113.8 % & 106 % statistically more significant respectively than the vaccinated challenged set (Fig - 10). The prophenol oxidase activity in the normal healthy shrimps (CUC), WSSV infected shrimps (CC), vaccinated unchallenged shrimps (VUC) &

vaccinated challenged (VC) shrimps remained almost the same and did not show significant variations statistically (Fig - 11). In the case of reactive oxygen intermediate (ROI) it was found to be very high in vaccinated challenged (VC) shrimps when compared to normal healthy (CUC), WSSV infected (CC), & vaccinated unchallenged (VUC) shrimps. It showed a statistically significant increase of 1052 % more compared to that of normal healthy shrimps (CUC), 248% more to that of WSSV infected shrimps (CC) & 337.2% more to that of vaccinated unchallenged (VUC) shrimps (Fig - 12).

On running an SDS-PAGE of the hemolymph collected from shrimps vaccinated unchallenged and vaccinated challenged, additional bands in the range of 14 to 43 KDa were demonstrated (Fig.- 13) in the vaccinated challenged group alone.

An interesting observation made was that the animals vaccinated in the field and maintained as unchallenged and challenged were proved 2-step PCR negative to WSSV Fig - 14 (a) & 14 (b).

A cost benefit analysis of the culture was made splitting in the whole expenditure in to two as the fixed cost and operational cost. Since a Government farm was used for the demonstration fixed cost was not considered and based on the variable cost, a net profit of Rs. 73616.00 (US$ 1534.00 @ Rs. 48.00 per USD) could be obtained from the 0.8 ha pond. The cost of production of Shrimpvac-1 used in this study was Rs 1,065 / L (US$ 22.19 @ Rs.48.00 per USD) (Table 9) with total cost of Rs 3486.06 (US$ 72.62@ Rs 48.00 per USD).

6.4 Discussion

Shrimpvac-I is the first generation Cocktail Vaccine designed to protect shrimp from WSSV, *Vibrio* and also to boost up the non-specific defense mechanism. However, WSSV alone was used as the pathogen to challenge post vaccination as challenging with *Vibrio* seldom proved to be successful under laboratory conditions due to unknown factors. Nevertheless, a satisfactory evaluation of the cocktail and its ingredients could be

made under laboratory conditions. On challenging with WSSV, the Shrimpvac-I and one of its ingredients, the inactivated virus, performed identical in according protection to the animals tested. Meanwhile the animals fed independently with bacterins and immunostimulants exhibited delay in the initiation and completion of mortality suggesting that they were able to impart partial protection by eliciting the non-specific defense mechanism. In a cocktail preparation of this sort this level of protection shall have greater impact in the overall performance of the vaccine under field conditions, where varying levels of stress factors and several opportunistic pathogens shall be operating together at any moment of time. The quantity of cocktail used and found effective under laboratory condition was 100 times less than the quantity of inactivated virus suspension used. This situation has been resulted due to the synergistic effect of all the three components in the preparation and especially due to the possible role of the immunostimulant (present in Shrimpvac-I) as the adjuvant.

For successful vaccination of shrimp against WSSV the vaccination programme must start from the larval - post larval stages as the exposure of young larval stages to inactivated viral particles or sub unit viral proteins would result in innocuous infections rather than mortality (Flegel and Pasharawipas, 1998). Moreover, vaccination at larval stages was essential in the sense that, at the very moment they are taken to grow-out system they are likely to be exposed to the pathogen. Therefore, the vaccination programme was scheduled in such a way that the post larvae could be taken out of the hatchery after completion of the immune activation phase and positively during the refractory phase.

It has been (previous chapter) worked out that 12.3ml inactivated WSSV suspension is required for vaccinating 1 Kg shrimp biomass. Shrimpvac-1, however, was proved effective under laboratory conditions in quantities 100times lesser than the quantity of inactivated WSSV suspension. Nevertheless, while administering under field condition (hatchery and grow-out) the quantity of Shrimpvac-1 was increased by 10 times. Accordingly 1.2ml/Kg body weight was administered in post larvae supplementing in the minimal quantity of larval feed (15.6g) required for 0.25million PL-10 having

0.004g weight per larvae. The feed was administered thrice a day, consecutively for 5 days. This mode of vaccination was effective as 100% survival of post larvae vaccinated with Shrimpvac-I was obtained with a cumulative mortality of 100% in the control set on challenging with WSSV over a period of 15 days. One of the major requirements for making the process of vaccinating larvae in hatchery fool-proof is to have an appropriate delivery system. In the present investigation the PL feed had been coated with the vaccine using Bindex and air-dried. Naturally over a period of two to three hours there could be varying levels of leaching out of the vaccine component. Therefore as one-step forward the vaccine has to be made in the form of micro encapsulated diet. It would also be required to have a feed attractant also incorporated so that there would be preferential feeding of the micro encapsulated particle.

In the grow-out system seven vaccinations were given, each vaccination lasting for three consecutive days. Along with the regular application of Shrimpvac-1, rigorous monitoring and correction of environmental quality were carried out. The application of probiotics as bioremediatiors and improvement of health of the animals by the application of gut probiotics and vitamins were required as the shrimp immune system was very much dependent on the environment, a phenomenon very much pertinent to aquatic animals (Newman, 2002). Maybe because of this and also due to the presence of bacterins and immunostimulants in Shrimpvac -I, no major disease other than occasional breakage of uropod setae, infestation with *Zoothamnium sp* and occasional fungal association could be noticed. As a cumulative effect of all these managerial measures it happened so that water exchange, that too partial, was required only towards the end of the culture.

The impact of repetitive vaccination of *P.monodon* starting from PL-10 to harvest was very much evidenced by the protective mechanism, which the animals could acquire against WSSV during the course of vaccination. Surprisingly the animals could withstand four consecutive challenges performed at an interval of 15 days in the bioassay system. It seems that every challenge functioned as discrete vaccination. The hematological parameters such as haemocyte count, prophenol oxidase and reactive oxygen

intermediates (ROIs) gave substantial evidence that the protective machinery triggered was not the one mediated by the non specific defense mechanisms. Evidently, the prophenol oxidase system remained without significant variation between the four groups of animals ($P > 0.05$). Moreover there was a depression in the circulating haemocyte count in the haemolymph of the vaccinated group when challenged with WSSV. This resulted in recording comparatively higher haemocyte counts on normal unchallenged, normal challenged and vaccinated unchallenged groups. Meanwhile, the steep significant rise in ROIs in the vaccinated-challenged group of animals ($P < 0.01$) strongly supports the view that there was cellular activity towards the removal of the virus from the body by phagocytic digestion. The elimination of phagocyted particles involves the release of degradative enzymes into the phagosome and the generation of ROIs (Rodriguez and Le Moullac, 2000). As reported in the previous section the formation of 'Viricidin' molecules, which specifically bind to WSSV antigen, facilitated their elimination by phagocytosis. On combining the data on the fall in the haemocyte count and steep rise in ROIs, it could be inferred that the haemocytes from the blood stream migrated to the tissues and organ systems in defense against the invading pathogen (WSSV), producing large quantity of the reactive oxygen intermediates. This inference is further supported by the negative nested PCR results with the gill tissue of these animals suggesting that the cascade of events which lead to the elimination of the virus are sufficient enough even to arrest the progression of viral multiplication. This is in agreement with Adams (1991) who had found 'bactericidins', a specific group of molecules, which could react specifically with the corresponding bacterial antigen in the hemolymph. Later in 1998, Teunissen *et al.*, mentioned that this would hold good only if there existed some memory for different pathogens in the shrimp immune system. It therefore could be postulated that the 'Viricidin' molecule interferes with the virus multiplication cycle and possibly even inhibiting its DNA synthesis as evidenced by negative nested PCR result. Cellular apoptosis is thereby prevented thus, saving the animal from White Spot Syndrome Virus. However, the eosinophilic partially hypertrophied nuclei with occlusion like bodies in HE-stained histological preparation within the nucleus remains to be explained. More ultrastructural studies in such nuclei are required.

A major difference which was observed in this study from that of the previous chapter is the absence of virus accommodation phase which was reported 10^{th} day post vaccination followed by a refractory phase. This inference was drawn from the hematological, histological and PCR based observations made in the vaccinated challenged animals. From the data it is apparent that the animals could be maintained in the refractory phase throughout the period by way of repeated vaccination under field conditions. However, as suggested earlier this is a grey area awaiting research with advanced molecular tools. But the question remains why the nuclei of vaccinated - unchallenged and vaccinated - challenged animals are partially hypertrophied and eosinophilic with occlusion like bodies. However, this study unequivocally proves that once vaccinated and protected from WSSV the situation can be prolonged by repeated vaccination every 10 days by oral administration of the inactivated virus.

It is more or less evident that 'viricidins' do play a major role in protecting the animals from WSSV. The additional bands formed in the range of 14 to 43 KDs on running SDS-PAGE of the hemolymph of the vaccinated and the challenged group of animals throws light on the nature of the above molecules as proteins. It was earlier speculated that this must be falling under IgSF, but do have much difference from those of vertebrates.

The quantity of Shrimpvac-I used was effectively 10 times lesser than the lowest quantity of the inactivated virus suspension used as vaccine during lab trials. It can be reasonably believed that this reduction in quantity could be achieved because of the presence of the immunostimulant in the preparation, which might have functioned as an adjuvant apart from its true nature. Maybe because of the addition of bacterins in the preparation, bacterial diseases especially vibriosis were rather less in incidence and intensity.

Shrimpvac-I in general, has been proved to be a fairly effective preparation for protecting shrimp from WSSV primarily and secondarily from *Vibrio* and other opportunistic pathogens. The product at field level is economically viable as it costs US $

105.59 for the production of 1 tonne shrimp biomass as the additional expenditure. For best results the following precautionary measures have to be taken.

1. Administration of the vaccine preparation should start from PL -10 onwards.
2. It must be applied through diet once in 10 days in such a way as to deliver 1.2 ml per Kg shrimp biomass.
3. Culture environment quality has to be maintained optimum in ranges to suite the requirements of the shrimp immune system to perform.
4. Gross parameters to be regulated are pH (Water and Sediment):6.8 to 8.2; E*h* (Sediment): > -100 mvolts; NH_3: < 0.01ppm; NO_2: < 0.1ppm; H_2S: <0.003 ppm; phytoplankton: 10^5 - 10^6 cells /ml; Zooplankton : 10^2 - 10^5 organisms /ml.

As this Centre has established a cell line from shrimp (*Pi*HTP-1, unpublished, patent pending) and could isolate and culture WSSV in it, commercial production of the Vaccine (Shrimpvac-I) is not much far away. An appropriate application of the vaccine preparation and strict aquaculture environment quality management shall relieve the shrimp industry from the grip of White Spot Syndrome Virus.

Table-1. Efficacy of Shrimpvac-1 and its components to immunize shrimp against WSSV

	Shrimpvac-1 and its components/ control	RPS after the challenge with WSSV on 5th day post completion of vaccination	RPS after the challenge with WSSV on 10th day post completion of vaccination
1	Shrimpvac-1	100	100
2	Inactivated virus	100	100
3	Bacterins	0 (5-8 days)	0 (5-8 days)
4	Immunostimulant	0 (9-15days)	0 (9-15days)
5	Inactivated tissue suspension	0 (1-7 day)	0 (1-7 day)
6	Uncoated control feed	0 (4-5days)	0 (4-5days)

N= 18; RPS : relative per cent survival

Figures in brackets: the duration of cumulative mortality

Table 2. Water quality monitored in the larval rearing system identified for vaccination during the period 3rd Dec 2001 to 5th Jan 2002

S.No	Parameters	Range	Managerial measures
1	Salinity (S%o)	35-36	Till attaining the stage of post larvae water level was gradually raised from 3 tonne to 12 tonne and henceforth 1/3rd water was regularly exchanged. From PL10 onwards, S %o was gradually lowered to suit that of the grow-out system.
2	Temperature (T°C)	28-29	
3	Ammonia (ppm)	0.001-0.098	
4	Nitrite (ppm)	BLD- 0.209	
5	Total heterotrophic bacterial population /ml water &/larva	1.0×10^3 to 1.0×10^6/ml 1.0×10^5 to 1.0×10^6/larvae	
6	Total *Vibrio* /ml water &/ larvae	3.6×10^2 to 5.5×10^5/ml 1.0×10^2 to 2.9×10^7/larvae	
7	Luminescent *Vibrio* /ml water & /larva	Nil	

Table 3. Efficacy of the vaccination against WSSV in *Penaeus monodon* post larvae as determined in a bioassay system.

S.No	Stock details	Description
1	Larvae used for vaccination	LRT-1 (Kollam)
2	Larval stage during vaccination	PL- 10 to PL - 15
3	Duration of vaccination at hatchery	1-1-02 to 5-1-02 (5 days)
4	Quantity of vaccine	62.5g vaccine coated PL feed / million larvae (4ml vaccine/ 100g feed)
5	Number of larvae in Tank. (LRT-1) at the time of vaccination	2.5 lakhs
6	Date of stocking the pond with vaccinated PL	10-1-02
7	Date on which larvae were brought to the lab for challenge test	6-1-02
8	Dates of challenge of larvae	10-1-02 (5^{th} day) 15-1-02 (10^{th} day)
9	Number of larvae challenged	100 (5^{th} day) 100 (10^{th} day)
10	Number of control larvae challenged	100
11	Quantity of inoculum in 10L water holding 100larvae	3.5 ml tissue suspension derived from 1.5g tissue macerated in 10ml 20ppt seawater
12	Remarks	No mortality to the vaccinated larvae even after 15 days post challenge, and 100% cumulative mortality in the control

Table 4. Relationship between the rate of feed consumption by the animal and the quantity of vaccine delivered per day under laboratory conditions and in hatchery.

