IMMUNE RESPONSE OF *PENAEUS MONODON* TO THE INACTIVATED WHITE SPOT SYNDROME VIRUS PREPARATION

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by

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March 2011

CERTIFICATE

This is to certify that the research work presented in this thesis entitled **"Immune Response of *Penaeus monodon* to the Inactivated White Spot Syndrome Virus Preparation"** is based on the original work done by Ms. Gigi Poulose under our guidance at National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Kochi 682016, in partial fulfilment of the requirements for the award of 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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Declaration

I hereby do declare that the work presented in this thesis entitled **"Immune Response of *Penaeus monodon* to the Inactivated White Spot Syndrome Virus Preparation"** is based on the original work done by me under the guidance of Prof. A. Mohandas (Emeritus Professor), Prof. I. S. Bright Singh (Co-ordinator), National Centre for Aquatic Animal Health, and Dr. Rosamma Philip, Asst. Professor, Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology, Kochi – 682 016 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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Gigi Poulose

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Chapter 1

GENERAL INTRODUCTION

CHAPTER - 1

General Introduction

Aquaculture, probably the fastest growing food-producing sector, now accounts for almost 50 percent of the world's food fish and is perceived as having the greatest potential to meet the growing demand for aquatic food. Given the projected population growth over the next two decades, it is estimated that at least an additional 40 million tonnes of aquatic food will be required by 2030 to maintain the current per capita consumption (FAO, 2006). Farming of fresh water and sea water fishes, molluscs, crustaceans and aquatic plants have been the strategies adopted in aquaculture.

Whilst a number of crustacean species are cultured, the predominant commercial species are brackishwater shrimps, freshwater prawns, and freshwater/brackish water crabs. Modern shrimp farming, the production of marine shrimp in impoundments, ponds, raceways and tanks, got started in the early 1970s, and, today, over fifty countries have shrimp farms. In the Eastern Hemisphere, Indonesia, India, Thailand, Vietnam and China are the leaders, and Malaysia, Taiwan, Bangladesh, Sri Lanka, The Philippines, Australia and Myanmar (Burma) have large shrimp farming industries. In the Western Hemisphere, Mexico, Belize, Ecuador and Brazil are the leading producers, and there are shrimp farms in Honduras, Panama, Colombia, Guatemala, Venezuela, Nicaragua and Peru. The shrimp importing nations--the United States, Western Europe and Japan--specialize in high-tech "intensive" shrimp farming but, thus far, their production has been insignificant. In the Middle East, Saudi Arabia and Iran produce the most farmed shrimp (Anon, 2008). In the world of shrimp culture, the main shrimp species produced is whiteleg shrimp, *Penaeus vannamei.* In the last few years, this species has replaced the giant tiger prawn – *Penaeus monodon* in many countries. The giant tiger shrimp was accounting for

more than half of the total shrimp aquaculture output. Other important commercial species are *P. indicus*, *P. merguiensis* and *P. chinensis*.

In India, commercial shrimp farming started gaining momentum during the mid-eighties. Shrimp is the most important commodity in India's fish export basket. The potential area available in the coastal region of the country for shrimp farming is estimated between 1.2 million to 1.4 million hectares. Presently, an area of about 1,57,000 ha is under farming with an average production of about 1,00,000 metric tonnes of shrimp per year. The average productivity has been estimated at 660 kg per hectare per year. Cultured shrimps contribute about 50per cent of the total shrimp exports from India (Anon, 2002). There is extensive shrimp culture in the Indian States of Tamilnadu, Maharastra, Gujarat, Orissa, Andhra Pradesh, Karnataka, Kerala, Goa, Pondicherry and West Bengal (FAO, 2007).

Shrimp aquaculture has exhibited a boom-and-bust pattern in many countries, ever since 1988 when the industry first collapsed in Taiwan due to disease problems. Other top-producing countries, such as China, Thailand, Indonesia and Ecuador, have also experienced a rapid expansion of shrimp farms that collapsed within 5-10 years of operation. Diseases that once were restricted to one region are now rapidly spreading over the world as a result of the expansion and globalisation of the shrimp industry by the international movement of live (for aquaculture) and dead shrimp (commodity for commerce), as well as due to complicated weather changes (Rönnbäck, 2001).

Disease in shrimp farming may be defined as a biotic or abiotic condition or factor that adversely affects culture performance (Lightner, 1996). Biotic diseases of shrimp are those that have living agents as the cause, while abiotic diseases may be caused by environmental or physical extremes (temperature, hypoxia, nitrogen super-saturation, extremes of pH, chemical toxicants, pesticides, nutritional deficiencies or imbalances, improper handing, etc.).

Within biotic diseases are diseases of infectious and noninfectious aetiologies. Shrimps have infectious diseases caused by viruses, bacteria, fungi, protozoan and helminth parasites, etc. (Lightner, 1988, 1993a, 1993b, 1996). Bacteria and fungi are mostly opportunistic parasites having specific infection mechanisms while the real menace in shrimp culture is posed by viral diseases.

1.1. White Spot Syndrome Virus (WSSV)

Worldwide, shrimp aquaculture has suffered substantial economic losses due to interplay of more than 20 viruses. In 2010, the Office International des Epizooties **(**OIE) or the World Organization for Animal Health listed viruses causing Taura syndrome (Taura Syndrome Virus - TSV), White spot disease (White Spot Syndrome Virus - WSSV), Yellowhead disease (Yellow Head Virus - YHV), Tetrahedral baculovirosis (*Baculovirus penaei* - BPV), Spherical baculovirosis (*Penaeus monodon*-type baculovirus - MBV), Infectious hypodermal and haematopoietic necrosis (Infectious Hypodermal and Haematopoietic Necrosis Virus - IHHNV), and Infectious myonecrosis (Infectious myonecrosis virus - IMNV) as the major pathogens affecting the booming business of shrimp culture.

Of the many viruses threatening the shrimp aquaculture, White Spot Syndrome Virus (WSSV), is considered to be a highly lethal, stress dependant and the most notorious aquatic virus. WSSV, one of the largest viruses, has a catastrophic and devastating impact of 100% mortality within one week in the shrimp farming industry.

Epidemiological investigations tracked the source of the WSSV to a batch of infected shrimp from the Fujian province of China in 1991/1992 (Nakano et al., 1994; Takahashi et al., 1994; Flegel, 1997; Sen et al., 1999; Pradeep et al., 2008). The viral agent was first isolated from an outbreak in

Japan in late 1993 (Inouye et al., 1994). Shrimp farms started getting affected simultaneously at different regions, and each was given specific name as:

- Hypodermal and Haematopoietic Necrosis Baculovirus (HHNBV) – China, 1993-1994 (Durand et al., 1996)
- Third *Penaeus monodon* Non-Occluded Baculovirus (Pm NOB III) – (Wang et al., 1995, Karunasagar et al., 1997)
- Rod shaped Nuclear Virus of *Marsupenaeus japonicus* (RV-PJ) – Japan (Inouye et al., 1994, 1996)
- Penaeid Rod shaped DNA virus (PRDV) (Venegas et al., 1999)
- Penaeid Acute Viremia (PAV) – Japan, 1995
- Systemic Ectodermal and Mesodermal Baculovirus (SEMBV) – Thailand and India, 1994 (Wongteerasupaya et al., 1995; Sahul-Hameed et al., 1998)
- Red Disease - Thailand and India, 1994
- Shrimp Explosive Epidermic Disease (SEED)
- White Spot Syndrome Virus – Taiwan, 1996
- White Spot Baculovirus complex (WSBV) (Chou et al., 1995; Lightner, 1996) – White Spot Baculovirus – Taiwan, 1996; White Spot Bacilliform Virus – China, 2001
- White Spot Virus – OIE, 2000

Later, it was recognized that a single viral agent was responsible for these reports. Eventually an informal consensus was reached to call it White Spot Syndrome Virus (WSSV). This pathogen is now recognized as the most serious for hampering shrimp aquaculture and its development world wide.

1.1.1. Taxonomic Rank

Phylogenetic analysis based on several enzyme genes including the ribonucleotide reductase large and small subunits, the protein kinases, the

endonuclease, the chimeric thymidine-thymidylate kinase and the DNA polymerase demonstrated that the WSSV is not closely related to Baculoviruses (Nadala et al., 1998; Tsai et al., 2000a,b; van Hulten et al., 2000a, 2000b; Witteveldt et al., 2001; Liu et al., 2001; Chen et al., 2002a,b; Huang et al., 2002a,b; van Hulten et al., 2002; Tsai et al., 2004; Zhan et al., 2004; Zhang et al., 2004a; Huang et al., 2005; Leu et al., 2005; Marks, 2005; Vlak et al., 2005; Xie and Yang, 2005; Zhu et al., 2005). Based on the morphological features, genomic structure and composition and phylogenetic analyses, the uniqueness of White Spot Syndrome Virus 1 (WSSV-1) was revealed, and is therefore now accommodated as the type species for the single new genus *Whispovirus* of the new virus family, *Nimaviridae*, in the 8th Report of International Committee on Taxonomy of Viruses (ICTV) (Vlak et al., 2005).

1.1.2. Morphology

The virion is a large, non-occluded, ellipsoid to bacilliform shaped, enveloped particle (210-380 nm length and 70-167nm width) with a tail like appendage at one end (Wongtterasupaya et al., 1995; Chang et al., 1996; Durand et al., 1997; Flegel and Alday-Sanz., 1998; Nadala et al., 1998; Park et al., 1998; Rajendran et al., 1999; Leu et al., 2005).

The viral envelope is 6-7nm thick and is a lipidic, trilaminar membranous structure with two electron transparent layers divided by an electron opaque layer (Wongteerasupaya et al., 1995; Durand et al., 1997; Nadala et al., 1998).

The rod shaped nucleocapsid with a tight fitting capsid layer located inside the envelope is a stacked ring structure composed of globular protein subunits of 10nm in diameter in two parallel rows arranged like 14-15 vertical striations located every 22nm along the long axis, giving it a cross-hatched appearance (Durand et al., 1997; Nadala and Loh, 1998). The size of the

nucleocapsid varies from isolate to isolate and ranges between 180nm and 420nm in length and 54-85nm in width with a 6nm thick external wall. When released from the envelope, the nucleocapsid increases in length indicating that it is tightly packed within the virion (Kasornchandra et al., 1998; Sahul-Hameed et al., 1998; Rajendran et al., 1999).

A proteinaceous layer referred to as the tegument has been reported as an intermediate layer between the envelope and the nucleocapsid as revealed from WSSV proteins identification studies by Tsai et al. (2006).

The tail-like appendage or the thread-like polar extension on the virus particle is sometimes observed at one end of the virion in negatively stained electron micrographs (Wongteerasupaya et al., 1995; Durand et al., 1996). The composition and function of this appendage is not identified so far. But, the presence of this tail-like extension is its most prominent feature for it to be included in the family- *Nimaviridae* ("nima" is Latin for "thread").

1.1.3. Genome of WSSV

The WSSV genome is a circular, supercoiled, double stranded DNA molecule ~300kb with an A+T content of 59% homogeneously distributed. The genome size varies according to the viral isolate. The genome of the three WSSV isolates has been fully sequenced: Thailand 293 kbp [WSSV-TH; Acc. No. AF369029] (van Hulten et al., 2000a; 2001a), Taiwan 307 kbp [WSSV-TW; Acc. No. AF440570] (Tsai et al., 2000a,b) and China 305 kbp [WSSV-CN; Acc. No. AF332093] (Yang et al., 2001). The nucleotide identity between these isolates is 99.3% (Marks, 2005).

Sequence analysis shows that the WSSV genome contains between 531 and 684 open reading frames (ORFs) with an ATG initiation codon. Of these, 181–184 ORFs are likely to encode functional proteins with sizes between 51 and 6077 aminoacids, which represent 92% of the genetic information contained

in the genome (van Hulten et al., 2001a; Yang et al., 2001). About 21–29% of such ORFs have been shown to encode WSSV proteins or share identity with other known proteins.

1.1.3.1. WSSV Genes

Transcriptional analysis of WSSV genome expression reveals that WSSV genes can be classified into **immediate early** (genes that are transcribed in the absence of viral proteins), **early** (genes involved in nucleotide metabolism, DNA replication and protein modification), and **late** genes (virion protein genes) (Marks et al., 2005; Sanchez-Paz, 2010), and they are regulated as coordinated cascades under the control of different promoters. Immediate early (IE) and early (E) genes are expressed before viral DNA replication, while expression of late (L) genes occurs after replication of the viral genome.

According to their assumed function, genes found in the WSSV genome have been classified into four groups by Sanchez-Martinez et al. (2007) as:

1.1.3.1.1. Temporal regulatory genes: which participate at specific times during infection.

WSSV temporal regulatory genes do not require viral proteins to be transcribed, and are expressed using the host molecular machinery in the first hours after infection. Three immediate-early genes from WSSV-TW, *ie1*, *ie2* and *ie3*, from ORF126, ORF242 and ORF418, respectively, have been characterized using cycloheximide-treated shrimp, a non-specific inhibitor of protein synthesis and a microarray analysis. Although the function of the immediate early genes remains unclear, the *ie1* gene product shares the common Cys2/His2 zinc finger DNA-binding domain (Liu et al., 2005). *ie1* gene expression has been shown to require shrimp's STAT1 to enhance its transcription (Liu et al., 2007a). These IE genes may be important to determine host range and also can function as regulatory trans-acting factors during

infection. Robust transcription of *ie1* upon WSSV infection may switch on expression of late genes (Liu et al., 2005).

1.1.3.1.2. Latency and sequester genes: whose expression can be detected even though the structural genes might not be active.

The latency stage of WSSV occurs before infection and proliferation. Latency genes show maximal activity rates of transcription following viral DNA synthesis, and are involved in the persistence of the virus within a host cell (Flint, et al., 2000). Their function is to keep a low number of viruses and inactivating host genes, until the optimal conditions of pH, salinity and temperature or population density are present. At least three genes have been described as being involved in WSSV genome latency. Using a microarray technique, these three genes, corresponding to the ORFs 151, 366 and 427 (510,210 and 900 bp, respectively), were found to be relatively highly expressed in SPF shrimp (SPF), which were then construed to be asymptomatic carriers (Khadijah et al., 2003). The amino acid sequences deduced from each of the three ORFs include an HLH motif and other more conserved regulatory regions. An ORF89 of 4436 bp long, transcribed by a promoter similar to that of the protein kinase and thymidinethymidylate kinase genes, was characterized and located in the shrimp cells'nuclei (Hossain et al., 2004). Looking for evidence of the role of ORF427 in the latency of WSSV, it was found that a new shrimp protein phosphatase (PPs) interacts with ORF427, suggesting that PPs play an important role in the latency of the virion (Lu and Kwang, 2004).

A sequester protein function has been observed in the WSSV249 protein, and there is evidence of four more genes containing a RING-H2 finger motif: WSSV199, WSSV222, WSSC249 and WSSV403 (Wang et al., 2005). The RING finger family has two subclasses: RING-HC and RING-H2, where a His residue replaces Cys4 in the motif that binds two zinc ions. This motif is characterized by a highly conserved spacing that binds two zinc ions in a cross

brace structure for conformational stability (Borden et al., 1995) and is present in poxvirus (Upton et al., 1994). Mutations of zinc residues in WSSV249 do not make conjugates with shrimp ubiquitin. In addition, the RING-H2 domain is capable of interacting with seven ubiquitination proteins from *Litopenaeus vannamei*, exhibiting a low degree of ubiquitin-conjugating enzyme (E2) specificity (Wang et al., 2005). In situ hybridization shows that WSSV249 and at least PvUbc display similar expression patterns. This suggests that the RING-H2 motif of WSSV249 may function as a sequestering ligase of PvUbc from the first hours of infection. More regulating roles of this motif might include RNA processing, apoptosis and viral replication (Fang et al., 2000).

1.1.3.1.3. Functional or physiological genes: which are involved in virus proliferation and life cycle functions such as replication and phosphorylation of host proteins, and nuclease activity.

These genes include the non-structural genes which encode proteins for enzymes involved in nucleic acid metabolism and DNA replication such as DNA polymerase – *dnapol* (Chen et al., 2002a), a small and a large subunit of ribonucleotide reductase - *rr* (Tsai et al., 2000a), a chimeric thymidine kinase – thymidylate kinase – *tk-tmk* (Tsai et al., 2000b), two protein kinase - *pk* (van Hulten and Vlak, 2001; van Hulten et al., 2001b; Yang et al., 2001), a dUTPase (Liu and Yang, 2005), a thymidylate synthase (Li et al., 2004a) and a non-specific nuclease (Witteveldt et al., 2001; Li et al., 2005b). Other proteins with a putative function include a collagen-like protein (Li et al., 2004b), flagellin, a chitinase, a pupal cuticle-like protein, a cell surface flocculin, a kunitz-like proteinase inhibitor, a class 1 cytokine receptor, a sno-like peptide, and a chimeric anti-apoptotic protein (van Hulten et al., 2001b; Yang et al., 2001; Marks, 2005). Genes for VP9 involved in transcriptional regulation (Liu et al., 2006a; 2006b), virus proliferation (WSV021) (Zhu et al., 2007) and/or

regulation of DNA replication (WSV477) (Han et al., 2007) have also been identified.

Transcriptional analysis of genes coding for proteins required in DNA replication and nucleotide metabolism are synthesized early during virus replication. Early transcribed WSSV genes in general have a TATA box 20–30 nucleotides upstream of the transcription initiation site (TIS) (A/C) TCANT which is also crucial for transcription. The distance between TIS and translational start site varies among these genes and is found to have some regulatory role. The cis-acting elements located upstream of TATA box also play roles in transcription regulation of some WSSV early genes. The putative binding sites for HSF, AP1, and HB transcription factors in the promoters of DNA polymerase, ribonucleotide reductase large subunit, and ribonucleotide reductase small subunit, respectively, appear to regulate basal transcription of these genes to some extent because deletions of these sequences moderately reduced their promoter activities (Chen et al., 2002b; Liu et al., 2005; Marks, 2005; Borirak, et al., 2009). By transcriptomic (WSSV-infected EST database and WSSV DNA microarray), proteomic (2D electrophoresis), X-ray diffraction, Far Western assays and indirect immunofluorescence approaches, ICP11 is identified as the most highly expressed WSSVgene/protein suggesting its importance to WSSV infection. It is found that ICP11 acts as a DNA mimic that prevents DNA from binding to histone proteins and, thus, disrupts nucleosome assembly (Wang et al., 2007a; 2007b; 2008a). WSSV has been found to contain an internal ribosome entry site (IRES) element for the internal initiation of mRNA translation. WSSV IRES efficiently co-expressed two proteins, one being a selectable marker, and the other the gene of interest (GFP protein and a glutathione-s-transferase) arranged in a dicistronic mRNA in vitro (Han and Zhang, 2006).

wsv112 gene or gene *wdut*, which encodes a dUTP pyrophosphatase (dUTPase) from WSSV-CN plays an important role during infection and replication, as it maintains low cellular dUTP:dTTP ratios, preventing the misincorporation of uracil into chromosomal DNA (Liu and Yang, 2005). The *wsv191* gene, which has a non-specific nuclease motif encoded inside, has been described as encoding a non-specific nuclease, whose product is homologous to non-specific nucleases of other organisms (van Hulten and Vlak, 2001). This gene (*wsv191*) was expressed in *Escherichia coli* mainly as a fusion protein, and the non-specific WSV191nuclease was detected using Western blotting, purified and able to hydrolyse DNA and RNA in vitro (Li et al., 2005a). ORF390 was identified as a novel anti-apoptotic gene. This ORF displays two putative caspase-cleavage sites, LLVETDGPS and VKLEHDGSK, and a caspase3 cleavage site EEDEVDGVP (Wang et al., 2004).