Description	**Quantity of feed demand Vs vaccine delivered**		
	Inactivated WSSV (vaccine) Lab trial	**Shrimpvac-I (Lab trial)**	**Shrimpvac-I (Hatchery)**
1. Rate of feed consumption	35g/Kg body weight	35g/Kg body weight	31.25g/0.25million larvae (1Kg larvae biomass @0.004g wet weight /larvae)
2. Quantity of Vaccine/Shrimpvac-I coated per unit weight	3.51ml/Kg feed	3.51ml/Kg feed	40.0ml/Kg feed
3. Quantity of Vaccine/Shrimpvac-I delivered to animal	12.3ml/Kg body weight	0.12ml/Kg body weight	1.25ml/Kg body weight

Table 5. Environmental quality of the grow-out system from the period of its preparation to harvest (7-12-2001 to 30- 3-2002)

S.No	Parameters	Range	Managerial measures
A. Water			
1	Salinity (S‰)	10- 25	By regular application of probiotics and fertilizers sufficient phytoplankton was maintained. Water exchange was restricted to topping up during the initial phase and 10-20% exchange towards the final stage
2	Temperature (T°C)	29.5 - 31.5	
3	pH	7-8.9	
4	Dissolved oxygen at 2 am (ppm)	4.5 - 5.0	
5	Ammonia (NH_3)ppm	0.07 - 0.90	
6	Nitrite (NO_2-N)ppm	0.003 - 0.040	
7	Phosphate (PO_4^-)ppm	0.002 - 0.20	
8	Secchi disc reading (cm)	25 - 40	
9	Colour	Light green to dark green	
10	Algal count/mL	1.4×10^3 to 2.1×10^5	
B. Sediment			
1	PH	7.47 - 8.73	
2	Eh (mVolts)	99 - 252	
3	Organic Carbon	5.33 +/- 0.5%	

Table 6. Vaccination schedule adopted in the grow-out system in response to growth increments and the periodic health assessment.

Dates of vaccination in pond	Survival	Average body weight (Gms)	Total biomass (Kg)	Quantity of feed to be given (Kg)[1]	Actual quantity of vaccinated feed supplied (Kg)*	Quantity of Vaccine coated (ml)	Ratio of quantity of vaccine applied to Kg standing shrimp biomass
15-1-02	100%	0.2	10.0	0.70	0.7	28.0	28/10 = 2.80
27-1-02	60%	4.0	120.0	6.00	2.1	85.0	85/120 = 0.70
9-2-02	60%	10.0	300.0	12.60	6.0	240.0	240/ 300 = 0.80
22-2-02	60%	17.0	530.0	14.84	14.1	564.0	564/530 = 1.06
4-3-02	60%	19.0	570.0	15.96	15.0	600.0	600/570 = 1.05
16-3-02	57.14%	24.0	686.0	15.08	16.5	660.0	660/686 = 0.96
28-3-02	57.14%	26.4	754.0	16.59	16.5	660.0	660/754 = 0.70

*Each Kg feed contains 40ml Shrimpvac-I

1. Based on the equation following Chen and William, 1988

Table 7. Details of size ranges and biomass under each category during harvest

Count of animals required for 1 Kg	**Weight in (gms)**	**Quantity in (Kgs)**
20	50	1.5
30	33.3	213.4
40	25	335.1
50	20	78.1
60	16.7	15.1
80	12.5	41.2
100.	10	3.3
Total quantity	687.7 Kg	
Total survival	56.5%	
Total Revenue	Rs. 1, 93, 433.50	

Table 8. Demonstration of efficacy of vaccination under field condition by challenge test

Number of vaccinations in field	7
Number of days of culture	90 days
Average body weight	30 gms
Average survival rate	56.5%
Number of animals brought for challenge test to the lab	15 nos
Number of challenges in lab	4 challenges
Number of animals challenged	5 numbers
General observations	The 5 animals challenged (4 times) were found healthy, feeding was normal and without any mortality even after 4 challenges.
PCR	Vaccinated (farm) animals were second step positive while the challenged ones were first step positive

Table 9. Cost benefit analysis in applying Shrimpvac-1 for protecting shrimp from white spot syndrome virus

Fixed cost	**Operational cost (Rs)**
Land cost - Nil (Govt farm)	Pond preparation 13,375.00
Construction - nil (not invested in present crop)	Seed cost 20,000.00
Aerator - nil (not invested in present crop)	Feed cost 24,000.00
Pump - 3 Hp- 1 no (not invested in present crop)	Medicine Cost Probiotics 6554.73 Vitamins 4960.87 Vaccine 4539.20 Enzymes 4532.20 Total 20,587.00
Farm equipments (feeding trays and other tools) - nil (not invested in present crop)	Salary (I technician + 2 workers) 33,954.00
Laboratory equipments - Nil (not invested in present crop)	Fuel cost 5,208.00
Farm house - nil (not invested in present crop)	Other expenses 2,478.00
Total - nil	Total 1,198,17.25
Total invest 1,19,817.25 Total revenue 1,93,433.50 Profit Rs. 73,616.25	

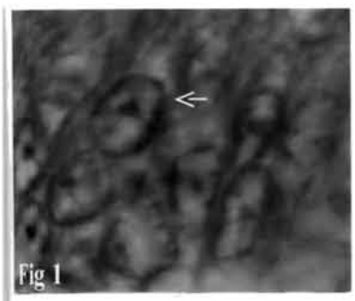
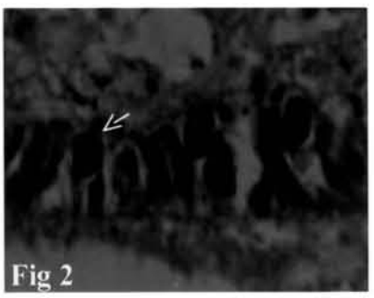


Fig 1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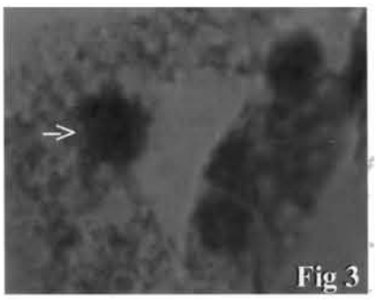
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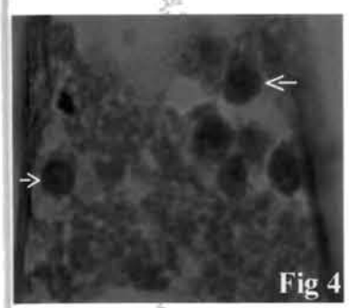
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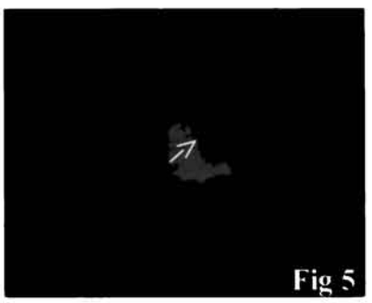
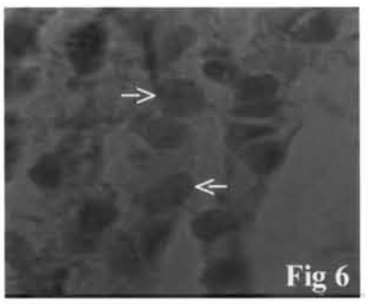


Fig 5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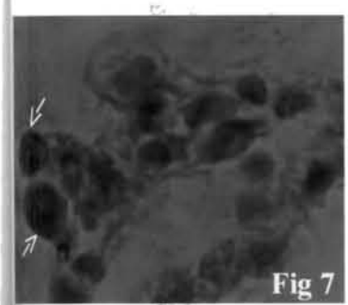
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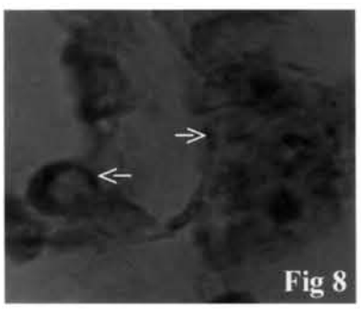


Fig 8

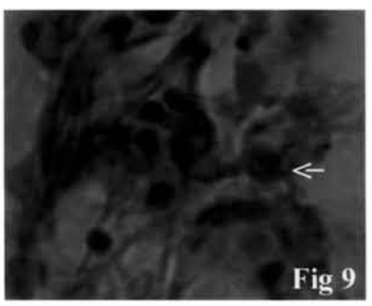


Fig 9

Fig 1 (1000X): Normal nucleus of epithelial lining (Arrow) (not infected with WSSV) Note nucleolus and clear nucleoplasm.

Fig 2 (400X): Basophilic hypertrophied nuclei (Arrow) characteristic of WSSV infection

Fig 3 & 4(1000X): Eosinophilic enlarged nuclei (arrows) of shrimp after 8 periodic vaccination during the culture period (hatchery & growout together)

Fig 5 to 9(1000X) : Eosinophilic enlarged nuclei (arrows) of vaccinated prawn which were subjected to 4 consecutive challenges at an interval of 15 days (Each figure represents an animal)

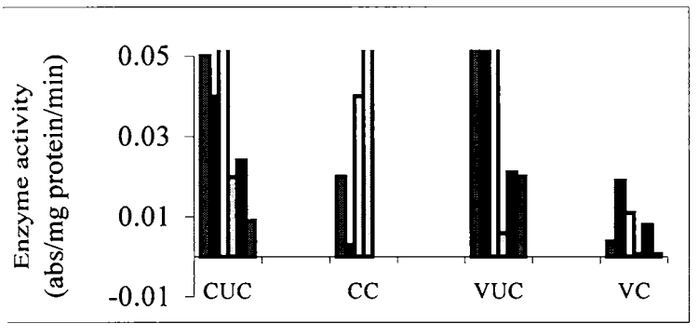


Fig.-10: Prophenol oxidase activity in normal healthy shrimp (CUC), WSSV infected shrimp (CC), Vaccinated shrimps (VUC) & Vaccinated unchallenged (VC) group of shrimps

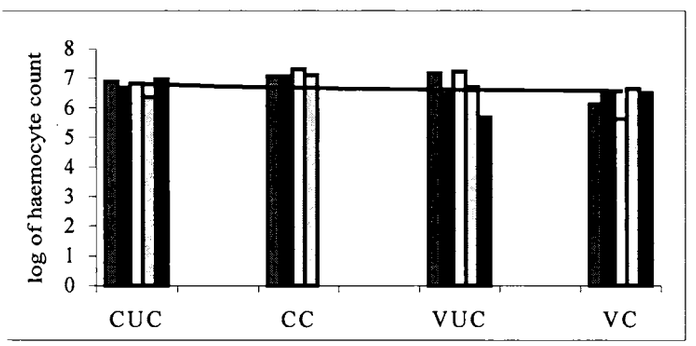


Fig.– 11: Haemocyte count in normal healthy shrimp (CUC), WSSV infected shrimp (CC), Vaccinated shrimps (VUC) & Vaccinated unchallenged (VC) group of shrimps

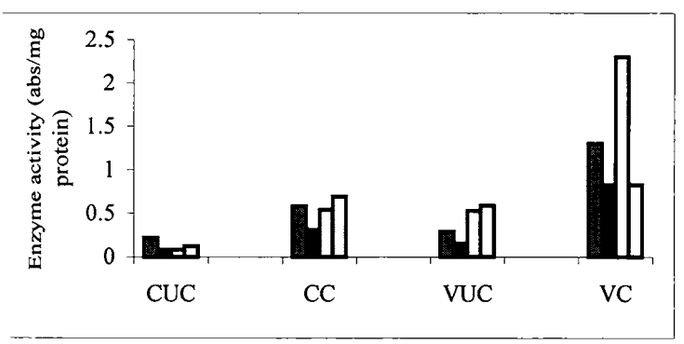
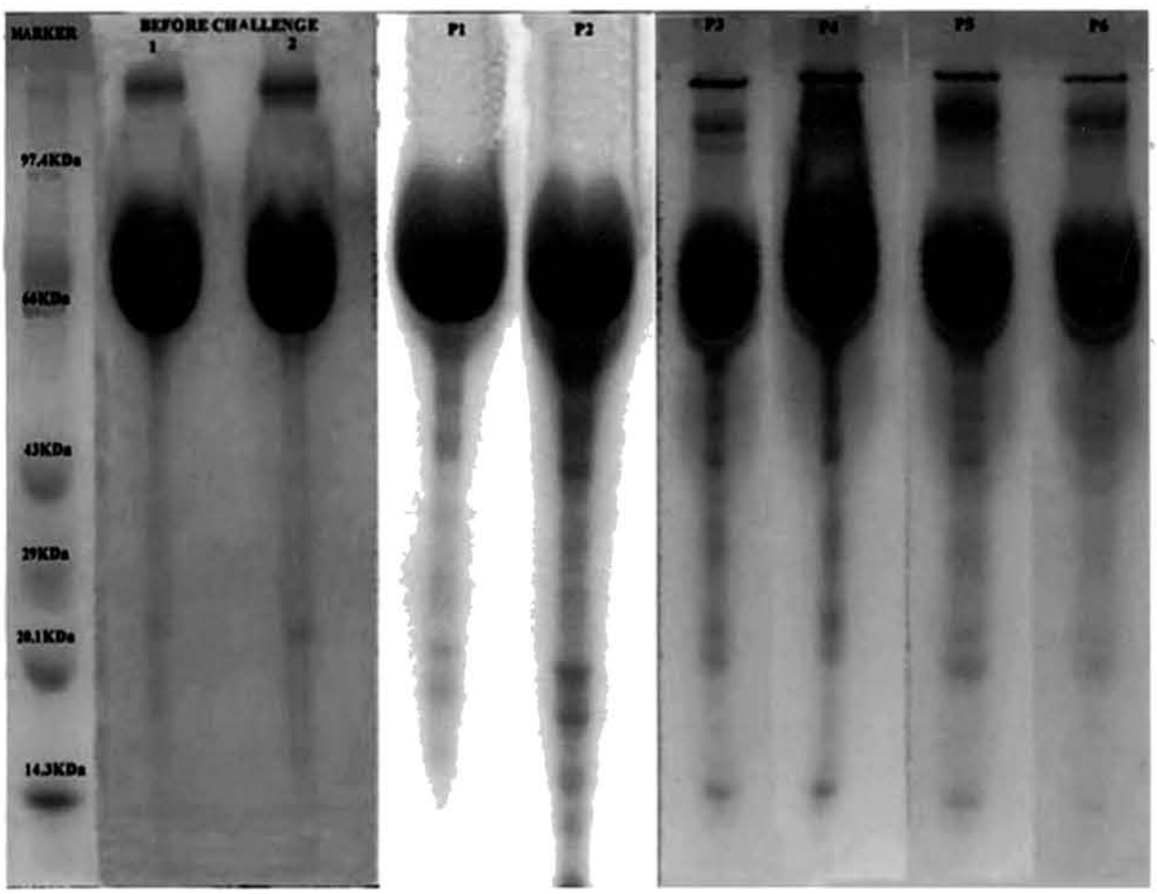