1.1.3.1.4. Structural genes: which encode the envelope and the nucleocapsid or tegument proteins.

The structural genes are highly expressed in natural host cells during white spot syndrome virus infection and hence include the late genes. It is likely that the promoters of the structural genes contain functional cis-acting elements as these gene products are the major structural components of WSSV, and are highly abundant during late stages (Marks et al., 2003; 2005) of infection. It is very likely that expression of the structural genes is enabled by virus-induced transcription machineries (Marks et al., 2006). For proper late gene expression, the degenerate TIS motif (A/TNAC/G) located 25 nucleotides downstream of an A/T rich region (Tsai et al., 2004; Marks, 2005) often plays a prominent role in the recognition by a virus-coded RNA polymerase (Davison and Moss, 1989; Morris and Miller, 1994; Garcia-Escudero and Viñuela, 2000; Weir, 2001; Kim et al., 2002).

Many of the structural genes encoding the envelope proteins – *vp28*, *vp19*, *vp73*, vp75, vp281, *vp18*, *vp76*, *vp31*, *vp110*, *vp36A*, *vp36B*, *vp31* (van Hulten et al., 2000a, 2000c, 2001b; Huang et al., 2005; Li et al., 2005b; Liang et al., 2005; Marks et al., 2006; Li et al., 2006a, 2006b); nucleocapsid proteins – *vp664*, *vp24*, *vp15*, *vp35* (Chen et al., 2002b; Leu et al., 2005; Xie and Yang, 2006; Li et al., 2009) and tegument protein – *vp26* (van Hulten et al., 2002; Tsai et al., 2006; Wan et al., 2008) have been studied.

RT-PCRs and/or Northern Blots of viral time courses confirmed that the 8 major structural virion genes – *vp28*, *vp19*, *vp73*, *vp75*, *vp664*, *vp24*, *vp26* and *vp15* were expressed in a late stage during infection (Marks et al., 2003; Marks et al., 2006; Leu et al., 2005). The analysis by mapping the TISs of these lacks a consensus TATA box. When the upstream sequences of all major structural protein genes are aligned by maximizing the identities around the transcriptional start sites, the TISs are present within or very near the nucleotide sequence ATNAC. The transcripts start 20–25 nucleotides downstream of an A/T rich region, which has an average A/T content of 79% compared to 61% of the 200 nt upstream regions of the 8 genes. *Vp15* and *vp19* contain a consensus TATA box, of which only the TATA box of *vp15* is at a functional distance of the TIS (Marks et al., 2003). The length of the TIS to the translational start codon is different for the various genes, ranging from 30 to 220 nt (nucleotide).

In addition to the 8 major structural proteins, the protein profile of WSSV particles shows a range of about 50 minor virion proteins (Li et al., 2007). Most of these have not been studied in detail. However, the corresponding messengers are supposed to be late, although 13 of them are clustered in the early class during microarray analysis (Marks et al., 2005). Remarkably, 36% of the minor virion protein genes contain a consensus TATA box within 300 nt of the translational start codon. This is in line with the MEME

analysis, which also suggested that the TATA box might be involved in late transcription (Marks et al., 2006).

1.1.4. Genetic variability of WSSV

OIE (2009) has reported that various WSSV isolates with small genetic polymorphisms have been identified (variants). It should be realized, however, that as Nimaviridae is a newly recognised family, the species concept will be subject to change after existing and new isolates have been studied in more detail. In silico restriction analysis with the enzyme KpnI predicts 27 fragments for the Chinese and Taiwanese isolates and 25 for the Thai isolate. Nine fragments of 0.3, 0.5, 0.7, 4.2, 4.7, 5.3, 5.4, 8.3 and 10.8 kbp are identical in size for all three isolates. Two fragments of approximate sizes of 9 and 20 kbp, respectively, are missing in the Thai isolate. The remaining 14–16 fragments vary in size from 1.2 to 18 kbp between the isolates.

The three sequences have an overall identity of 99.32% and five major differences among them: (i) a large deletion of about 13.2 kb in WSSV-TH and about 1.2 kb in WSSV-CN genome relative to WSSV-TW, (ii) a variable region prone to recombination, (iii) a transposase sequence present only in WSSV-TW, (iv) variation in the number of repeat units within homologous repeats (hrs) in the noncoding region and direct repeats in coding region and (v) single nucleotide mutations, including deletion, insertion or single nucleotide polymorphisms (SNPs) (Marks et al., 2004; Dieu et al., 2004; Shekar et al., 2005; Pradeep et al., 2008). Based on analysis of the two polymorphic loci, ORF 14/15 and ORF 23/24 that are prone to recombination and deletion events, Marks et al. (2005) suggested that the putative ancestral type for WSSV (WSSV-TH-96-II) originated in Thailand with the largest genome size (~312 kb) so far reported.

Experimental restriction analysis with HindIII in several WSSV isolates also found differences in restriction fragment length polymorphism (RFLP) between a Chinese isolate (*F. chinensis*), two isolates from Indonesia (*P. monodon*) and one from the USA (*Farfantepenaeus setiferus*). The latter two isolates were more similar to each other (Nadala and Loh, 1998). Other WSSV isolates from China (*F. chinensis*), India (*P. monodon*), Thailand (*P. monodon* and *L. vannamei*) and the USA (crayfish, *Orconectes punctimanus* from Washington, and *L. vannamei* from South Carolina and Texas) were compared by dot blot hybridization using a DNA probe from a Taiwanese isolate. With this method, negative results or a very faint signal were found in some samples from India, Thailand and Texas. This finding suggests important differences between these isolates. Further RFLP analysis of PCR products from 10 different primer sets showed that the Texas isolate was very different from the others (Lo et al., 1999). Different regions of the WSSV genome display important sequence variations which can be used to establish the origin of a WSSV isolate and its spread within a certain area (Dieu et al., 2004; Hoa et al., 2005) and also to differentiate isolates in the field (Wongteerasupaya et al., 2003; Marks, 2005). Such variability may also induce false negative results when using certain PCR primers (Claydon et al., 2004; Kiatpathomchai, et al., 2005). An unstable region of 9.6 kbp of the Chinese WSSV genome appears to undergo spontaneous deletions of different sizes depending on the host species. This observation has led to the suggestion that such deletions may play an important function in WSSV virulence (Lan et al., 2002).

Pradeep et al. (2008), characterized 81 WSSV isolates from India by sequencing polymerase chain reaction (PCR) amplicons of two polymorphic loci, ORF 14/15 and ORF 23/24 of WSSV. The Indian strains carried a 10,970 bp deletion in the ORF 23/24 region relative to WSSV-TW and WSSV-TH-96-II. Analysis of the ORF 14/15 regions revealed two novel strains of WSSV with unique sequences which could have evolved by recombination. None of the

WSSV isolates had a transposase sequence (13 kb deletion region) or VP35 gene as reported for Taiwan isolates. The Indian strains were closely related to Thailand strains suggesting movement of a putative ancestor from Thailand to other parts of the world including India.

1.1.5. Virulence variability and Antigenic variability in response to Genetic variability

Of the three isolates sequenced, WSSV-TH (first to be sequenced) is identified to be the most virulent, with 50% mortality reported in *Penaeus monodon* within 2 days (Marks et al., 2005). Differences in virulence of six WSSV isolates were found in post-larvae of *L. vannamei* and juveniles of *F. duorarum* inoculated per os. Virulence was determined as the time required to induce 100% mortality in *L. vannamei*. The Texas isolate was the most virulent while the Washington isolate (from crayfish) was the least virulent. The shrimp *F. duorarum* is known to be more resistant to WSSV infection. In this species, cumulative mortality was 60% with the Texas isolate and 35% with the WSSV isolate from crayfish (Wang et al., 1999a). Another study showed that differences in virulence and competitive fitness may be dependent on the genome size. A putative ancestral WSSV isolate (WSSV-TH-96-II) with the largest genome size recorded (312 kbp), showed a lower virulence [median lethal time (LT50) = 14 days] and competitive fitness compared with another WSSV isolate (WSSV-TH) with a smaller genome size (292 kbp) (LT50 = 3.5 days). This study indicated that WSSV isolates with a smaller genome size may represent an advantage for virus replication (Marks, 2005).

The protein profiles of the six WSSV isolates described in Lo et al. (1999) and isolates from India and Korea were very similar as all of them displayed at least three major structural proteins (VP28, VP24 and VP19). An additional band corresponding to VP15 was found in four isolates. The sequence of the amino-terminal portion of these proteins was identical between isolates

(Wang et al., 2000; Rajendran et al., 2004). Several WSSV isolates from the USA (*F. setiferus* and *L. vannamei*), Panama, China (*F. chinensis* and *M. japonicus*), Indonesia (*P. monodon*), Japan (*M. japonicus*), Thailand, Malaysia, Taiwan or different isolates from India were shown to have low antigenic variability using polyclonal or monoclonal antibodies (from whole WSSV virions or raised against full or truncated recombinants of VP28) in different immunoassays such as immunodot assays (Nadala and Loh, 2000; You et al., 2002), Western blot (Shih et al., 2001; Yoganandhan et al., 2004), indirect immunofluorescence (IIF) (Poulos et al., 2001), immunohistochemistry (IHC) (Anil et al., 2002) or enzyme-linked immunosorbent assay (Zhang et al., 2001).

1.1.6. Proteome of WSSV

With the completion of the WSSV genomic sequence, attention has been focused on the functional analysis of the encoded proteins, particularly the structural proteins as these are the first molecules to interact with host and, therefore, play critical roles in cell targeting as well as in triggering the host defenses.

The WSSV proteome has been unraveled by using the techniques such as gradient SDS-PAGE, 2D SDS-PAGE, coupled with western blotting, trypsin digestion and/or protein N-terminal sequencing, iTRAQ (isobaric tags for relative and absolute quantification), the application of mass spectrometry such as matrix assisted laser desorption/ionization time-of-flight (MALDITOF) or nano-electrospray ionization quadrupole time of flight (ESI Q-TOF) or liquid chromatography–nano-electrospray ionization tandem mass spectrometry (LC-nanoESI-MS/MSLC-ESI) using a Q-TOF mass spectrometer followed by database searches of sequenced genomes (Sahul-Hameed et al., 1998; van Hulten et al., 2000a, 2000c; Huang et al., 2002a; Tsai et al., 2004; Wang et al.,2007a, Wang et al., 2007b). In a recent study, in order to achieve a better understanding of the structural proteome of WSSV, shotgun proteomics using

offline coupling of LC system with MALDI TOF/TOF MS/MS as a complementary and comprehensive approach to investigate the WSSV proteome was done. The resulting data from shotgun proteomics has identified 45 viral proteins, 13 of which are reported for the first time, the remains were identified in the previous studies (Li et al., 2007). Therefore, the overall number of viral proteins that have been identified are 59.

1.1.6.1. WSSV Proteins

A comprehensive determination of the localization of structural proteins in the virion is being done by techniques such as immunogold electron microscopy (IEM), western blot analysis and by other proteomics approach using offline coupling LC system with MALDI-TOF/TOF MS/MS (Chen et al., 2007a). In total, through different proteomic methods, 35 proteins are currently identified as envelope proteins (including tegument proteins) and 9 as nucleocapsid proteins (Tan and Shi, 2008).

More than 50 structural proteins and one non structural protein VP9 (Liu et al., 2006a) have been detected in WSSV up till now. They were named according to the estimated molecular weights of the protein bands in SDS-PAGE or the number of amino acids. Proteins located in the envelope are: VP12B, VP19, VP22, VP24, VP28, VP31, VP36B, VP37, VP38A, VP39B, VP41, VP41A, VP41B, VP51A, VP51B, VP51C, VP52A, VP52B, VP53, VP53A, VP68, VP76, VP110, VP124, VP150, VP187, VP180, VP281, VP292, VP466 (van Hulten et al., 2000b; van Hulten et al., 2001b; Huang et al., 2002a; Huang et al., 2002b; van Hulten et al., 2002; Zhang et al., 2002; Tsai et al., 2004; Yi et al., 2004; Wu et al., 2005; Zhu et al., 2005; Liang et al., 2005; Li et al., 2005b, 2006a, 2006b; Tsai et al., 2006; Xie and Yang, 2006; Wu and Yang, 2006; Zhu et al., 2006), in the nuclocapsid: VP15, VP35, VP51C, VP60B, VP388, VP664 (van Hulten et al., 2002; Tsai et al., 2004; Leu et al., 2005; Witteveldt et al., 2005; Tsai et al., 2006; Xiao et al., 2006) and in the tegument:

VP26, VP36A, VP39A, VP95 (Tsai et al., 2006). Locations of other proteins are not known. The functions of most of these proteins have not been fully elucidated.

Envelope proteins play vital roles in initiating a virus infection, including binding to receptors or penetrating into host cells by membrane fusion. VP28 is the most abundant envelope protein located on the surface of the virus particle and is supposed to play a key role in WSSV binding to shrimp cells as an attachment protein facilitating virus enter the cytoplasm (van Hulten et al., 2000a; Yi et al., 2004). VP28 is involved in attachment and penetration into cells (Yi et al., 2004) and systemic infection (van Hulten et al., 2001b; Wu et al., 2005). It has been reported that VP28 can bind to shrimp cells in low-pH environment and interact with host cells (Sritunyalucksana et al., 2006). Using far-western and coimmunoprecipitation experiment, Xie and Yang (2005) reported that VP28 interact with both VP26 and VP24 by forming a complex. Recently, WSV010 has been identified as a novel envelope protein and has interaction with a major viral structural protein VP24 (Chen et al., 2007a). Thus, VP24 may also be acting as a linker protein for VP28, VP26 and VP24 to form a complex, which plays an important role in viral morphogenesis and infection. VP664, a remarkable large protein of around 664 kDa, was thought to be the major core protein responsible for the striated appearance of the nucleocapsids (Leu et al., 2005) and is quite evenly distributed at intervals on the outer surface of the nucleocapsid (Tsai et al., 2004). Moreover, VP664 molecules evidently extend from the nucleocapsid to the outside surface of the tegument, which may increase the flexibility of the nucleocapsid and allows it to assume its olive-like shape in the mature virion (Tsai et al., 2006). VP15, a highly basic protein with no hydrophobic regions, is a histone-like, double-stranded DNA-binding protein that tends to binds double-stranded DNA with a clear preference to supercoiled DNA, suggesting that VP15 is involved in packing the viral genome within the nucleocapsid (Witteveldt et al., 2005). ICP11 is the most highly expressed

WSSV non-structural protein in WSSV infection. This protein acts as a DNA mimic that prevents DNA from binding to histone proteins and thus, disrupts nucleosome assembly (Wang et al., 2008a).

Neutralization assays suggested that envelope proteins VP24, VP28, VP31, VP36B, VP68, VP76, VP281 and VP466 are involved in early stages of WSSV replication (van Hulten et al., 2001b; Huang et al., 2005; Li et al., 2005b; Wu et al., 2005; Li et al., 2006c; Li et al., 2006b; Xie and Yang, 2006).

1.1.7. Survival (outside the host) and Stability (effective inactivation methods) of WSSV

The agent is viable for at least 30 days at 30°C in seawater under laboratory conditions (Momoyama et al., 1998); and is viable in ponds for at least 3–4 days (Nakano et al., 1998). The agent is inactivated in <120 minutes at 50°C and <1 minute at 60°C (Nakano et al., 1998). WSSV is inactivated by various physical and chemical treatments including heat treatment at 55°C for 90 min, 70°C for 10 min, desiccation in filter paper within 3 h at 26°C, very acidic pH 1 for 10 min, pH 3 for 1 h, very alkaline pH 12 for 10 min, UV irradiation at 9 x 10^5 μW s/cm^2 for 60 min, different concentrations of disinfectants - ozone 0.5 and 0.8 μg/ml, formalin 200 parts per million (ppm), 25% sodium chloride within 24 h, and chloroform within 15 min (Chang et al., 1998; Nakano et al., 1998; Balasubramanian et al., 2006). The effective concentrations of sodium hypochlorite, povidone iodine, benzalkonium chloride were between 75 - 200 ppm (Chang et al., 1998a; Balasubramanian et al., 2006).

1.1.8. Morphogenesis

In-vitro studies with primary cell culture and in-vivo studies with postlarvae (PL) show that the replication cycle is approximately 20 hours at 25°C.

The stages of WSSV morphogenesis have been characterized and are directly related to the development of cellular lesions (Durand et al. 1997; Wang et al., 1999b; Tsai et al., 2006; Escobedo-Bonilla et al., 2008).

Stage 1: The early stage of cell infection:. Infected cells show slightly hypertrophied nuclei. A viral nucleosome appears before the formation of viral particles. It is composed of viral proteins organized in fibrillar fragments. In the cytoplasm, the endoplasmatic reticulum (ER) becomes enlarged with abundant free ribosomes.

Stage 2: In the nucleus, the fibrillar material induces the formation of circular membranes that are soon filled with viral core material starting viral assembly. At this stage, Cowdry-A type inclusions appear as a translucent zone between the virogenic stroma and the very electron-dense marginated chromatin. The nuclei become hypertrophied and rounded.

Stage 3: In the nucleus, the nucleocapsids appear with low electron density and gradually grow from one end towards the other, still open. The central intranuclear inclusion appears smaller than in cells in stage 2 and is more electron dense because of the presence of abundant viral particles. When the marginated chromatin disappears, the nuclear membrane is disrupted and the marginal transparent zone is fused with the lucent cytoplasm. Most organelles are abnormal, disintegrated or forming membranous structures.

Stage 4: In the nucleus, the nucleocapsid is completed with 12–14 rings of globular protein units arranged in a stacked series. Each nucleocapsid has one round and one square end. The nucleocapsid becomes completely enclosed by the envelope.

Stage 5: A late stage of viral morphogenesis. The viral particles become ovoid in shape and a long tail-like projection derived from the envelope is

observed. The inner material of the tail is separated from the nucleocapsid. Afterwards, the nucleocapsids become shorter, thicker and more electron - dense because of the packing of the viral DNA-VP15 complex.

Stage 6: The final phase of morphogenesis. The mature virions are elliptical with complete smooth envelopes enclosing an electron-dense nucleocapsid and with a tail-like projection at the last enclosed end. Sometimes assembly of nucleocapsids occurs completely separated from the envelopes and later they are wrapped by the envelopes. At this final stage, infected cells are severely damaged and disrupted. Void spaces are observed in tissues as cells disintegrate.