Fig.-12: Reactive Oxygen Intermediates (ROIs) in normal healthy shrimp (CUC), WSSV infected shrimp (CC), Vaccinated shrimps (VUC) & Vaccinated unchallenged (VC) group of shrimps

Each bar in the figures represent an animal



SDS-PAGE analysis of the prawn hemolymph drawn from the vaccinated unchallenged (Before challenge 1 & 2) and vaccinated challenged (P1 to P6) group of shrimps

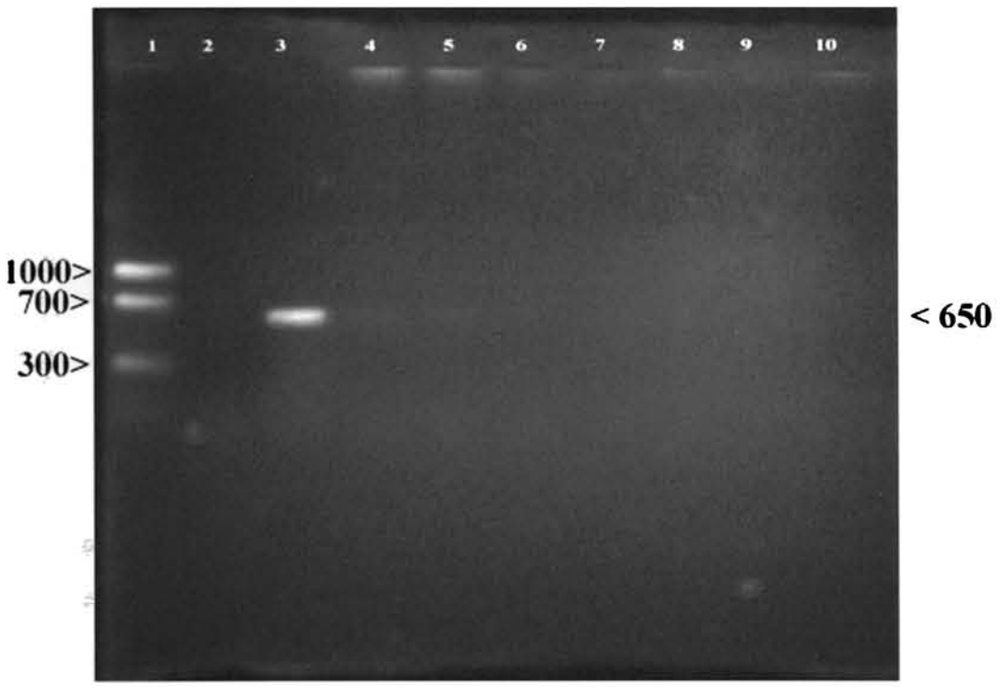


Fig 14 (a) PCR detection of WSSV DNA by the amplification of 650bp fragment of the viral genome (1st step) Lane- 1 Marker, Lane -2 PCR -ve control, Lane - 3 PCR +ve control, Lane- 4 & 5 Vaccinated unchallenged Lane - 6 to 10 Vaccinated challenged

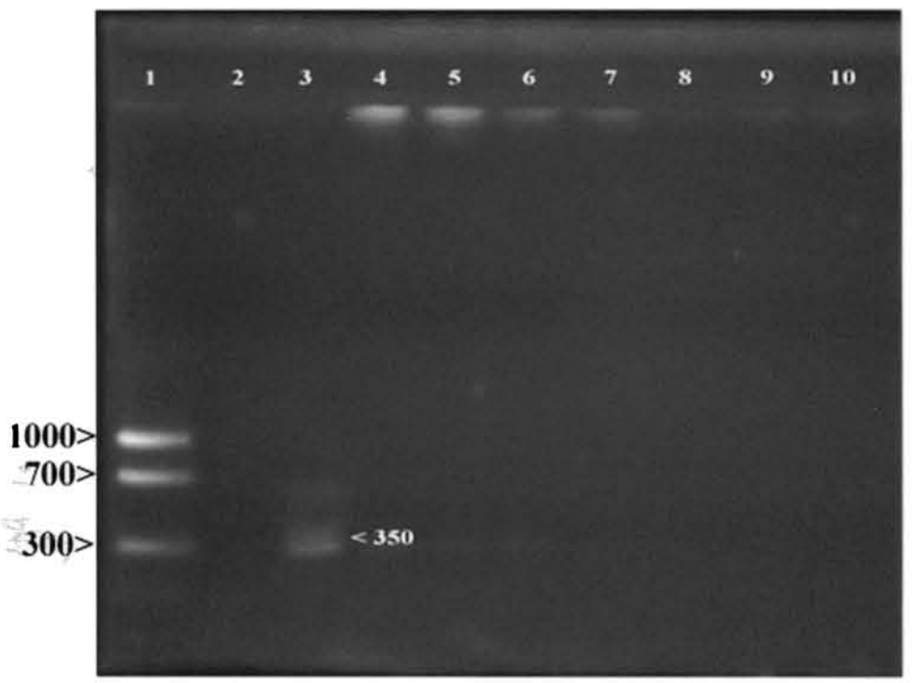


Fig 14 (b) PCR detection of the WSSV DNA by amplyfying the 300bp fragment of the viral genome Lane - 1 Marker, Lane - 2 PCR -ve control, Lane - 3 PCR (Nested) +ve control, Lane -4 & 5 vaccinated unchallenged, Lane - 6 to 10 vaccinated challenged

SUMMARY

SUMMARY

Shrimp forms a major constituent of World Fisheries. But the alarming rate of the dwindling landings from the seas around the globe threatens the very sustenance of shrimp industry and among the varying options putforth to recover it from the loosing ground, aquaculture along with conservation of the existing stock are proposed as the two major futuristic approaches. Any culture activity is bound to have diseases as one of the impediments. Among the various microbial diseases of shrimps, viral diseases cause the most extensive mortalities in cultured populations. To add to this scenario was the outbreak of white spot virus syndrome, which has been attributed to the mass moralities of cultured shrimps in Asia during the past one decade. First reported in Thailand in 1992 and later spreading throughout East and Southeast Indonesia, India, and other shrimp growing regions of the world, white spot syndrome virus was responsible for the devastating conditions with a mortality rate of 95-100% in 2-3 days after the onset.

It appeared in India in 1994 and since then it has been damaging the Indian aquaculture industry and still continues to be a serious threat. It is a primary viral disease, caused by an enveloped, rod shaped double-stranded DNA virus called white spot syndrome virus. Affected shrimp become weak, body pink in colour, refuse to feed and have prominent white spots on the carapace at the moribund stage. The virus replicates in the nucleus of all cells of ectodermal and mesodermal origin resulting in hypertrophy of nuclei and subsequent degeneration of the cell. Studies on prawn immune system being in its infancy, a comprehensive approach to tackle the pathogen inside the host body could not be attained till date. As it says, "Prevention is better than cure", better management strategies, various disease control measures and early detection proved to be the best to tackle this viral disease. The present study focus on three precise objectives such as,

1 Histological demonstration, histopathology, transmission electron microscopic evidence and ultrastructure of white spot syndrome virus in various tissues of *Penaeus indicus*. and viral morphogenesis.

2. Development of polyclonal antisera to shrimp cell cultured white spot syndrome virus.
3. Development and demonstration of a cocktail vaccine 'Shrimpvac-I' for the management of white spot syndrome virus and vibrios in shrimp grow-out systems.

A. The first phase of the study was confined to the histopathology of White spot syndrome virus and its electron microscopic demonstration. Histopathological changes brought about by the virus in various organs like gill, foregut, stomach, midgut, hindgut, hepatopancreas, nerve, ovary, eye and integument of *Penaeus indicus* were investigated by light microscopy in comparison with the histology of normal tissue. Subsequently, ultrastructural details of the virus and viral morphogenesis were worked out by electron microscopy. It is observed that WSSV has tissue tropism and infects tissues of mesodermal and ectodermal origin. Infected tissue shows severe nuclear hypertrophy, cellular degeneration, multifocal necrosis and hemocytic encapsulation in general. Of the various tissues examined, the foregut and gill was found to be specifically suitable for the postmortem demonstration of the virus, because of the severity of infection and ease of tissue processing. The present study is the first comprehensive approach towards histopathology of WSSV in *Penaeus indicus*. WSSV could be demonstrated in the nuclei of all tissues, except those of midgut, subjected for electron microscopic observation. They were the nuclei of gill, foregut, heart, hepatopancreatic connective tissue, hindgut, nerve and dorsal aorta. A comparison was made between the electron microscopic and histopathological observations and a greater degree of correlation between the two in depicting the severity of the infection was unraveled. The study also illustrated variations in response and susceptibility of various tissues to WSSV infection. Accordingly, out of the tissues investigated, gill, foregut, hindgut and dorsal aorta exhibited advanced viral multiplication than the other tissues such as heart, midgut, nerve and hepatopancreas. Even though hepatocytes were not infected the connective tissue nuclei were packed with virions.

During viral morphogenesis it was observed that the viral nuclear material, capsid and envelope were synthesized with in the virogenic stroma of the nucleus. They migrate

to various loci for virus assembly. The trilaminar envelop with one open-end sliding over to the single open-ended capsid is an unique feature recorded with this virus. The virus nuclear material gets surrounded by a trilaminar vesicle and carried to the open end of the enveloped capsid. An unknown process pushes the spherical nuclear material to the core of the capsid. Capsid gets closed, the vesicular structure fuses with the envelope to form fully assembled virions. While the virus assembly takes place they assume a paracrystalline structure which later gets loosened facilitating migration of the virions towards the base of the nuclear membrane within the marginated chromatin. The nuclear membrane lyse, releasing the virions to cytoplasm from where the virus gets released when the animal dies and cells get decayed.

B. The second phase of the work was confined to the generation of polyclonal antisera as reagents for the development of immunodiagnostics. Accordingly, a polyclonal antisera against WSSV was developed in rabbit by using the purified antigen (WSSV) generated in a prawn hepatopancreatic cell line (*Pi* HPT- 1) established in this lab. Simple agarose diffusion technique, single radial immunodiffusion and Ouchterlony technique confirmed the presence of antibodies. The polyclonal antibody developed indicated a titre value of $1{:}10^8$ after the 3rd booster dose by microwell precipitation test. Immunodiffusuion of antibody against the background protein (Cell culture supernatant) showed a very faint band which indicated the presence of small quantity of immunogenic background molecules in the antigen preparation used for immunizing rabbit. However, on reacting with wild samples and PCR negative samples, no cross-reaction was observed. This absence of bands against prawn muscle extract and PCR negative samples suggests that in the practical sense the small fraction of cross reacting antibodies might not lead to any false positive reactions.