1.1.9. Host range

White spot syndrome virus has an extremely wide host range. At least 18 cultured and/or wild penaeid shrimp (Wongteerasupaya et al., 1996; Durand et al., 1997; Lu et al., 1997; Chou et al., 1998; Lightner et al., 1998; Park et al., 1998), eight caridean species (Sahul-Hameed et al., 2000; Shi et al., 2000; Pramod-Kiran et al., 2002), seven species of lobster (Chang et al., 1998b; Rajendran et al., 1999), seven species of crayfish (Wang et al., 1998; Corbel et al., 2001; Jiravanichpaisal et al., 2001; Edgerton, 2004; Jiravanichpaisal et al., 2004), 38 crab species (Lo et al., 1996a; Kanchanaphum et al., 1998; Kou et al., 1998; Sahul-Hameed et al., 2001; Sahul-Hameed et al., 2003), six non-decapod crustacean species (Supamattaya et al., 1998; Otta et al., 1999; Hossain et al., 2001), members of the phyla Chaetognata and Rotifera (Yan et al., 2004; Ramı´rez-Douriet et al., 2005; Yan et al., 2007), polychaete worms (Supak et al., 2005; Vijayan et al., 2005) and some aquatic insect larvae (Lo et al., 1996b; Flegel, 1997; Ramı´rez-Douriet et al., 2005) have been found to be WSSV-positive by PCR. Although many of these species have been confirmed to support WSSV replication under experimental conditions, some other species collected from the wild have only been found WSSV positive by PCR. This

indicates that many such species are not necessarily the natural hosts of WSSV, but may only be vectors. Such vectors include rotifers (Yan et al., 2004), marine molluscs, polychaete worms (Vijayan et al., 2005) and non-decapodal crustaceans including *Artemia salina* (Chang et al., 2002) and the copepods, as well as non-crustacean aquatic arthropods such as sea slaters (Isopoda) and Euphydradae insect larvae. All these species can accumulate high concentrations of viable WSSV, although there is no evidence of virus replication (Lo and Kou, 1998).

1.1.10. Pathogenesis

The route of WSSV entry and development mechanism of Whit Spot Disease has recently been shown by Escobedo-Bonilla et al. (2008). Apparently, shrimp becomes susceptible to infection from PL 6 (Venegas et al., 1999), PL 10 (Flegel, 2007) or PL 30 onwards (Pérez et al., 2005). The different findings in these studies could be due to shrimp species, inoculation procedure, infectious titer used, virulence of the WSSV isolates used or detection limit. Susceptibility differences for WSSV between the life stages of a host and between the host species were indicated in earlier studies (Momoyama et al., 1999; Wang et al., 1999a).

Experimental methods of WSSV inoculation that simulate natural routes of virus entry developed are: (i) waterborne, by immersing animals in water containing WSSV cell-free lysates (Chou et al., 1998; Supamattaya et al., 1998) and (ii) oral administration of WSSV-infected tissue for a single time or once daily for up to 7 days (Lightner et al., 1998; Wang et al., 1999b; Rajan et al., 2000). The latter route is considered the most important in natural and culture conditions (Chou et al., 1998; Wu et al., 2001; Lotz and Soto 2002; Pramod-Kiran et al., 2002).

The portals of WSSV entry into shrimp have not yet been clearly defined. The major targets of WSSV infection are tissues of ectodermal and mesodermal embryonic origin, especially the cuticular epithelium and subcuticular connective tissues (Momoyama et al., 1994; Wongteerasupaya et al., 1995). According to experimental data on feeding shrimp with WSSV-infected tissues, the primary sites of WSSV replication in early juvenile *P. monodon* are the subcuticular epithelial cells of the stomach and cells in the gills, in the integument and in connective tissue of the hepatopancreas as determined by in situ hybridization (ISH) (Chang et al., 1996). Another study on *M. japonicus* indicated that epithelial cells in the midgut trunk may be a transient site of WSSV replication which would allow the virus to cross the underlying basal lamina (Di Leonardo et al., 2005). In *P. monodon*, a WSSV challenge by immersion showed that haemocytes migrating to gills and midgut were WSSV-negative at late stages of infection [48–72 h post-inoculation (hpi)]. Many WSSV-positive cells were found in gills and only a few in midgut epithelium. Electron microscopy showed that epithelial cells in the midgut were VP28-positive in supranuclear vacuoles early during infection (8 hpi), suggesting lysis of WSSV particles. VP28-positive nuclei were never seen in the epithelial cells of the midgut (Arts et al., 2007a).

The infectivity titres of a WSSV stock solution was determined by challenge through oral route (Escobedo-Bonilla et al., 2005) by way of a standardized oral inoculation procedure that delivered an exact amount of virus titre to all inoculated shrimps (Escobedo-Bonilla et al., 2006). With this standardized inoculation technique, the primary sites of WSSV replication was determined with immunohistochemistry (IHC) as the epithelial cells in the foregut, cells in the gills (only with a high dose, 10,000 SID50 - shrimp infectious dose with 50% endpoint), and cells in the antennal gland (Escobedo-Bonilla et al., 2007).

The mechanism of viral spread from the primary replication sites to other target organs has been controversial. Some studies have indicated that WSSV infects haemocytes in crayfish and travels throughout the body in these cells to reach distant target organs (Wang et al., 2002; Di Leonardo et al., 2005). Other studies have shown by in situ hybridization (ISH), IHC and indirect immunofluorescence (IIF) that circulating haemocytes in freshwater prawns and shrimp are refractory to WSSV infection (van de Braak et al., 2002a; Shi et al., 2005; Escobedo-Bonilla et al., 2007), thus indicating that WSSV might reach other target organs through haemolymph circulation in a cell-free form (Escobedo-Bonilla et al., 2007). It is possible that these mechanisms of spread may be host species-dependent.

White spot syndrome virus targets cells of organs of ectodermal and mesodermal origin, including those of the epidermis, gills, foregut, hindgut (Wongteerasupaya et al., 1995; Chang et al., 1996), antennal gland, lymphoid organ (Durand et al., 1996; Chang et al., 1998b), muscle, eye-stalk, heart (Kou et al., 1998), gonads (Lo et al., 1997), haematopoietic cells and cells associated with the nervous system (Rajendran et al., 1999; Wang et al., 1999b). Epithelial cells of organs of endodermal origin such as the hepatopancreas, anterior and posterior midgut caeca and midgut trunk are refractory to WSSV infection (Sahul- Hameed et al., 1998). In the late stages of infection, the epithelia of the stomach, gills and integument may become severely damaged (Chang et al., 1996; Wang et al., 1999b). This may cause multiple organ dysfunctions and probably leads to death. Although WSSV infects the underlying connective tissue in the shrimp hepatopancreas and midgut, the hepatopancreatic tubular epithelial cells of these two organs are of endodermal origin, and they do not become infected.

A number of techniques such as two-dimensional gel electrophoresis, expressed sequence tags (Wang et al., 2007a), microarray chips (Wang et al.,

2006a), suppression subtractive hybridization (Zhao et al., 2007) and differential hybridization (He et al., 2005) are useful tools to better understand the host response to mechanisms of WSSV virulence and pathogenesis. These methods measure the altered abundance of host and/or viral mRNA and/or protein expression levels after WSSV infection. Molecules with important biological functions that showed variations in response to WSSV infection included those involved in energy production, nucleic acid synthesis, calcium homeostasis and/or cellular signalling.

1.1.11. Disease Pattern and Clinical Signs

Infection of WSSV sometimes causes disease and sometimes not (Tsai et al., 1999), depending on factors as yet poorly understood but related to species tolerance and environmental triggers. With an appropriate infectious dose to allow sufficient time before mortality, animals susceptible to disease show large numbers of virions circulating in the haemolymph (Lo et al., 1997), but this may also occur for tolerant species that show no mortality. Thus, high viral loads *per se* do not cause disease or mortality for all susceptible species.

White spot syndrome virus infections of penaeid shrimp are characterized by a rapid mortality accompanied by gross signs in moribund shrimp. WSD is characterized by the display of white spots or patches of 0.5–3.0 mm in diameter embedded in the exoskeleton (Lo et al., 1996a; Kasornchandra et al., 1998; Wu et al., 2001). The exact mechanism of white spot formation is not known. It is possible that a WSSV infection may induce the dysfunction of the integument resulting in the accumulation of calcium salts within the cuticle and giving rise to white spots (Wang et al., 1999b). Other signs of disease include a reddish discolouration of the body and appendages because of the expansion of chromatophores (Lightner et al., 1998; Nadala et al., 1998), a reduction in feed uptake (Chou et al., 1995; Durand et al., 1996; Flegel, 1997),

preening and response to stimulus (Wongteerasupaya et al., 1995; Durand et al., 1997), loose cuticle (Lo et al., 1996b), swelling of branchiostegites because of accumulation of fluid (Otta et al., 1999), enlargement and yellowish discolouration of the hepatopancreas (Sahul-Hameed et al., 1998), and thinning and delayed coagulation of haemolymph (Wang et al., 2000; Kiatpathomchai et al., 2001). In the field, WSSV-infected shrimps gather near to the pond edge and display clinical signs 1 or 2 days before the first mortality occur (Kou et al., 1998). Cumulative mortality may reach 100% within 10 days after the onset of disease (Karunasagar et al., 1997; Lotz and Soto, 2002). In grow-out ponds, juvenile shrimp of all ages and sizes are susceptible to the disease but massive mortality usually occurs 1 or 2 months after stocking (Kasornchandra et al., 1998).

Histopathology reveals that in the early stages of WSSV infection, the inclusions are intranuclear and eosinophilic, showing a clear halo under the nuclear membrane called Cowdry type-A inclusions. However, shrimp with a well-developed WSSV infection show inclusion bodies without a halo and display a weakly basophilic colour (Lightner, 1996). The lymphoid organ of diseased shrimp may be distended, and haemolymph infiltration in the enlarged haemal sinuses and interstitial spaces may result in a hypertrophied yellowish hepatopancreas (Wang et al., 1995). In the late stages of infection, karyorrhexis and cellular disintegration may occur, leading to the formation of necrotic areas characterized by vacuolization (Karunasagar et al., 1997; Kasornchandra et al., 1998; Wang et al., 1999b).

1.1.12. Transmission

WSSV infection in shrimp arises from many sources; a classic mechanism of virus transfer between regions results from the movement of live shrimp or other hosts from infected to non-infected areas.