C. Third phase of the work was oriented towards developing an appropriate vaccine preparation to protect shrimp from WSSV. To generate the vaccine, the virus stock was produced by infecting healthy prawns with white spot infected prawn meat in a bioassay system in large quantity. The infected meat was macerated making it into slurry and inactivated, checked inactivation in bioassay by feeding vaccine coated pelleted feed to

the test animals. WSSV could be inactivated within duration of 48 hours in the presence of 0.2% formalin (v/v final concentration) at room temperature. The vaccine coated feed preparation was fed to test animals at a rate of 1gm vaccine to 10 gm feed for 7 days, challenged on 1st, 5th, 10th and 15th day post vaccination with a positive and negative controls for each challenge. Complete mortality in positive controls and those challenged on 1st and 15th day post vaccination and 100% survival in the tanks challenged on 5th and 10th day post vaccination could be observed. Survived animals could be maintained more than 3 months with periodic challenges without the manifestation of the disease. The minimum required quantity of vaccine without compromising with the effectiveness of immunization was determined. For this, test animals were fed with lower concentration of vaccine such as 0.25g/10g feed, 0.5gm/10gm feed and 0.75gm/10gm feed for 7 continuous days and later challenged on 5th and 10th post vaccination. It was found that a minimum quantity of 0.25 g/10g feed was sufficient enough to protect the animals during 5th and 10th day post vaccination. (Maximum 10 days). Histology of gill tissue of the 5th day challenged animal showed no significant variation than that of healthy gill, but the epidermal nuclei of the gill arch were slightly enlarged but eosinophilic in appearance in the case of 10th day challenged group. The PCR of gill tissue of 5th day challenged group showed no amplification of viral DNA neither in first step nor nested, while the 10th day challenged sample showed amplification of 650bp (1st step) and 300bp (nested). But the animals did not exhibit any manifestation of the disease. Subsequent to the administration of the inactivated virus preparation four discrete time depended phases could be observed in the immune cycle of penaeids. They were the immune activation phase, the refractory phase, virus accommodation phase and the immune-declining phase. During the refractory phase it is postulated that specific 'viricidin' molecules which may specifically react with the virus antigen are produced facilitating their elimination by subsequent phagocytosis. During the virus accommodation phase, specific adhesion molecules or a new class of molecules belonging to immunoglobulin superfamily are released which protect the animals from cellular apoptosis, tissue damage and mortality in spite of multiplication of the virus in the tissue. The study suggested that the situation could be prolonged by repeatedly vaccinating the shrimp once in 10 days.

Based on these findings, a cocktail vaccine named 'Shrimpvac-I' was designed by incorporating bacterins derived from pathogenic strains of *Vibrio* and an immunostimulant along with the vaccine developed earlier. Being it a cocktail preparation, concentration of the vaccine could be reduced by 10 times (0.025g/10gm feed). Efficacy of each component of the cocktail as well as 'Shrimpvac-I' as such was tested in bioassay system. Bacterins and immunostimulant when used singly could not elicit complete protection against WSSV but could lead to the prolongation of the duration required for mortality. Meanwhile 'Shrimpvac-I' gave complete protection to the test animals against WSSV. Based on the results it could be concluded that 40ml of 'Shrimpvac-I' /kg feed was sufficient to impart protection to the animals from WSSV. On calculating the quantity required per Kg body weight of the animal it was observed that 1.2ml of 'Shrimpvac-I' / kg shrimp biomass was the minimum quantity required for eliciting and sustaining the immune response which could render protection. Following was the vaccination protocol designed based on the study: A batch of larvae negative to diagnostic PCR for WSSV has to be identified and vaccinated for 5 days in hatchery at the rate of 1.25ml/Kg biomass. Later they have to be stocked in a well-prepared pond and subjected to 7 periodic vaccinations at an interval of 10 days for a complete culture period of 90 days. By this method, the culture could be completed with an overall survival of 56.5% and a total biomass of 687.7 kg could be harvested, which fetched a price of Rs 1,93,434.00 (US $ 4030.00 @ Rs 48.00 per USD). During harvest, live samples of prawns were collected, brought to the laboratory sacrificed, hemolymph collected and subjected to hematological analysis, SDS-PAGE and the tissues were subjected to diagnostic PCR and histology. Animals were maintained in the bioassay system and subjected to periodic challenges at an interval of 15 days. All the animals survived 4 consecutive challenges after which they were sacrificed and subjected to hematology, SDS-Page, diagnostic PCR and histological studies. Hematological analysis showed a reduced hemocytic count in the vaccinated - challenged group when compared to the control as well as the vaccinated group. The ROI was 1052 % high in the vaccinated - challenged group when compared to the normal prawn and 248% higher than an infected prawn and 337.2% more than vaccinated shrimps, the values statistically significant. Prophenol oxidase activity did not show much variation in the sets. SDS-

PAGE, showed additional bands in the range of 14 KDa to 43KDa in the vaccinated-challenged group when compared to the vaccinated and control groups. No amplification of the WSSV DNA could be observed by diagnostic PCR. Histological analysis, showed slightly enlarged, eosinophilic granulated nuclei in all the gill tissues examined which showed marked difference with that of the characteristic basophilic hypertrophied nucleus of WSSV infection.

The impact of repetitive vaccination of *P.monodon* starting from PL-10 to harvest was very much evidenced by the protective mechanism, which the animals could acquire against WSSV during the course of vaccination. Surprisingly, the animals could withstand four consecutive challenges performed at an interval of 15 days in the bioassay system. It seems that every challenge functioned as discrete vaccination. The hematological parameters such as haemocyte count, prophenol oxidase and reactive oxygen intermediates (ROIs) gave substantial evidence that the protective machinery triggered was not the one mediated by the non-specific defense mechanisms. Evidently, the prophenol oxidase system remained without significant variation between the four groups of animals ($P > 0.05$). Moreover there was a depression in the circulating haemocyte count in the haemolymph of the vaccinated group when challenged with WSSV. This resulted in recording comparatively higher haemocyte counts on normal unchallenged, normal challenged and vaccinated unchallenged groups. Meanwhile, the steep significant rise in ROIs in the vaccinated-challenged group of animals ($P < 0.01$) strongly supports the view that there was cellular activity towards the removal of the virus from the body by phagocytic digestion. The elimination of phagocyted particles involves the release of degradative enzymes into the phagosome and the generation of ROIs. As reported in the previous section the formation of 'Viricidin' molecules, which specifically bind to WSSV antigen, facilitated their elimination by phagocytosis. On combining the data on the fall in the haemocyte count and steep rise in ROIs, it could be inferred that the haemocytes from the blood stream migrated to the tissues and organ systems in defense against the invading pathogen (WSSV), producing large quantity of the reactive oxygen intermediates. This inference is further supported by the negative nested PCR results with the gill tissue of these animals suggesting that the cascade of

events which lead to the elimination of the virus are sufficient enough even to arrest the progression of viral multiplication. Therefore, it could be postulated that the 'Viricidin' molecule interferes with the virus multiplication cycle and possibly even inhibiting its DNA synthesis as evidenced by negative nested PCR result. Cellular apoptosis is thereby prevented thus, saving the animal from White Spot Syndrome Virus. However, the eosinophilic partially hypertrophied nuclei with occlusion like bodies in HE-stained histological preparation within the nucleus remains to be explained. More ultrastructural studies in such nuclei are required.

From the data it is apparent that the animals could be maintained in the refractory phase throughout the period by way of repeated vaccination under field conditions. However, as suggested earlier this is a Grey area awaiting research with advanced molecular tools. But the question remains why the nuclei of vaccinated - unchallenged and vaccinated - challenged animals are partially hypertrophied and eosinophilic with occlusion like bodies. However, this study unequivocally proves that once vaccinated and protected from WSSV the situation can be prolonged by repeated vaccination every 10 days by oral administration of the inactivated virus.

It is more or less evident that 'viricidins' do play a major role in protecting the animals from WSSV. The additional bands formed in the range of 14 to 43 KDs on running SDS-PAGE of the hemolymph of the vaccinated and the challenged group of animals throws light on the nature of the above molecules as proteins. It was earlier speculated that this must be falling under IgSF, but do have much difference from those of vertebrates.

The quantity of Shrimpvac-I used was effectively 10 times lesser than the lowest quantity of the inactivated virus suspension used as vaccine during lab trials. It can be reasonably believed that this reduction in quantity could be achieved because of the presence of the immunostimulant in the preparation, which might have functioned as an adjuvant apart from its true nature. Maybe because of the addition of bacterins in the

preparation, bacterial diseases especially vibriosis were rather less in incidence and intensity.

Shrimpvac-I in general, has been proved to be a fairly effective preparation for protecting shrimp from WSSV primarily and secondarily from *Vibrio* and other opportunistic pathogens. The product at field level is economically viable as it costs US $ 105.59 for the production of 1 ton shrimp biomass as the additional expenditure. For best results the following precautionary measures have to be taken.

1. Administration of the vaccine preparation should start from PL -10 onwards.
2. It must be applied through diet once in 10 days in such a way as to deliver 1.2 ml per Kg shrimp biomass.
3. Culture environment quality has to be maintained optimum in ranges to suite the requirements of the shrimp immune system to perform.
4. Gross parameters to be regulated are pH (Water and Sediment):6.8 to 8.2; E*h* (Sediment): > -100 mvolts; NH_3: < 0.01ppm; NO_2: < 0.1ppm; H_2S: <0.003 ppm; phytoplankton: 10^5 - 10^6 cells /ml; Zooplankton : 10^2 - 10^5 organisms /ml.

As this Centre has established a cell line from shrimp (*Pi*HTP-1, unpublished, patent pending) and could isolate and culture WSSV in it, commercial production of the Vaccine (Shrimpvac-I) is not much far away. An appropriate application of the vaccine preparation and strict aquaculture environment quality management shall relieve the shrimp industry from the grip of White Spot Syndrome Virus.

REFERENCES

Adams, A. 1991. Response of penaeid shrimp to exposure to vibrio species. Fish and shellfish Immunol. 1: 59-70.

Adams, J.R and J.T. Mc Clintock. 1991. Baculoviridae. Nuclear polyhedrosis virus Part – I. Nuclear polyhedrosis viruses of insects. In: Adam, J.R and Bonami J.R. (Eds.). Atlas of Invertebrate viruses. CRC Press, Boca Raton, p. 87-204.

Adams, J.R. and J.R. Bonami. (Eds). 1991. Atlas of Invertebrate viruses. CRC Press, F.L. Bacca Raton, p. 1-53.

Alapide – Tendencia, E.V and L.A. Dureza. 1997. Isolation of *Vibrio* sp. from *P. monodon* (Fabricus) with red disease syndrome. Aquaculture. 154(2): 107-114.

Amirante, G.A and F.G. Mazzalai. 1978. Synthesis and localization of hemagglutinins in hemocytes of the cockroach *Leucophaea maderae.L.* Dev.Comp.Immunol. 2: 735-740.

Amirante, G.A. 1986. Agglutinins and lectins of crustacea. In: Gupta, A.P (Ed.) Haemocytic and humoral immunity in Arthropods. Wiley, New York, pp. 359-380.

Anderson, R.S. 1996. Production of reactive oxygen intermediates in haemocytes. Immunological significance. In: Soderhall, K., Sadaaki, I., Vasta, G. (Eds). New directions in invertebrate immunology. SOS Publication, Fair Hauen, p. 109-129.

Andres Soto, M., V.R. Shervette and J.M. Lotz. 2001. Transmission of white spot syndrome virus (WSSV) to *Litopenaeus vannamei* from infected cephalothorax, abdomen, or whole shrimp cadaver. Diseases of Aquatic Organisms. 45: 81-87.

Anil, T.M., K.M. Shankar and C.V. Mohan. 2002. Monoclonal antibodies developed for sensitive detection and comparision of white spot syndrome virus isolates in India. Dis. Aquat.Org. 51: 67-75.

Anonymous. 1995. SEMBV – an emerging viral threat to cultured shrimp in Asia. Asian Shrimp News, 20: 2- 3.

Arala- Chaves, M and T. Sequeira. 2000. Is there any kind of adaptive immunity in invertebrates? Aquaculture 191: 247-258.

Arimoto, M., T. Yamazaki, Y. Mizuta, I. Furusawe. 1995. Characterisation and partial cloning of the genomic DNA of a baculovirus from *P. japonicus*. (Pj NOB – BMNV). Aquaculture. 132: 213.

Armstrong, P.B. 1991. Cellular and humoral immunity in the horse shoe crab, *Limulus polyphemus* .In Ayodya P.Gupta (Ed.) Immunology of insects and other arthropods, CRC Press. 3-17.

ASCC (Asian Shrimp Culture Council). 1995. Mass mortality caused by systemic bacilliform virus in cultured penaeid shrimp, *Penaeus monodon* in Thailand. Asian shrimp News. 21: 2-4.

Barracco, M.A., B. Duvic and K. Soderhall. 1991. The β-1-3 glucan-binding protein from the crayfish, *Pacifastacus leniusculus* when reacted with a β-1-3 glucan, induces spreading and degranulation of cray fish granular cells. Cell Tissue Res. 266: 491- 497.

Barteling, S.J and R. Woortmeyer. 1984. Formaldehyde inactivation of foot and mouth disease virus, conditions for the preparation of safe vaccine. Archives of virology. 80: 103-117.

Bell, K.L. and V.J. Smith. 1993. *In vitro* superoxide production by hyaline cells of the shore crab *Carcinus maenas* (L). Dev. Comp. Immunol. 17: 211-219.

Bell, T.A and D.V. Lightner. 1988. A handbook of normal penaeid shrimp histology. World Aquaculture Society. Baton Rouge. Louisiana. USA. 2-6.

Bettencourt, R., H. Lanz-Mendoza and I. Faye. 1997. Cell- adhesion properties of hemolin, an insect immune protein in the Ig superfamily. Eur.J.Biochem 250: 630-637.

Bonami, J.R., J. Mari, B.T. Poulos and D.V. Lightner. 1995. Characterization of hepatopancreatic parvo-like virus, a second unusual parvovirus pathogenic for penaeid shrimps. J. Gen. Virol. 76(4): 813-817.

Bottiger, M., E. Lycke, B. Melen and G. Wrange. 1958. Inactivation of poliomylitis virus by formaldehyde. Incubation time in tissue culture of formalin treated virus. Arch.Gesamte Virusforsch.3: 259-266.

Bradford, M.M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. Anal.Biochem. 72: 248-254.

Bruce, L.D., R.M. Redman, D.V. Lightner and J.R. Bonami. 1993. Application of gene probes to detect a penaeid shrimp baculovirus in fixed tissue using *in situ* hybridization. Dis. Aquat. Org. 17(3): 215 – 221.

Bruce, L.D., D.V. Lightner, R.M. Redman and K.C. Stuck. 1994. Comparision of traditional and molecular detection methods for baculovirus penaei infections in larval *P. vannamei*. J. Aquat. Animal Health. 6(4): 355-359.

Cai, S., J. Huang, C. Wang, X. Song, X. Sun, J. Yu, Y. Zhang and C. Yang. 1995. Epidemological studies on explosive epidemic disease of prawn in 1993-94. J. Fish. China, 19: 112-117.

Chang, C.F., M.S. Su, H.Y. Chen, C.F. Lo, G.H. Kou and I.C. Liao. 1999. Effect of dietary β-1-3 glucan on resistance to white spot syndrome virus (WSSV) in post larval and juvenile *Penaeus monodon*. Dis. Aquat. Org., 36: 163-168.

Chang, P.S., C.F. Lo, Y.C. Wang and G.H. Kou. 1996. Identification of white spot syndrome associated baculovirus (WSBV) target organs in the shrimps (*P. monodon*) by *in situ* hybridication. Diseases of Aquatic organisms. 27(2): 131-139.

Chang, P.S., D.H. Tasi and Y.C. Wang. 1998 (b). Development and evaluation of a dot blot analysis for the detection of white spot syndrome baculovirus (WSBV) in *Penaeus monodon*. Fish Pathology: 33: 45-52.

Chang, P.S., H.C. Chen and Y.C. Wang. 1998 (c). Detection of white spot syndrome associated baculovirus in experimentally infected wild shrimp, crab and lobsters by *in situ* hybridization. Aquaculture. 164; 233-242.

Chang, Y.S., C.F. Lo, S.E. Peng, K.F. Liu, C.H. Wang and G.H. Kou. 2001. White spot syndrome virus (WSSV) PCR – positive Artemia cysts yield PCR – negative nauplii that fail to trasmit WSSV when fed to shrimp postlarvae. Diseases of Aquatic Organisms 49: 1-10.

Chanratchakool, P. 1996. White patch disease of black tiger shrimp. Aquaculture Asia. 1: 36-37.

Chen K.J and W.G.Co. 1988. Prawn culture scientific and practical approach. Westpoint Aquaculture Corporation, p 323.

Chen, L.L., C.F. Lo, Y.L. Chiu, C.F. Chang and G.H. Kou. 2000. Natural and experimental infection of white spot syndrome virus (WSSV) in benthic larvae of mud crab *Scylla serrata*, Dis. Aquat. Org. 40: 157-161.

Chen, S.N. 1992. Coping with diseases in shrimp farming. Shrimp '92, Hongkong, Proc. 3rd Global conference on the shrimp industry, Hong Kong, 14-16, Sep. 1992, p 113-117.

Chen, S.N and G.H. Kou 1994. Viral infection and mass mortality of cultured shrimps in Taiwan. Abstract book, Interntional symposium of Biotechnology aplications in aquaculture, 5 -10 Dec, 1994. Taipei, Taiwan, Asian Fisheries Soc.

Chen, S.N. 1995. Current status of shrimp aquaculture in Taiwan. In: Browdy, C.L., Hopkins, J.S. (Eds) Swimming through troubled waters. Proceedings of the special session of shrimp farming. Aquaculture '95. World Aquaculture Society. Baton Rouge, LA, p 29-34.

Cheng C.F, Chen H.U, Su M.S and Liao. 2000. Immunomodulation by dietary β-1-3 glucan in the brooders of the black tiger shrimp *Penaeus monodon*. Fish and Shellfish Immunology. 10: 505-514.

Chondar, S.L. 1996. 'SLC-URINUM' therapy to control white spot viral disease in penaeid shrimp. Fishing Chimes, July. p 21.

Chou, H.Y, C.Y. Huang, C.H. Wang, H.C. Chiang, C.F. Lo. 1995. Pathogenicity of a baculovirus infection causing WSS in cultured penaeid shrimp in Taiwan. Diseases of Aquatic Organisms. 23(3): 165-173.

Chou, H.Y., C.Y. Huang, C.F. Lo and G.H. Kou. 1998. Studies on transmission of white spot syndrome associated baculovirus (WSBV) *in Penaeus monodon* and *P. Japonicus* via waterborne contact and oral ingestion. Aquaculture. 164: 263-276.

Destoumieux, D., P. Bulet, D. Loew, A. Van Dorsselaer, J. Rodriguez and E. Bachere. 1997. Penaedins: a new family of antimicrobial peptides in the shrimp *Penaeus vanammei* (Decapoda). J. Biol. Chem. 272: 28398-28406.

Direkbusarakom, S and L. Ruangpan. 1998. Protective efficacy of *Clinacanthus rutans* on yellow head disease in black tiger shrimp (*P. monodon*) Fish pathology, Vol. No. 33 (2) Issue No. 4, p 401-405.

Durand, S., D.V. Lightner, L.M. Nunan, R.M. Redman, J. Mari and J.R. Bonami. 1996. Application of gene probes as diagnostic tools for white spot baculovirus of penaeid shrimp. Diseases of Aquatic Organisms. 27(1): 59-66.

Durand, S., D.V. Lightner, R.M. Redman and J.R. Bonami. 1997. White spot syndrome baculovirus (WSBV) ultrastructure, morphogenisis and the use of gene probes as a diagnostic tool. In Abstract: European Associatin of Fish Pathologists VIII International Conference on 'Disease of Fish and Shell Fish' Edinburg, p. 69.

Durand, S., D.V. Lightner, R.M. Redman and J.R. Bonami. 1997. Ultrastructure and morphogenesis of white spot syndrome baculovirus (WSSV). Diseases of Aquatic Organisms. 29: 205-211,

Ed Harlow and L. David. 1988. Antibodies: A laboratory manual. Cold Spring Harbour Laboratory. NY. p 37-147

Edgerton, B. 1996. A new bacilliform virus in Australian *Cherax destructor* (Decapods: Parastacidae) with notes on *Cherax quadricarinatus* bacilliform virus (*Cherax baculovirus*). Diseases of Aquatic Organisms. 27(1): 43-58.

Faye I. 1990. Acquired immunity in insects: the recognition of non-self and the subsequent onset of immune protein genes. Res Immunol 141: 927-32

Fegan, D.F., T.W. Flegel, S. Sriurairatana and M. Waiyakruttha. 1991. The occurrence, development and histopathology of monodon baculovirus in *P.monodon* in southern Thailand. Aquaculture 96: 205- 217

Flegel, T.W., S. Sriwairatana, C. Wongteerasupaya, V. Boonsueng, S. Panyim and B. Withyachumnarnkul. 1995 (a). Progress in characterization and control of yellow head virus of *Penaeus monodon.* In Browdy.C and S. Hopkins (Eds) Swimming through troubled water; proceedings of the special session on shrimp farmeng, Aquacehure 95, San Dings, Feb. 1995, World Aquaculture Society, Baton Rouge, LA, p. 76-83.

Flegel, T.W., S. Sriwairatana, C. Wongteerasupaya, J.F. Vickus, G.L., Nash, A. Anutara, V. Boonsaeng, S. Panyim and B. Withyachumnarnkul. 1995 (b). Progress in characterisation and control of yellow head virus and white spot virus in Thailand. World Aquaculture Society, Bangkok, Thailand, p 126.

***Flegel, T.W., S.Boonyaratpalin & B .Withyachumnarnkul. 1996.** Progress in research on yellow head virus and white spot virus in Thailand. In proceedings of Aquaculture,96.

Flegel, T.W. 1996. A turning point for sustainable aquaculture. The white spot virus in Asian shrimp culture. Aquaculture Asia. 1: 29-34.

Flegel, T.W. 1996. The white spot virus crisis in Asian shrimp culture. Aquaculture Asia July-Sep. 1996. p. 29-34.

Flegel, T.W. 1997. Major viral diseases of the black tiger prawn (*Penaeus monodon*) in Thailand. World J. Microbiol. Biotechnol. 13: 433- 442.

Flegel, T.W and T. Pasharawipas. 1998. Active viral accomodation: a new concept for crustacean response to viral pathogens. In: Flegel T.W. (ed.) Advances in shrimp

biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok, p 245-250

Fragkiadakis, G and E. Stratakis. 1995. Characterization of haemolymph lectins in the prawn *Parapenaeus longirostris*. J. Invertebr. Pathol. 65: 111-117.

Gollas-Galvan, T., J. Hernandez-Lopez and F. Vargas-Albores. 1999. Prophenoloxidase from brown shrimp (*Penaeus californiensis*) hemocytes. Comp.Biochem.Physiol. 122B: 77-82.

Hameed, A.S.S, M. Anil Kumar, M.C. Stephen Raj and Kunthala Jayaraman. 1998. Studies on the pathogenicity of systemic, ectodermal and mesodermal baculovirus and it's detection in shrimp by immunological methods. Aquaculture. 160: 31-45.

Hameed, A.S.S, M. X. Charles and M.A. Kumar. 2000. Tolerance of *Macrobrachium rosenbergii* to white spot syndrome virus. Aquaculture. 183(3-4): 207-213.

Hameed, A.S.S, K. Yoganandhan, S. Sathish, M. Rasheed, V. Murugan and K. Jayaraman. 2001. White spot syndrome virus (WSSV) in two species of freshwater crabs (*Paratelphura hydrodomous* and *P. pulvinata*). Aquaculture. 201: 179-186.

Hameed, A.S.S, B.L.M. Murthi, M. Rasheed, S. Sathish, K. Yoganandha, J. Murugan and K. Jayaraman. 2002. An investigation of Artemia as a possible vector for white spot syndrome virus (WSSV) transmission to *Penaeus indicus*. Aquaculture. 204: 1-10.

Hernandez-Lopez, J., T. Gollas-Galvan and Vargas-Albores. F. 1996. Activation of the prophenol oxidase system of the brown shrimp (*Penaeus californiensis*, Holmes) haemolymph. Comp.Biochem.Physiol. 104B: 407-413.

Hetru, C., P. Bulet, S. Cociancchi, J.L. Dimarcq, D. Hoffman, and J.A. Hoffman. 1994. Antibacterial peptides/ polypeptides in the insect host defense, a comparison with vertebrate antibacterial peptides/ polypeptides. In: Hoffman,J., Janeway.A., Natori, A. (Eds). Phylogenetic perspectives in immunity, the insect host defense, CRC press Boca Raton, 43-66.

Hossain, M.S., A. Chakraborty, B. Joseph, S.K. Otta, I. Karunasagar and I. Karunasagar. 2001. Detection of new hosts for white spot syndrome virus of shrimp using nested polymerase chain reaction. Aquaculture. 198 (1-2): 1-11.

Hsu, H.C., C.F. Lo, S.C. Lin, K.F. Liu, S.E. Peng, Y.S. Chang, L.L. Chen, W.J. Liu and G.H. Kou. 1999. Studies on effective PCR screening strategies for white spot syndrome virus (WSSV) detection in *Penaeus monodon* brooders. Diseases of Aquatic Organisms. 39: 13-19.

Huang, C. H., L.R Zhang, J.H. Zhang, L.C. Xiao, Q.J. Wu, D.H. Chen, K. Joseph and K. Li. 2001. Purification and characterization of white spot syndrome virus (WSSV) produced in an alternate host: crayfish, *Cambarus clarkii*. Virus Research 76: 115-125.

Huang, J., X.L. Song, J. Yu and C.H. Yang. 1994. Baculoviral hypodermal and haematopoietic necrosis-pathology of the shrimp explosive epidemic disease. Yellow Sea Fishery Research Institute, Qingdao, P.R. China, (Abstract).

Huang, J., J. Yu, X. Song, J. Kong and C. Yang. 1995. Studies on fine structure, nucleic acid, polypeptide and serology of hypodermal and hematopoietic necrosis baculovirus of penaeid shrimp – Yellow Sea Fisheries Research Institute, Chinese Academy of Fisheries Sciences Qingdao 266071 People's Rep. China. Mar. Fish. Res; Haiyang – Shuichas – Yanjice. 16(1): 11-23.

Ingild, 1983. A. Single Radial Immunodiffusion. Scand. J. Immunol. 17(Supple - 10): 41-56.

Inouye, K., S. Miwa, N. Oseko, H. Nakano and T. Kimura. 1994. Mass mortalities of cultured kuruma shrimp, *Penaeus japonicus*, in Japan in 1993. Election microscopic evidence of the causative virus (In Japanese). Fish pathology. 29: 149 -158.

Inouye, K., K. Yamano, N. Ikeda, O. Kimura, H. Nakano, K. Momoyama, J. Kobayashi and S. Miyajima. 1996. The penaeid rod shaped DNA virus (PRDV) which causes penaeid acute viremia (PAV) Fish. Pathol. 31: 39-45.