Infections by WSSV have been reported in many species of cultured penaeids including: *P. monodon*, *L. vannamei*, *L. stylirostris*, *Marsupenaeus japonicus*, *Fenneropenaeus chinensis*, *Macrobrachium rosenbergii* and *Procambarus clarkii* (Chang et al., 1998b; Lightner and Redman, 1998; Lo et al., 1999; Wang et al., 1999a; Flegel, 2001). Rapid transmission of WSSV on culture systems may occur from infected shrimp, through water and by way of cannibalism of moribund shrimp (Chang et al., 1996); however, the major source of infection for shrimp farms is from infected spawners and postlarvae. There are reports that show the relation between batches of infected postlarvae in a farm and the subsequent infection of a neighbour's pond and farm, leading to the general practice of batch-testing of postlarvae for WSSV using PCR before stocking. There are reports of a transovarial or vertical transmission of WSSV by infected gonads, oogonia and follicle cells in *P. monodon* ovarian tissues (Kou et al., 1997). However, other studies could not find any WSSV-infected mature eggs, suggesting that infected egg cells were killed by the virus before maturation (Lo et al., 1997).

Potential sources for WSSV transmission include human activities; seabirds or other animal's (insects, fish) migration; infected frozen food products; infected pond sediments; contaminated aquaculture tools or instruments; and untreated infected shrimp by-products (liquid and solidwastes) from processing plants (Lightner et al., 1997a, 1997b; Kanchanaphum et al., 1998; Supamataya et al., 1998; Sahul-Hameed et al., 2002).

Birds, arthropods or other organisms exposed to contaminated effluent discharge from shrimp packing plants may transfer the pathogen to areas not contaminated with the virus. The introduction of WSSV-infected organisms to the areas where the pathogen was previously unknown may be possible through ballast water from cargo ships (Flegel and Fegan, 2002). Polymerase chain reaction results show that different arthropods, including copepods, and insects

can act as vectors of WSSV virus (Chou et al., 1996; Lo et al., 1996a; Flegel, 1997; Maeda et al., 1997; Sahul-Hameed et al., 2003). Furthermore, some of these arthropods, such as *Portunus pelagicus*, and *Acetes* sp., are common in shrimp culture areas and may transmit the WSSV (Supamattaya et al., 1998). Other vectors can enter shrimp ponds through pumped water, favouring management strategies that reduce water exchange rates or rely in closed cycles and recirculation (Sahul-Hameed et al., 2003).

1.1.13. Host-viral interaction

Field observations and results of laboratory experiments in the past decade indicate that interaction of shrimp and WSSV show specific tolerance to single or multiple viral infections without gross or histological signs of disease. The research results, with respect to shrimp and IHHNV, suggested that tolerance was due more to genetic change in the host than to change in the type of virus (Flegel, 2007). It is noted that it cannot be identified as 'latent' as analysis revealed that infectious virions are produced continuously. The tolerance also seemed to show specificity (Tsai et al., 1999; Withyachumnarnkul, 1999; Moss, 2002; Moss et al., 2005). On the information gathered from recent research, the viral accommodation concept (Flegel and Pasharawipas, 1998; Flegel, 2007) can apparently take place at virtually all life stages of shrimp and not just at larval stages. It has also been shown, contrary to early indications, that shrimp larval stages can be infected with WSSV (Yoganandhan et al., 2003a) although they do not show signs of disease. Indeed, several publications have shown that the viral infections are frequently transmitted vertically from persistently infected adults to their offspring that also develop persistent infections (Walker et al., 2001; Hsu et al., 1999; Cowley et al., 2002). It is possible that the persistent infections themselves provide the ''specific memory'' that is involved in reducing the severity of disease. Thus, the updated viral accommodation concept is stated as follows: ''crustaceans and other arthropods actively accommodate viral pathogens as persistent infections

that act as a kind of memory that functions to specifically reduce the severity of disease and to dampen viral triggered apoptosis'' (Flegel, 2007).

Observations of viral sequences in shrimp genome have been published where in WSSV-like sequences have also been reported from the genome of *P. monodon* from Australia (de la Vega, 2006). Flegel (2009) has hypothesized that shrimp uses the reverse transcriptase (RT) in their genome to recognize "foreign" mRNA of both RNA and DNA viruses and uses integrases (IN) to randomly insert short cDNA sequences into their genomes and by chance, some of these sequences result in production of immunospecific RNA (imRNA) capable of stimulating RNAi that suppresses viral propagation and the individuals with protective inserts would pass these on to the next generation.

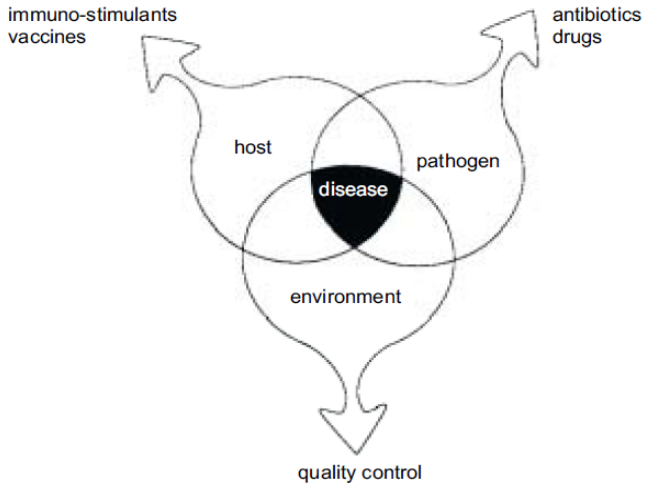
1.1.14. Antiviral response in shrimps

Viral components like genomic DNA and RNA or dsRNA generated in virally infected cells can be sensed by host pattern recognition receptors (PRRs) after which the appropriate antiviral response is triggered (Liu et al., 2009). Such antiviral responses have been reviewed by Liu et al. (2009). The higher antiviral activities of apoptosis (Wang et al., 2008b) and phagocytosis observed in *Marsupenaeus japonicus*, than that of prophenoloxidase (proPO) system, suggest that cellular responses play more important role in the immune defence of invertebrates against WSSV infection (Wang and Zhang, 2008). However, in the resistance shown by *P. japonicus* to WSSV challenge, apoptosis was not found to play an important role (Wu and Muroga, 2004). The proteins or genes involved in anti-WSSV response have been briefed by Liu et al. (2009) and explained in Chapter 4. WSSV immediate early gene (*ie1*) was shown to employ shrimp STAT as a transcription factor to enhance its expression resulting in its high promoter activity in the host cells (Liu et al., 2007a). Another study showed cytokine activation mediated antiviral response in which shrimp STAT was activated in response to WSSV infection and that WSSV does not disrupt JAK-

STAT (Janus kinase/signal transducer and activator of transcription) pathway but benefits from STAT activation in shrimp (Chen et al., 2008a). RNA interference (RNAi) has been proven to be an alternative and more specific approach for the antiviral mechanism in shrimps as this effect has been confirmed by injection of WSSV specific dsRNA/siRNA targeting WSSV proteins (Robalino et al., 2005; Kim et al., 2007; Xu et al., 2007; Liu et al., 2009).

1.2. Prophylaxis for White Spot Disease

Disease can be seen as the resultant of a complex interaction between host, pathogen and environment and hence disease control itself depends on a complex of three factors; diagnosis, treatment and preventive measures (Sindermann and Lightner, 1988). Prophylaxis refers to all the preventive steps adopted into the routine husbandry practices that are taken during a hatchery and farming operation to minimize the load of pathogens and to prevent the occurrence of diseases.



The classic three circles of host- pathogen- environment interactions as described for fish (Schnieszko, 1974) were modified to show how to minimize the risk of disease (van de Braak, 2002). The pathogens can be treated with antibiotics or other drugs, the environment can be improved by hygiene and the host response can be augmented by immuno-stimulation (Anderson, 1992).

1.2.1. Environmental Management and WSSV

Environmental management interventions in shrimp culture (e.g., proper pond preparation, keeping carriers out, reduced stocking densities, use of healthy seed, use of low exchange systems, not exchanging water during periods of outbreak, minimizing feed wastage, pond bottom management, etc.) have not prevented outbreaks, but seem to have helped reduce the impact of disease.

However, for a shrimp aquaculture venture to be profitable and disease free, the environmental management mainly include deep attention to the following: **pond bottom and water preparation** [sludge removal, ploughing on wet soil, water filtration using twin bag filters of 300µ mesh size, water depth of atleast 80cm. and water conditioning for 10-15 days before stocking]; **seed selection and stocking practices** [use of specific pathogen free (SPF) for biosecurity, uniform size and coloured PLs actively swimming against the water current, nested PCR negative PLs for WSSV, weak PL elimination before stocking using formalin(100ppm) stress for 15-20 min. in continuously aerated water, on-farm nursery rearing of PLs for 15-20 days, seed transportation time of less than 6 hrs. and stocking into green water depending on seasonal conditions] and **post-stocking/ grow out** [use of water reservoirs with 10-15 days aging, regular usage of agricultural lime, no harmful/banned chemicals, using of feed check trays to avoid local waste accumulation, regular removal of benthic algae, water exchanges only in critical periods, weekly checking of pond bottom mud for blackish organic waste accumulation, regular health and growth assessment using cast net, removal and safe disposal of sick and dead shrimps, emergency harvesting after proper decision making, no draining or abandoning of disease affected stocks].