Itami, T., Y. Takahashi and Y. Nakamura. 1989. Efficacy of vaccination against vibriosis in cultured kuruma prawns *Penaeus japonicus*. Aquatic Anim Health 1: 234-242

Itami, T., Y. Yan and Y. Takahashi. 1992. Studies on vaccination against vibriosis in cultured prawn *Penaeus japonicus*. I J Shimonoseki Univ 40: 83-87

Itami, T., Y. Takahashi, E. Tsuchihira, H. Igusa. 1994. Enhancement of Disease resistance of Kuruma prawn *Penaeus japonicus* and increase in phagocytic activity of prawn hemocytes after oral administration of b-1-3-glucan. (Schizophyllun).In. Chou.L.M, Munro. A.D, Lam T.J, Chen T.W, Cheong L.K.K, Ding J.K, Hooi K.K, Khoo H.W, Pang V.P.E, Shim K.F, Tan C.H (eds) The Third asian Fisheries forum. Asian Fisheries Society, Manila, Philippines, p 375- 378.

Itami, T., M. Asano, K. Tokushige, K. Kubono, A. Nakagawa, N. Takeno, H. Nishimura, M. Maeda, M. Kondo and Y. Takahashi. 1998. Enhancement of disease resistance of kuruma shrimp Penaeus japonicus, after oral administration of peptidoglycan derived from Bifidobacterium thermophilum. Aquaculture 164: 277-288.

Iwanaga, S., S.L. Kawabata, and T. Muta. 1998. New types of clotting factors and defense molecules found in horseshoe crab haemolymph: Their structures and functions. J.Biochem. 123: 1-15.

Jasmin, K.J and K.M. Mary. 2000. Occurrence of white spot syndrome (WSS) in a prawn form at Cochin, Indian J. Fish. 47 (3): 243-243.

Jayashree, L., P. Janakiram and R. Madhavi. 2000. Characteristics, Pathogenicity and Antibiotic sensitivity of Bacterial isolates from white spot diseased shrimp. Asian Fisheries Science. 13: 327-334.

Jiravanichpaisal, P., E. Bangyeakhun, K. Soderhall and I. Soderhall. 2001. Experimental infection of white spot syndrome virus in freshwater crayfish *Pacifastacus leniusculus*. Diseases of Aquatic Organisms. 47: 151-157.

Johansson, M.W and K. Soderhall. 1988. Isolation and purification of a cell adhesion factor from crayfish blood cells. J Cell Biol 160: 1795-1803

Johansson, M.W and K. Soderhall. 1989. Exocytosis of the prophenoloxidase activating system from crayfish haemocytes. J.Comp.Physiol. 156B: 175-181.

Johansson, M.W and K. Soderhall. 1989. Cellular immunity in crustaceans and the Pro PO system. Parasitol. Today. 5: 171- 176.

Johansson, M.W., M.L. Lind, T. Holmblad, P.O. Thornqvist and K. Soderhall. 1995. Peroxinectin a novel cell adhesion protein from crayfish blood. Biochem Biophys Res Commun 216: 1079-1087.

Johansson, M.W. 1999. Cell adhesion molecules in invertebrate immunity. Dev Comp Immunol 23: 303-315.

Kaitpathamchai, W., V. Boonsaeng, A. Tassanakajon, C. Wongteerasupaya, S. Jitrapakdee and S. Panyim. 2001. A non-stop, single tube, semi-rested PCR technique for grading the severity of white spot syndrome virus infections in *Penaeus monodon*. Diseases of Aquatic Organisms. 47: 235-239.

Kanchanaphum, P and C. Wongteerasupaya, N. Sitidilokartana, V. Boonsaeng, S. Panyim, A. Tassanakajon, B. Withyachumnarnkul and T.W. Flegel. 1998. Experimental transmission of white spot syndrome virus (WSSV) from crabs to shrimps *Penaeus monodon*. Diseases of Aquatic Organisms. Vol. No. 34(1), 1- 7.

Karunasagar, I., S.K. Otta, Biju Joseph, G. Subha, P. Tauro and I. Karunasagar. 1996. Successful management of white spot disease in shrimp immunostimulants, Fishing Chimes. Nov.

Karunasagar, I., S. K. Otta and I Karunasagar. 1997. Histolopathological and bacteriological study of white spot syndrome of *Penaeus monodon* along the West Coast of India. Aquaculture. 153: 9-13.

Kasornchandra, J., S. Boonyaratpalin, R. Khongpradit and U. Aekpanithanpong. 1994. A bacilliform virus, the causative agent of red disease with white patch in black tiger shrimp (Penaeus monodon). Dept of Fisheries, Bangkok, Technical paper no:3 p 1-11.

Kasornchandra, J., B. Boonyaratpalin, R. Khongpradit and U. Akpanithanpong. 1995. Mass mortality caused by systemic bacilliform virus in cultured penaeid shrimp, *P. monodon* in Thailand. Asian Shrimp New. 5. p. 2-3.

Kasornchandra, J and B. Boonyaratpalin. 1998. Primary shrimp cell culture: Application for studying white spot syndrome virus (WSSV) In : Flegel, T.W. (Ed.) Advances in shrimp biotechnology. National Centre for Genetic Engineering and Biotechnology, Bangkok, pp. 273-276.

Kasornchandra, J., S. Tantivanit, K. Supamattaya, S. Boonyaratpalin and T. Itami. 1997. Recent progress in research on the white spot disease in penacid shrimp in Thailand. In International symposium on disease in marine Aquaculture, Oct. '97, Japan.

Kasornchandra, J., S. Boonyaratpalin and T. Itami. 1998. Detection of white spot syndrome in cultured penacid shrimp in Asia: Microscopic observation and PCR. Aquaculture. 164: 243-251.

Kiatpathomchai, W., V, Boonsaeng, A. Tassanakajon, C. Wongteerasupaya, S. Jitrapakdee and S. Panyim. 2001. A non-stop, single tube, semi-nested PCR technique for grading the severity of white spot syndrome views infections in *Penaeus monodon*. Diseases of Aquatic organisms. 47: 235-239.

Kim, C.K., P.K. Kim, S.G. Sohn, D.S. Sim, M.A. Park, M.S. Heo, T.H. Lee, J.D. Lee, H.K. Jun and K.L. Jang. 1998. Development of polymerase chain reaction (PCR) procedure for the detection of baculovirus associated with white spot syndrome (WSBV) in penaeid shrimp. J. Fish. Dis. 21: 11-17.

Kimura, T., K. Yamano, H. Nakano, K. Momoyama, M. Hiraoka M and K. Inouye. 1996. Detection of penaeid rod shaped DNA (PRVD) by PCR (In Japanese). Fish Pathology. 31: 93-98.

Kondo, M., T. Itami, Y. Takahashi, R. Fujii and S. Tomonaga. 1998 (a). Ultrastructural and cytochemical characteristics of phagocytes in Kuruma prawn. Fish Pathology. 33(4). 421 - 427.

Kondo, M., T. Itami and Y. Takahashi. 1998 (b). Preliminary characterization of lectin in the hemolymph of kuruma prawn . Fish pathology Vol no: 33, Issue no:4 429-435

Kopaeck, P., M. Hall and K. Soderhall. 1993. Characterization of a clotting protein, isolated from plasma of the freshwater crayfish, *Pacifastacus leniusculas*. Eur. J. Biochem. 213: 591-597.

Kou, G.H and C.H. Lo. 1997.Tissue tropism analysis of white spot syndrome virus in latently and patently infected *Penaeus monodon*. In International Symposium on disease in marine aquaculture Oct. 97. Japan. 1997.

Kou, G.H., S.N. Chen and S.L. Huang. 1989. Studies on bacterial infection and vaccination trials for culture of *Penaeus monodon* in Taiwan. Diseases of Fish and shellfish. Abstract of the 4th EAFP International conference. 24-28 September 1989 Santiago de compostela Spain p 94.

Krishna, R.R., K.G. Rao, P. Rao and P.H. Babu. 1997. White Spot Disease, World Aquaculture, December, p 14 - 17.

Kumar, G.S., I.S.B. Singh and R. Philip. 2001. A cell culture system from the eye stalk of *Penaeus indicus*. In: E. Lindner. Olsson. N. Chatzissavidou and E. Lullau (eds.). Animal Cell Technology: From Target to Market. Proceedings of the 17th ESACT Meeting, Tylosand, Sweden, Kluwer Academic Publishers, June 10-14 2001. p. 261-265.

Ladenhorff, N.E and M.R. Kanost. 1991. Bacteria- induced protein P4 (hemolin) from Manduca sexta: a member of the immunoglobulin super-family which can inhibit hemocyte aggregation. Arch Insect Biochem Physiol 18: 285-300.

Laemmli, U K. 1970. Cleavage of structural protein during the assembly of the head of bacteriophage. Nature 227, 680-685

Lan, J., P. Pratanpipat, G. Nash, S. Wongwisansri, C. Wongteerasupaya, B. Withyachumnarnkul, S. Thammasart and C. Lohawattanakal. 1996. Carrier and susceptible host of the systemic ectodermal and mesodermal baculovirus, the causative agent of white-spot disease in penaeid shrimp. In world Aquaculture '96 Book of Abstracts, The 1996 Annual Meeting of the World Aquaculture Society January 29 – February 2 1996, Queen Sirikit National Connection Center, Bangkok, p. 213–214.

Lightner, D.V and R.H. Redman. 1981. A baculovirus disease of Penaeid shrimp, *P. monodon*, J. Invertebrate Pathol. 38: 299.

Lightner, D.V., R.H. Redman and T.A. Bell. 1983. Observations on the geographic distribution, pathogenesis and morphology of the baculovirus for *P. monodon* Fabricus. Aquaculture, 32:209-233.

Lightner, D.V. 1993. Diseases of cultured penaeid shrimp In: J.P. Mcrey (ed.). Handbook of Mariculture, Crustacean Aquaculture, 1, CRC Press, Boca Raton, p. 393-486.

Lightner, D.V. 1996. Handbook of diagnostic procedures for diseases of penaeid shrimp. Special publication of the world Aquaculture Society, Baton Rouge, LA (1996).

Lightner, D.V., R.M. Redman, B.T. Poulos, L.M. Nunan, J.L. Mari and K.W. Hasson. 1997. Risk of spread of penaeid shrimp viruses in the America by international movement of line and frozen shrimp. Rev. Sci. Tech. Off Int. Epz., 16: 146-160.

Lightner, D.V., K.W. Hasson, B.L. White, R.M. Redman. 1998. Experimental of white spot syndrome of Western hemisphere penaeid shrimp with Asian white spot syndrome virus and Asian yellow head virus. J.Aquat. animal.Health.10. 271-281

Lightner, D.V. 1999. The penaeid shrimp viruses TSV, INHNV, WSSV and YHV: current status in the America, Available Diagnostic Methods and management strategies. J. Phil. Aquacult. 9 (2): 27-52.

Lo, C.F., C.H. Ho, S.E. Peng, C.H. Chen, H.C. Hsu, Y.L. Chiu, C.F. Chang, K.F. Liu, M.S. Su, C.H. Wang and G.H. Kou. 1996 (a). White spot baculovirus (WSBV) detected in cultured and captured shrimp, crabs and other arthropods. Diseases of Aquatic Organisms. 27(3): 215-225.

Lo, C.F., J.H. Leu, C.H. Ho, C.H. Chen, S.E. Peng, Y.T. Chen, C.M. Chou, P.Y. Yeh, C.J. Huang, H.Y. Chou, C.H. Wang and G.H Kou. 1996 (b). Detection of baculovirus associated with white spot syndrome virus (WSSV) in penaeid shrimps using polymerase chain reaction. Dis. Aquat. Org. 25: 133-141.

Lo, C.F., K.F. Lin, C.H. Ho, Y.L. Chu, C.H. Chen, P.Y. Yeh and S.E. Peng. 1997. Detection and tissue tropism of white spot syndrome baculo virus (WSBV) in captured brooders of *P. monodon* with a special emphasis on reproductive organs. Diseases of aquatic organisms. 30(1): 53-72.

Lo, C.F and G.H. Kou. 1998. Virus associated with white spot syndrome of shrimp in Taiwan: A Review. Fish. Pathology. 33(2): 365.

Lo, C.F., Y.S. Chang, C.T. Cheng and G.H. Kou. 1998. Monitoring cultured shrimp for white spot syndrome virus (WSSV) infection during their growth period by polymerase chain reaction. In 5th Asian Fisheries Forum, International conference on fisheries and food security beyond and year 2000. Chiengmai, Thailand. p.127.

Lo, C.F., H.C. Hsu, M.F. Tsai, C.H. Ho, S.E. Peng, G.H. Kou and D.V. Lightner. 1999. Specific genomic DNA fragment analysis of different geographical clinical samples of shrimp white spot syndrome virus. Diseases of Aquatic Organisms. 35: 175-185.

Lu, Y., L.M. Tapay, P.C. Loh, R. Gose and J.A. Brock. 1997 (a). The pathogenicity of a baculo-like virus isolated from diseased penacid shrimp obtained from China for cultured penaeid shrimp species in Hawaii. Aquacult. Int. 5: 277-282.

Lu, Y., L.M. Tapay., R.B. Gose., J.A. Brock and P.C. Loh. 1997 (b). Infectivity of yellow head virus (YHV) and the Chinese baculo-like virus (CBV) in two species of penaeid shrimp, *Penaeus stylirostris* (Stimpson) and *P. vannamei* (Booae). In: T.W.

Flegel and I. Mac Rae (eds). Diseases in Asian Aquaculture III. Asian Fisheries Society, Flegel, Manila.

Magbanua, F.O., K.T. Natividad, V.P. Migo, C.G. Alfafara, F.O. de la Pena, R..O. Miranda, J.D. Albaladejo, E.C.B. Nadala, P.C. Loh and L.M. Tapay. 2000. White spot syndrome virus (WSSV) in cultured *Penaeus monodon* in the Philippines. Diseases of Aquatic Organisms. 42: 77-82.