It is important that the entry of disease into the pond system should be prevented and the disease effects be minimized by reducing stress to the shrimp. On-farm sampling programs to monitor health and performance of the shrimp

and detect the onset of disease should be carried out. These include visual inspection of the ponds (looking for signs of oxygen problems, dead or moribund shrimp, birds wading/ circling overhead, uneaten feed, phytoplankton crash, erratic swimming to the surface, water quality problems or disease); monitoring feed consumption on daily basis(virulent shrimp disease like WSSV, evoke abrupt and drastic reductions in feed consumption during the acute phase of the disease, usually due to loss of appetite in affected animals); sampling of growth and survival once a week (by randomly collecting 5-10 shrimp from 3-5 sampling stations per ha.) and monitoring disease(by visual observation, checking the gut fullness index or pink/reddish coloration of body, soft shell, white spots on cuticle, haemolymph coagulation time etc.). Appropriate check on water parameters are advised as parameters such as temperature and ammonia are reported to have a significant effects on the disease and mortality of WSSV infected animals (Vidal et al., 2001; Guan et al., 2003; Jiravanichpaisal et al., 2004; Jiang et al., 2004; Du et al., 2006).

Appropriate management measures with efforts on prevention, on better management practices and on maintaining healthy shrimp are to be effectively implemented in the environment for WSSV to be under control in shrimp farming.

1.2.2. Immunostimulants, AMPs, Antivirals & Vaccination in WSD management

1.2.2.1. Immunostimulants and WSSV

In cases where disease outbreaks are cyclic and can be predicted, immuno-stimulants may be used in anticipation of events to elevate the nonspecific defence mechanism, and thus prevent losses from diseases. An immuno-stimulant is a chemical, drug, stressor or action that enhances the defence mechanisms or immune response (Anderson, 1992), thus rendering the

animal more resistant to diseases. However, caution should be taken as a number of the potent immuno-stimulants may suppress or alter certain biological pathways if used inappropriately (van de Braak, 2002).

Diets containing microbial components such as β-1,3 -glucan (Song et al., 1997) from *Schizophyllum commune* (Chang et al., 1999; Chang et al., 2003), lipopolysaccharide from *Pantoea agglomerans* (Takahashi et al., 2000) or extracts of plants (*Cyanodon dactylon*, *Aeglemarmelos*, *Tinospora cordifolia*, *Picrorhiza kurooa*, *Eclipta alba*) (Citarasu et al., 2006) and marine isolate of *Candida sake* (Sajeevan et al., 2006) have been shown to improve the immunity and reduce the mortality of WSSV infected shrimp. The mechanism was attributed to an activation of innate immunity. The best results were obtained when immunostimulants were applied before exposure to the pathogen. Excess or indiscriminate use of immunostimulants may not be useful or even cause negative effects (Horowitz and Horowitz, 2001). Immuno-stimulation will certainly continue to play an important role in disease control in intensive shrimp culture. For immunostimulants to be included in the effective management of WSD, the timing, dosages and methods of administration as well as the side effects are needed to be investigated (Sakai, 1999).

1.2.2.2. Antimicrobial peptides and WSSV

Antimicrobial peptides (AMPs) have a broad range of antimicrobial activity against Gram-positive and Gram-negative bacteria, filamentous fungi and, in some cases, viruses and protozoa (Bachere, 2003). AMPs are part of the innate immunity of shrimp. In two studies, WSSV was incubated *in vitro* with a synthetic AMP 'mytilin' before inoculation in shrimp *Palaemon serratus*. Mortality of *P. serratus* was lowered by the pretreatment with AMPs (Dupay et al., 2004; Roch et al., 2008). Anti-lipopolysaccharide factor (ALF) is one of the putative antimicrobial peptides identified in penaeid shrimp (Somboonwiwat et al., 2005). de la Vega et al. (2008) reported that injection of dsRNA

corresponding to *Lv*ALF1 (*Litopenaeus vannamei* Anti-lipopolysaccharide factor 1) activated the sequence-independent innate anti-viral immune response giving increased protection from WSSV infection in *Litopenaeus vannamei*.

1.2.2.3. Antivirals and WSSV

An antiviral is a compound that suppresses the ability of a virus to replicate at any step of the replication cycle (attachment, entry, uncoating, transcription, translation, and assembly).

Natural extracts like fucoidan from marine algae *Sargassum polycystum* was incorporated to the diet and was fed to *P. monodon* juveniles for four days before and after challenge with WSSV. It was observed that shrimp receiving the highest amount of fucoidan in the diet (100, 200 and 400 mg/ kg body weight of shrimp) showed increased survival rate (Chotigeat et al., 2004). An ethanolic extract bis(2-methylheptyl)phthalate from *P. pinnata* leaves incorporated into shrimp diet (200 and 300 µg/g body weight of shrimp/ day) and tested for antiviral activity against WSSV infection in *P. monodon* was found to increase survival (40 to 80%) (Ramesthangam and Ramasamy, 2007). Increased survival of *P. monodon* was also observed after administration of a mixture of WSSV and extracts of *Aegle marmelos*, *Cynodon dactylon*, *Lantana camara*, *Mimosa charanita*, *Phyllanthus amarus* plants (Balasubramanian et al., 2007; 2008). The mechanisms of the antiviral activity of these plant extracts against WSSV are not known.

A phage display peptide was claimed to be effective against WSSV *in vivo* in crayfish and *in vitro* in primary culture of lymphoid organ of shrimp (Yi et al., 2003). Nucleic acid based therapeutics for viral infections in shrimp aquaculture has been reviewed by Shekhar and Lu (2009). Injection of double stranded RNA (dsRNAs) induced protection in *L. vannamei* depending on the WSSV inoculation dose (Robalino et al., 2004). The degree of protection

differed between dsRNAs targeted genes (Robalino et al., 2005). dsRNAs corresponding to viral proteins VP28, VP281, protein kinase gene of WSSV and green fluorescence protein (GFP) gene gave protection to *P. chinensis* (Kim et al., 2007). Injection of the short interfering RNAs (siRNA) VP15-siRNA or VP28-siRNA was found to be effective in *P. monodon* (Westenberg et al., 2005). Multiple injections of VP28-siRNA delayed and reduced mortality of WSSV infected *P. japonicus* due to the inhibition of the virus replication (Xu et al., 2007). Oral administration of pellet feed coated with inactivated bacteria containing overexpressed dsRNA of the WSSV VP28 gene and VP28dsRNA-chitosan complex nanoparticle-coated feed challenged with WSSV showed higher survival compared to control shrimp (Sarathi et al., 2008).

1.2.2.4. Vaccination and WSSV

In search for the practical strategies to overcome viral infection, recently, vaccination of shrimps is under trials. Defence stimulation in invertebrates is often called 'vaccination' too, but this 'vaccination' is not equivalent to vertebrate vaccination where lymphocytes and/or specific antibodies produced constitute an adaptive immune mechanism. Therefore, the term vaccination when applied to invertebrate immunity concept should be understood as a more complex mechanism than that was previously envisaged for shrimp immunity. The notion of shrimp vaccination is strongly supported by studies within the last decade in which a body of literature has been building that indicates that shrimp and other crustaceans can be vaccinated with either inactivated virus, protein "sub-unit" or DNA vaccines and thereby protecting from mortality induced by WSSV. Preventative health management of viral diseases in shrimp aquaculture would appear to be a real possibility, as evidenced by the increasing number of published experimental vaccine trials (Johnson et al., 2008). These reports suggest that contrary to the previous belief that invertebrates rely entirely on innate immune system, some aspects of specific immunity like, inducibility, appear to be present in some cases (Loker et al., 2004; Kurtz and Franz, 2003)

and a specific immune response and protection could be induced in shrimps (Rout et al., 2007).

Types of aquaculture shellfish vaccines for combating WSD include administration of heat inactivated WSSV (Namikoshi et al., 2004); formalin inactivated WSSV (Namikoshi et al., 2004; Singh et al., 2005; Melena et al., 2006); recombinant viral proteins of WSSV - VP19, VP28, VP26, VP292, VP466 (Witteveldt et al., 2004a, 2004b; Namikoshi et al., 2004; Vaseeharan et al., 2006; Ha et al., 2008) and DNA vaccine for WSSV (Rout et al., 2007; Rajeshkumar et al., 2008; Li et al., 2010).

The route of administration for vaccines for WSSV has been injection, immersion and oral. Majority of research on shrimp viral vaccination is centred around delivery by injection (Namikoshi et al., 2004; Witteveldt et al., 2004a; Vaseeharan et al., 2006; Rout et al., 2007; Ha et al., 2008). In these studies, vaccination was performed via intramuscular injection to ensure the application of a consistent amount of protein per shrimp. Even though this technique is far from practical under shrimp farming conditions, it is very suitable in determining the vaccinating potential of proteins. In contrast, clearly injection is not feasible for animals as small as marine shrimp. The immersion way of vaccination is simple but requires large quantities of vaccine and has been tried by Melena et al. (2006) for WSD. Clearly, any vaccination strategy must be designed with the key considerations of minimising immunomodulatory stresses before, during and postvaccination. The most practical means of vaccine administration is its incorporation into the food. This method is adequate for mass administration to animals of all sizes, does not interfere with the normal routine husbandry, and thus imposes no extra stress and not much extra labour. Oral vaccination of shrimp against WSD has been tried out using inactivated and recombinant WSSV proteins (Namikoshi et al., 2004; Witteveldt et al., 2004b, 2006; Singh et al., 2005). In general, limitations of the oral vaccination route are

the large amount of stimulants needed, thus increasing the cost and the uncertainty about the individual dosage. Furthermore, this method often results in low and inconsistent protection levels, which might be due to degradation of the antigens in the digestive tracts. The molecules may reach the immune system in various degrees of alteration, which depend on the base material, the encapsulation method and the animal. However, when oral vaccination is cost-effective, it would obviously be the ideal method in aquaculture. A number of studies have also indicated protective efficacy following oral administration of feed coated with killed bacteria expressing recombinant viral proteins (Witteveldt et al., 2004b). It would therefore seem feasible to transfer recombinant - killed bacteria into the gut by the same route. Enhancement of the defence system in the practice of shrimp culture is most feasible by oral administration.