Mancini, G., A.O. Carbonara and J.R. Heremans. 1965. Immunochemical quantification of antigens by single radial immunodiffusion. Immunochemistry 2. 235.

Marielle, C.W., Van Hulten, M.F. Tsai, C.A. Schipper, C.F. Lo, G.H. Kou and J. M. Vlak. 2000. Analysis of a genomic segment of white spot syndrome virus of shrimp containing ribonucleotide reductase genes and repeat regions. Journal of General Virology. 81: 307-316.

Martin, G.G., D. Pooles, C. Poole, J.E. Hose, M. Aris, L. Reynolds, N. Mackel and A. Whang. 1993. Clearence of bacteria injected into the hemolymph of the penaeid shrimp, *Sicyonia ingentis*. J.Inv.pathol. 62: 308-315.

Mendoza, H.L and I. Faye. 1996. Immunoglobulin superfamily proteins in invertebrates. In: Soderhall, S. Iwanaga and G.R. Vasta. (Eds). New directions in invertebrate immunology. SOS Publications, Fair Hauen, p. 285-302.

Mohan, C.V and K.M. Shankar. 1997. White spot viral disease management in shrimps (Need for scientific approach). Fishing chimes. 17: 41-43.

Mohan, C.V., P.M. Sudha, K.M. Shankar and A. Hegde. 1997. Vertical transmission of white spot baculo virus in shrimps - a possibility? Current science 73(2): 109-110.

Mohan C.V and K.M. Shankar. 1998. Recent findings on white spot syndrome (WSS) of cultured shrimp: management implications. Fishing Chimes, Vol. 18(6): 35-37.

Mohan, C.V., K.M. Shankar, S. Kulkarni and P.M. Sudha. 1998. Histopathology of cultured shrimp showing gross signs of yellow head syndrome and white spot syndrome during 1994 Indian epizootics. Diseases of Aquatic Organisms 34: 9-12.

Momoyama, K., M.M. Heraoka, H. Nakano, H. Koabe, K. Inouye and N. Oseka. 1994. Mass mortalities of cultured kuruma shrimp, *Penaeus japonicus*, in Japan in 1993. Histopathological studies (In Japanese) Fish pathology. 29: 141-148.

Moralles, M.S., Covarrubias and C.C. Sanchez. 1999. Histopathological studies on wild broodstock of white shrimp *Penaeus varamei* in the plantations. Area adjacent to San-Blas, Nayarit, Mexico. Journal The world Aquaculture Society. 30(2): 192-200.

Murphy, F.A., C.M. Fauquet, D.H.L. Bishop, S.A. Ghabrial, A.W. Jarvis, G.P. Martilli, M.A. Mayo and M.D. Summers. 1995. Vius Taxonomy. Archives of Virology, Springer-Verlag, Vienna.

Nadala, C.B.E., B. Lourdes, M. Tapay and P.C. Loh. 1997. Yellow head virus: a rhabdo virus - like pathogen of penaeid shrimp. Dis. Aquat. Org. 31: 141-146.

Nadala, E.C.B., L.M. Tapay, S. Cao and P.C. Loh. 1997. Detection of yellow head virus and Chinese baculo virus in penaeid shrimp by the western blot technique. J. Virol. Methods. 69: 39-44.

Nadala, C.B.E and P.C. Loh. 1998. A comparative study of three different isolates of white spot virus. Diseases of Aquatic Organisms. 33: 231-234.

Nadala, C.B.E., B. Lourdes, M. Tapay and P.C. Loh. 1998. Characterization of a non-occluded baculovirus like agent pathogenic to penaeid shrimp. Diseases of Aquatic Organisms. 33: 221-229.

Nadala, E.C.B and P.C. Loh. 2000. Dot-blot nitrocellulose enzyme immunoassays for the detection of white spot virus and yellow head virus of penaeid shrimp. J. Virol. Methods. 84: 175-179.

Nakano, H., H. Koube, S. Umezaea, K. Momoyama, M. Hiraoka, K. Inouye and N. Oseka. 1994. Mass mortalities of cultivated kuruma shrimp, *Penaeus japonicus*, in Japan in 1993: Epizootiological survey and infection trials. Fish Pathol. 29: 135-139 (in Japanese).

Nash, G. 1995. SEMBV – an emerging viral threat to cultured shrimp in Aisa, Asian Shrimp . 20.

Newman, S.G. 1999. Advances in the prevention of disease is shrimp with particular emphasis on the use of lipopolysaccharide (LPS) based non-specific immune stimulants. Conferencia Regional de Camaronicultura Herramientas Y soluciones para combatir el white spot en American Latina. July 7-8, 1999, p 25.

Newman, S.G. 2000. Management and prevention of stree in aqucculture with a focus on farmed shrimp. URL:http://www.aqua-in-tech.com.

Ninawae, A.S. 1997. How white spot disease devastated Indian fish farms. Fish Farming International. 24 (3): 12-13.

Noga, J.E., A.A. Thomas, A.R. Bullis and K. Lester. 1996. Antibacterial activity in hemolymph of white shrimp, *Penaeus setiferns*. Journal of Marine Biotechnology. 4 (3): 181-184.

Nunan, L.M and D.V. Lightner. 1997. Development of a non-radioactive gene probe by PCR for detection of white spot syndrome virus (WSSV). J. Virol. Methods. 63: 193-201.

Nunan, L.M., B.T. Poulos and D.V. Lightner. 1998. The detection of white spot syndrome virus (WSSV) and Yellow Head Virus (YAV) in imported commodity shrimp. Aquaculture. 160: 19-30.

Omori, S.A., G.G. Martin and J.E. Hose. 1989. Morphology of haemocytic lysis and iso-clotting in the ridge back prawn, *Sicyorea ingeatis*. Eu. Cell. Tissue Res. 255: 117-123.

Otta. S. K., I. Karunasagar, P. Tauro and I. Karunasagar. 1998. Microbial diseases of shrimp. Indian J. Microbiology. 38. 113 –125.

Otta, S.K., G. Shubha, B. Joseph, A. Chakraborthy, I. Karunasagar and I. Karunasagar. 1999. Polymerase chain reaction (PCR) detection of white spot syndrome virus (WSSV) in cultured and wild crustaceans in India. Dis. Aquat. Org. 38: 67-70.

Ouchterlony, O and L.A. Nilsson. 1978. Immunodiffusion and immunoelectrophoresis: In Handbook of Experimental Immunology, 3rd Ed. (Weir, D.H. ed), Blackwell, Oxford, UK. p. 19.1-19.44.

Overstreet, R.M., K.C. Stuck, R.A. Karol and W.E. Hawkins. 1998. The detection of white spot syndrome virus (WSSV) and yellow head virus (YHV) in imported commodity shrimp. Aquaculture 160: 19-30.

Park J.H., Y.S. Lee, S. Lee, Y. Lee. 1998. An infectious viral disease of penaeid shrimp newly found in Korea. Diseases of Aquatic Organisms. 34: 71-75.

Peng, S.E., C.H. Chen, C.F. Lo and G.H. Kou. 1995. Detection of white spot syndrome associated non-occluded baculovirus in penaeid shrimp. In: Abstracts of seventh

International conference in Diseases of Fish and Shellfish 10-15, Sep. 1995, Palme de Mallora, Spain p 8.

Peng, S.E., C.H. Lo, K.G. Liu and G.H. Kou. 1997. Investigation on the transition from talent to patent infection of white spot syndrome virus (WSSV) in penaeus monodon using PCR and in situ hepbridisation (Abstract). In International Symposium on diseases in marine Aquaculture Oct. 97, Japan

Peng S.E., and C.F. Lo. 1998. The transition from pre-patent to patent infection of white spot syndrome virus (WSSV) in *P. monodon* triggered by pereiopod excision. Fish Pathology. 33(4): 395- 401.

Peng, S.E., C.F. Lo, C.H. Ho, C.F. Chang and G.H. Kou. 1998. Detection of white spot baculovirus (WSBV) in giant freshwater prawn, *Macrobrachium rosenbergii* using polymerase chain reaction. Aquaculture. 164: 253-262.

Perazzolo, L.M and M.A. Barracco. 1997. The prophenoloxidase activating system of the shrimp *Penaeus paulensis* and associated factors. Dev. Comp. Immunol. 21: 385-395.

Poulos, B.T., C.R. Pantoja, D. Bradley-Dunlop, J. Aguilar and D.V. Lightner. 2001. Development and application of monoclonal antibodies for the detection of white spot syndrome virus of penaeid shrimp. Diseases of Aquatic Organisms. 47: 13-23.

Prabhu, N.M., A.R. Nazar, S. Rajagopal and S.A. Khan 1999. Use of probiotics in water quality management during shrimp culture. J. Aqua.Trop. 14: 227- 236.

Pratanpipat, P., C. Nithimethachoke, A. Akarajamoron, G. Nash, B. Withyachumnarnkul, S. Thammasart and C. Lohawattanakul. 1996. The efficacy of formalin for disinfection of systemic ectodermal and medodermal baculovirus. In: World Aquaculture 96 Book of Abstracts, the 1996 annual meeting of the World Aquaculture

Society, January 29 – February 2, 1996, Queen Sirikit National Convention Centre, Bangkok, p. 318.

Rabin, H. 1970. Hemocytes, hemolymph and defense reaction in crustaceans. J. Reticulo endothel.Soc. 7: 195-207.

Rajan, P.R., P. Ramaswamy, V. Purushothaman and G.P. Brennan. 2000. White-spot baculovirus syndrome in the Indian shrimp *P. monodon* and *P. indicus*. Aquaculture. 184 (1-2): 31-44.

Rajendran, K.V., K.K. Vijayan, T.C. Santiago and R.M. Krol. 1999. Experimental host range and histopathology of white spot syndrome virus (WSSV) infection in shrimp, prawns, crabs and lobster from India. J. Fish Dis. 22: 183-193.

Rao, J. 1996. The use of immunostimulatory substances in fish and shellfish farming. Reviews in fishery science 4(3): 229-288.

Ratanapo, S and M. Chulavatnatol. 1990. Monodin, a new sialic acid – specific lectin from black tiger prawn, *Penaeus monodon*. Comp. Biochem. Physiol. 97B: 515-520.

Ratcliffe, N.A., A.F. Rowley, S.W. Fitzgerald and C.P. Rhodes. 1985. Invertebrate immunity: Basic concepts and recent advances. Int.Rev.Cytol. 97: 183-350.

Richman, L.K., R.J. Montali, D.K. Nichols and D.V. Lightner. 1997. A newely recognized fatal baculovirus infection in fresh water cray fish. Proc.A.m.Assoc.Zoo.Vet.262- 264.

Scholz, U., G. Garcia Diaz, D. Ricque, L.E. Cruz Suarez, F.Vargas Albores and J. Latchford. 1999. Enhancement of vibriosis resistance in juvenile *Penaeus vannamei* by supplementation of diets with different yeast products. Aquaculture. 176, 271-283.

Sen, A., I.S. B. Singh, R. Rengarajan, R. Philip, G.S. Kumar and A. Sen. 1999. Evidence of a bacilliform virus causing outbreaks of white spot disease in *Penaeus monodon* H. Milne Edwards in India. Asian Fisheries Science. 12: 41-47.

Shankar, K.M and C.V. Mohan. 1998. Epidemiological aspects of shrimp viral diseases in India – A review. J. Aqua. Trop 13(1): 43-49.

Shi, Z., C. Huang, J. Zhang, D. Chen and J.R. Bonami. 2000. White spot syndrome virus (WSSV) experimental infection of the freshwater crayfish, *Cherax quadricarinatus*. Journal of Fish Diseases. 23: 285-288.

Shih, H.H., C.S. Wang, L.F. Tan and S.N. Chen. 2001. Characterisation and application of monoclonal antibodies against white spot syndrome virus. J. fish. Dis. 24: 143-150.

Soderhall, K. 1982. Phenoloxidase activating system and melanization - a recognition mechanism of arthropods? A review. Dev Comp Immunology 6: 601-611.

Smith, V.J and K. Soderhall. 1983. β-1-3 glucan enhancement of protease activity in the cray fish haemocyte lysate. Comp.Biochem. Physiol.74 B. 221-224.

Soderhall, K., L. Hall, T. Unestam and L. Nyhlen. 1979. Attachment of phenoloxidase to fungal cell walls in arthropod immunity. Journal of invertebrate pathology. 34: 285-294.

Soderhall, K., A. Wingren, M.W. Johansson and K. Berthussen. 1985. The cytotoxic reaction from the freshwater crayfish, *Astacus astacus*. Cell. Immunol. 94: 326-332.

Soderhall, K and L. Cerenius. 1998. Role of the prophenoloxidase activating system in invertebrate immunity. Curr Opin Immunology 10(1): 23-28.

***Soderhall, K and L. Cerenius. 1992.** Crustacean immunity. Annual Rev of Fish Diseases. 3-23.

Soderhall, K., L. Cerenius and M.W. Johansson. 1996. The prophenoloxidase activating system in invertebrates. In: Soderhall K, Iwanaga S, Vasta G.R. (Eds) New directions in Invertebrate Immunology, SOS publication, Fair Haven, 229-253.

Soderhall, K and P.O. Thornqvist. 1997. Crustacean immunity - A short Review. In. Gudding. R. Lillehaug A, Midtyling P.J, Brown F (Eds). Fish Vaccinology. Dev.Biol.Stand. Basel, Karger. 90: 45-51.

Song, Y.L and Y.T. Hsieh. 1994. Immunostimulation of tiger shrimp (*Penaeus monodon*) haemocytes for generation of microbicidal substances: analysis of reactive oxygen species. Dev. Comp. Immunol. 18: 201-209.

Sudha, P.M., C.V. Mohan, K.M. Shankar and A. Hegde. 1998. Relationship between white spot syndrome virus infection and clinical manifestation in Indian cultured penaeid shrimp. Aquaculture. 167: 95-101.

Sun, S.C., I. Lindstrom, H.G. Boman, I. Faye and O. Schmit. 1990. Hemolin: an insect immune protein belonging to the immunoglobulin superfamily. Science 250: 1729-1732.

Sung, H.H., G.H. Kuo and Y.L. Song. 1994. Vibriosis resistance induced by glucan treatment in tiger shrimp (*Penaeus monodon*). Fish Pathology. 29: 11-17.

Sung, H.H., H.J. Chang, C.H. Her, J.C. Chang, and Y.L. Song. 1998. Phenoloxidase activity of hemocytes derived from *Penaeus monodon & Macrobrachium rosenbergii*. J. Invertbs. Pathol. 71: 26-33.

Supamattaya, K., R.W. Hoffmann, S. Boonyaratpalin and P. Kanchanaphum. 1998. Experimental transmission of white spot syndrome virus (WSSV) from black tiger shrimp *Penaeus monodon* to the sand crab *Postunus pelagicus*, mud crab, *Scylla serrata* and krill *Acetes* sp. Diseases of Aquatic Organisms. 32: 79-85.

Suzuki, N., I. Mizumoto, T. Itami, Y. Takahashi, R. Tanaka, H. Hatate, T. Nomoto and A. Kozawa. 2002. Virucidal effect of singlet oxygen on penaeid white spot syndrome virus. Fisheries Science. 66: 166-168.

Takahashi, Y., T. Itami, M. Kondo, M. Maeda, R. Fuji, S. Tomonaga, K. Supamattaya and S. Boonyaratpalin. 1994. Electron microscopic evidence of bacilliform virus infection in kuruma shrimp (*Penaeus japonicus*). Fish Pathology. 29(2): 121-125.

Takahashi, Y., T. Itami, M. Maeda, N. Suzuki, J. Kasornchandra, K. Supamattaya, R. Khongpradit, S. Boonyaratpalin, M. Kondo, K. Kawai, R. Kusuda, I. Hirono and T. Aoki. 1996. Polymerase chain reaction (PCR) amplification of bacilliform virus (RV – PJ) DNA in *P. japonicus* Bate and systemic ectodermal and mesodermal baculovirus (SEMBV) DNA in *P. monodon* Fabricus. Journal of Fish Diseases. 19: 399- 403.

Takahasi, Y., K. Uchara, R. Watanabe, T. Okumura, T. Yamashita, H. Omura, T. Yomo, T. Kawano, A. Kanemitsu, H. Narasaka, N. Suzuki and T. Itami. 1998. Efficacy of oral administration of Fucoidan, a sulphated polysaccharide in controlling white spot syndrome in kuruma shrimp in Japan. Advances in shrimp biotechnology National center for genetic engineering and biotechnology, Bangkok. p. 171-173, Flegel (ed).

Tan, L.T., S. Soon, K.J. Lee, M. Shariff, M.D. Hassan and A.R. Omar. 2001. Quantitative analysis of an experimetnal white spot syndrome virus (WSSV) infection in *Penaeus monodon* Fabricius using competitive polymerase chain reaction. J. Fish. Dis. 24: 315-323.

Tang, K., F.J. and D.V. Lightner. 2000. Quantification of white spote syndrome virus DNA through a competitive polymerase chain reaction. Aquaculture. 189: 11-21.

Tapay, L.M., Y. Lu, J.A. Brock, Nadala Jr. E.C.B. and P.C. Loh. 1997. Transformation of primary cell culture of shrimp (*Penaeus stylirostris*) lymphoid (oka) organ with simian virus – 40 (T) antigen. Suc. Exp. Biol. Med. 209: 73-78.

Tapay, L.M., E.C.B. Nadala and P.C. Loh. 1999. A polymerase chain reaction protocol for the detection of various geographical isolates of white spot virus. J. Virol. Meth. 82: 39-43.

Teunissen, O.S.P., R. Faber, G.H.R. Booms, T. Latscha and J.H. Boon. 1998. Influence of vaccination on vibriosis resistance of the giant black tiger shrimp *Penaeus monodon* (Fabricius). Aquaculture. 164: 359-366.

Tournut, J. 1989. Applications of probiotics to animal husbandry. Rev.Sci.Tech.Off. Intnl.Epiz. 8: 551- 66.

Tsai, M.F., G.H. Kou, H.C. Liu, K.F. Liu, C.F. Chang, S.E. Peng, H.C. Hsu, C.H. Wang and C.F. Lo. 1999. Long-term presence of white spot syndrome virus (WSSV) in a cultivated shrimp population without disease outbreaks. Dis. Aquat. Org. 38: 107-114.

Tyson, C.J and C.R. Jenkin. 1974. The cytotoxic effect of haemocytes from the crayfish (*Parachaeraps bicarinatus*) on tumour cells of vertebrates. Austr. J. Exp. Biol. Med. Sci. 52: 915-923.

Van Hulten. 1999. Virus Taxonomy at the XI International Congress of Virology, Sydney, Australia, 1999.

Van Hulten, M.C.W., S.D. Goodal and J.M. Vlak. 2000 (a). Three functionally diverged major structural proteins of white spot syndrome virus evolved by gene duplication. J. Gen. Virology. 81: 2525-2529.

Van Hulten, M.C.W., M. Westernberg, , S.D. Goodal and J.M. Vlak. 2000 (b). Identification of two major virion protein genes of white spot syndrome virus of shrimp. Virology. 266: 227-236.

Van Hulten, M.C.W., M. Reijns, A.M.G. Vermeesch, F. Zandbergen and J.M. Vlak. 2002. Identification of VP 19 and VP 15 of white spot syndrome virus (WSSV) and glycosylation status of the WSSV major strctural proteins. J. Gen. Virology. 83: 257-265.

Vargas-Albores, F., M.A. Guzman and J.L. Ochoa. 1993. An anticoagulant solution for haemolymph collection and prophenoloxidase studies of Penaeid shrimp (*P. californiensis*). Comp.Biochem.Physiol. 106A: 299-303.

Vargas-Albores, F. 1995. The defense system of brown shrimp (*P. californiensis*): Humoral recognition and cellular response. J.Mar.Biotechnol. 3: 153-156.

Venegas, C.A., L. Nonaka, K. Mushiake, T. Nishizawa and K. Muroga. 2000. Quasi-immune response of *Penaeus japonicus* to penaeid rod- shaped DNA virus (PRDV) Dise. Aqua. Org. 42: 83-89.

Wang, C.H., C.F. Lo, J.H. LIu, C.M. Chou, P.Y. Yeh, H.Y. Chou, M.C. Yung, C.F. Chang, M.S. Su and G.H. Kou. 1995. Purification and genomic analysis of baculovirus associated with white spot syndrome (WSBV) of *Penaeus monodon.* Diseases of Aquatic Organisms. 23(3): 239-242.

Wang, C.S, K.F.J. Tang, G.H. Kou and S.N. Chen. 1996. Yellow head disease – like virus infection in the kuruma shrimp *P. japonicus* cultured in Taiwan. Fish pathology. 31(4): 177-182.

Wang, T. X., Hu-chaogun, Hu-chaogun *et al.* 1996. Preliminary studies in the developing mechanism of pathologic white spots on the shell of *P. monodon.* Trop. Oceanol. Redai – Haiyang. 15(1): 24-29.

Wang, C.S., G.H. Kou, K.F.J. Tang, and S.N Chen. 1997 (a). Light and electronmicroscopic evidence of white spot disease in the giant tiger shrimp, *Penaeus monodon* (Fabricius) and the kuruma shrimp, *Penaeus japonicus* (Bate) cultured in Taiwan. J. Fish Dis. 20(5): 323 –331.

Wang, C.S., Y.J. Tsai, G.H. Kou and S.N. Chen. 1997 (b). Detection of white spot disease virus infection in wild caught greasy back shrimp, *Metapenaeus crisis* (de Haan) in Taiwan. Fish Pathology. 32(1): 35-41.

Wang, Y.C., P.S. Chang, C.F. Lo and G.H. Kou. 1998. Experimental infection of white spot baculovirus in some cultured and wild decapods in Taiwan. Aquaculture 164: 221-231.

Wang, C.H., C.F. Lo, P.S. Chang and G.H. Kou. 1998. Experimental infection of white spot baculovirus in some cultured and wild decapods in Taiwan. Aquaculture 164: 221-231.

Wang, Q., B.L.White, R.M. Redman and D.V. Lightner. 1999 (b). Per se Challenge of *Litopenaeus vannamie* post larvae and *Farfantepenaeus duorarum* juveniles with six geographic isolates of white spot syndrome virus. Aquaculture 170: 179-194.

Wang, Y.G., M.D. Hassan, M. Shariff, S.M. Zamri and X. Chen. 1999 (a). Histopathology and cytopathology of white spot syndrome virus (WSSV) in cultured *Penaeus monodon* from peninsular Malaysia with emphasis on pathogenesis and the mechanism of white spot formation. Dis.Aquat .Org. 39(1), 1-11.

Wang, C.H., H.N. Yang, C.H. Tang, C.H. Lu, G.H. Kou and C.F. Lo. 2000. Ultra structure of white spot syndrome virus development in primary lymphoid organ cell cultures. Dis. Aquat. Org., 41: 91-104.

Wang, Q.L., B.T. Paulos and D.V. Lightner. 2000 (a). Protein analysis of geographic isolates of shrimp white spot syndrome virus. Arch. Virol. 145(2): 263-274.

Wang, Q.L., L.M. Nunan and D.V. Lightner. 2000(b). Identification of genomic variations among geographic isolates of white spot syndrome virus using restriction analysis and southern blot hybridization. Diseases of Aquatic Organisms. 43: 175-181.

Wang, Y.G., K.L. Lee, M. Najiah, M. Shariff and M.D. Hussan. 2000. A new bacterial white spot syndrome (BWSS) in cultured tiger shrimp *Penaeus monodon* and its comparison with white spot syndrome (WSS) caused by virus. Diseases of Aquatic Organisms. 41: 9-18.

Wesslen T, E. Lycke, S. Gard and G. Olin. 1957. Inactivation of poliomyelitis virus by formaldehyde. Arch.Ges.virusf. 7. 125-135.

Wongteerasupaya, C., J.E. Vickers, S. Sriurairatana, G.L. Nash, A. Akarajamorn, V. Boonsaeng, S. Panyim, A. Tassanakajon, B. Withyachumarnkul and T.W. Flegel. 1995. A non-occuluded systemic baculovirus that occurs in cells of ectodermal and mesodermal origin of *Peanaeus monodon*. Diseases of Aquatic Organisms. 21: 69-77.

Wongteerasupaya, C., S. Panyim, S. Wongwisansri, P. Pratanpipat, V. Boonsirm, G.L. Nash, B. Withyachumnarnkul and T.W. Flegel. 1996. DNA fragment of *P. monodon* bacilovirus Pm NOB II, gives positive *in situ* hypridization with white spot viral infections in six penaeid shrimp species. Aquaculture. 143: 23-32.

Wongteerasupaya, C., S. Wongwisansri, V. Boensaeny and S. Panyim. 1996. DNA fragments of *P. monodon* Baculovirus (Pm NOBH) gives the *in situ* Hybridization with white spot viral infections in 6 penaeid shrimp species. Aquaculture. 143(1): 23-32.

Yaligar, M.D and L.N. Pai. 1996. Remedy for white spot Disease of shrimp (Latex therapy) 'SLC – Urinum' therapy to control white spot viral disease in penaeid shrimp, Fishing Chimes, July. p 21.

Yang. F., W. Wang, R.Z. Chen, X. Xu. 1997. A simple and efficient method for purification of prawn baculovirus DNA. J.Virol. Methods. 67: 1-4.

Yang, F., H.E. Jun, X. Lin, Q. Li, D. Pan, X. Zhang and X.U. Xun. 2001. Complete genome sequence of the shrimp white spot bacilliform virus. J. Virol. 75: 11811-11820.

Yoshida, H., K. Kinoshita and M. Ashida. 1996. Purification of a peptidoglycan recognition protein from haemolymph of the silk worm, *Bombyx mori*. J. Biol. Chem. 271: 13854 -13860.

You, Z., E.C.B. Nadala, J. Yang, M.C.W Van Hulten and P.C. Loh. 2002. Production of polyclonal antiserum specific to the 27.5 k Da envelop protein of white spot syndrome virus. Dis. Aquat. Org. 51: 77-80.

Zhan, W.B., Y.H. Wang, J.L. Fryer, K. Okubro, H. Fukuda, K.K. Yu and Q. X. Meng. 1999. Production of monoclonal antibodies (Mab's) against white spot syndrome virus (WSSV) Journal of Aquatic Animal Health. 11: 17-22.

Zhang, X., L. Xu and X. Xu. 2001 (a). Detection of prawn white spot bacilliform virus by immunoassay with recombinant antigen. Journal of Virological Methods 92: 193-197.

Zhang, X., X. Xu and C.L. Huo. 2001 (b). The structure and function of a gene encoding a basic peptide from prawn white spot syndrome virus. Virus Research. 79: 137-144.