1.2.2.4.1. Global scenario in WSSV Vaccine

Namikoshi et al. (2004) studied efficacy of vaccines made of inactivated WSSV with and without immunostimulants (β-1,3glucan or killed *Vibrio penaeicida*) and of recombinant proteins of WSSV (rVP26, rVP28) and tested these by intramuscular vaccination followed by intramuscular challenge of kuruma shrimp with WSSV. The shrimp vaccinated with formalin inactivated WSSV showed resistance to the virus on 10^{th} day post vaccination (dpv) but not on 30^{th} dpv. Heat inactivated WSSV did not induce a resistance in the shrimp even on 10^{th} dpv. Additional injections with glucan or *V.penaeicida* enhanced the efficacy of formalin inactivated WSSV vaccine; however the relative percentage survival (RPS) values did not exceed 60% even when shrimp were vaccinated three times at 10 day intervals. On the other hand, two injections with rVP26 or rVP28 induced a higher resistance with RPS values 60% and 95%, respectively in the shrimp on 30^{th} dpv. Their results indicated that VP26

and VP28 are 'protective antigens' which can evoke protection of shrimp by vaccination and is of assistance in elucidating the mechanism of 'quasi-immune response' or induced resistance in shrimps.

Witteveldt et al. (2004a) evaluated the potential to vaccinate *P.monodon* against WSSV using the WSSV envelop proteins VP19 and VP28. Shrimps were vaccinated by intramuscular injection of the purified WSSV proteins and challenged 2 and 25 days post-vaccination to assess the onset and duration of protection. VP19 vaccinated shrimp showed a significant better survival as compared to vaccinated control shrimp with a relative percentage survival (RPS) of 33% and 57% at 2 and 25 days post-vaccination, respectively. Groups vaccinated with VP28 and a mixture of VP19 and VP28 showed a significantly better survival when challenged 2 days after vaccination (RPS of 44% and 33%, respectively), but not after 25 days. This suggests that the shrimp immune system is able to specifically recognize and react to proteins and that vaccination of shrimp may be possible despite the absence of a true adaptive immune system. In studies conducted by Witteveldt et al. (2004b) *P.monodon* shrimp were fed food pellets coated with inactivated bacteria over expressing two WSSV envelope proteins, VP19 and VP28. Vaccination with VP28 showed a significant lower cumulative mortality compared to vaccination with bacteria expressing the empty vectors after challenge via, immersion (relative survival, 61%), while vaccination with VP19 provided no protection. To determine the onset and duration of protection, challenges were subsequently performed 3, 7 and 21 days post-vaccination. A significantly higher survival was observed both 3 and 7 days post-vaccination (relative survival, 64% and 77% respectively), but the protection was reduced to 21 days after the vaccination (relative survival, 29%). Hence, Witteveldt et al. (2004b) suggests that in contrary to current assumptions that invertebrates do not have a true adaptive immune system, a specific immune response and protection can be induced in *P.monodon.*

From the studies conducted by Singh et al. (2005) for an attempt to make a cocktail vaccine for WSSV, it has been found from laboratory studies that the administration of $0.025g^{-1}$ inactivated virus preparation (IVP) at the rate of 0.035g feed g^{-1} body weight day^{-1} for 7 days continuously, provided an acquired resistance to virus invasion in *Fennerpenaeus indicus* for 5^{th} day challenge post-vaccination schedule as evidenced from histopathological and negative nested PCR results. But, the 10^{th} day challenge gave another picture in which histological studies showed slightly enlarged eosinophilic nuclei and positive nested PCR for WSSV. The results suggested that *F. indicus* can be protected from WSD by simple oral administration of IVP.

Melena et al. (2006) submitted the larvae and post-larvae of *Penaeus vannamei* (Boone) to primary challenge with formalin-inactivated white spot syndrome virus (WSSV). Survival rate and viral load were evaluated after secondary per os challenge with WSSV at post-larval stage 45 (PL45). Only shrimp treated with inactivated WSSV at PL35 were alive (4.7%) at 10 days post-infection (p.i.). Moreover, at 9 days p.i. there was 100% mortality in control, while there was 94% mortality in shrimp treated with inactivated WSSV at PL35. Based on viral genome copy quantification by real-time PCR, surviving shrimp previously exposed to inactivated WSSV at PL35 contained few WSSV ($0–2 \times 10^3$ copies μg^{-1} of DNA). Consequently, pre-exposure to inactivated WSSV resulted in slower WSSV replication and delayed mortality. This evidence suggests a protection obtained by inactivated WSSV might result from non-specific antiviral immune response.

Vaccination trials show significant resistance in the shrimp vaccinated twice with recombinant VP292 (Vaseeharan et al., 2006). A recombinant protein vaccine has the advantage that animals can be vaccinated with a large amount of specific antigens. This mode of vaccination is convenient especially for shrimp viruses including WSSV because they have not been yet generated in cell lines

due to lack of a valid cell line for crustaceans. Vaseeharan et al. (2006) have been using *E. coli* transformed with VP292 for administration. Vaccination trial results show significant resistance in the shrimp injected twice with rVP292 (RPS: 52%) against WSSV by the 30th day post-initial vaccination. On the other hand, single vaccination of shrimp with rVP292 at a 20-day interval resulted in significantly lower RPS values (41%) compared with two times vaccination. This suggests that the generation of a more specific immune response (long-term protection) provided by injection of virus-specific proteins like VP292 is possible to prevent the entry of the virus. This immune response may also have long-term effects by enhancing the response to WSSV. VP292 is identified as one of the ideal antigens, which can evoke protection of shrimp by vaccination.

Rout et al. (2007) have explored the protective efficacy of DNA vaccination for WSD and tissue distribution of the immunised recombinant plasmid in black tiger shrimp (*Penaeus monodon*). Four recombinant constructs were generated by inserting four genes encoding the WSSV structural proteins VP15, VP28, VP35 and VP281 individually into DNA vaccine vector pVAX1. For vaccination experiments, shrimp were immunized with these DNA constructs and later challenged with WSSV. A significant level of protection was offered by the plasmids encoding VP28 or VP281 till 7 weeks whereas protein vaccination failed to protect vaccinated shrimp after 3 weeks of first immunization. In addition, the tissue distribution study revealed the persistence of immunized DNA at least upto 2 months in the injected shrimp muscle. Nevertheless, in this study, failure of nucleocapsid proteins (VP15 and VP35) to provide protective immunity, unlike envelope proteins, suggests that the protective mechanism operates by preventing the viral attachment to host and/or decreasing viral load by phagocytosis. This also raises the possibility that the mechanism behind protection involves recognition of virus particles prior to cell entry. In other words, it hints the absence of vaccine mediated intracellular protective mechanisms like synthesis of antiviral peptides preventing viral

assembly, at least by these two antigens. The results herein demonstrate for the first time that DNA vaccination could elicit protective response in shrimp against WSSV infections.

Two structural protein genes, VP19 and VP466, of white spot syndrome virus (WSSV) were cloned and expressed in Sf21 insect cells using a baculovirus expression system by Ha et al. (2008) for the development of injection and oral feeding vaccines against WSSV in *Penaeus chinensis*. The cumulative mortalities of the shrimps vaccinated by the injection of rVP19 and rVP466 at 15 days after the challenge with WSSV were 50.2% and 51.8%, respectively. For the vaccination by oral feeding of rVP19 and rVP466, the cumulative mortalities were 49.2% and 89.2%, respectively. This study showed that the shrimp defense system was able to recognize WSSV structural proteins, and thus the vaccination of shrimp against WSSV could be possible by the oral feeding of the protein vaccine, rVP19, and the injection of rVP19 and rVP466. This opens the way for the design of practical strategies to control WSSV.

The protective efficacy of oral delivery of a DNA construct containing the VP28 gene of WSSV encapsulated in chitosan nanoparticles was investigated in black tiger shrimp (*Penaeus monodon*) by Rajeshkumar et al. (2008). The results showed that significant survival was obtained in WSSV-challenged shrimp at 7, 15 and 30 days post-treatment (relative survival, 85%, 65% and 50%, respectively). This protection level is significantly higher when compared to previous reports of shrimp treated orally (inactivated bacteria coated with pellet feed) with VP28 KDa recombinant protein against WSSV (Witteveldt et al., 2004b). This study also observed a high level of phenol oxidase and super oxide dismutase activity in the vaccinated animals.

The protective effect of lpv28 (VP28 gene was cloned in the eukaryotic expression vector pVAX1, and the construct vector was named as lpv28) against WSSV was evaluated in *L. vannamei* by injecting lpv28 construct and later

challenging with WSSV was studied by Li et al. (2010). The result of these vaccination trials showed that the survival rate in shrimp vaccinated with lpv28 (a vector constructed with VP28 gene) was 52.5% when compared to control groups (100% mortality). The immunological parameters analyzed in the vaccinated and control groups showed that the vaccinated groups had high levels of lysozyme, alkaline phosphatase, and total superoxide dismutase when compared to the control group. Furthermore, protein expression analysis indicated that VP28 can be detected in gill, muscle and head soft tissue of the shrimps in the immunized group after 14^{th} day injection. Thus, the result indicated that DNA vaccination strategy has a potential utility against WSSV.

Protection against WSSV was observed following prior exposure of shrimp to formalin inactivated virus via intramuscular injection (Namikoshi et al., 2004) or oral feeding (Singh et al., 2005) which shows that protective immune responses are not reliant on the presence of viable virus. The protective response was significant at 10 days post-vaccination but not 30 days post-vaccination (Namikoshi et al., 2004). The timing of the protective response gives some indication that mechanisms involved in this response may differ from those involved in protection by exposure to live virus. With early indications that vaccination was indeed possible in shrimp, several groups investigated the efficacy of subunit vaccines (Namikoshi et al., 2004; Singh et al., 2005; Witteveldt et al., 2004a, 2004b; Jha et al., 2006). Several different viral proteins have been expressed in bacterial expression vectors, but each of them is reported to be associated with the envelope of the virus and thus expected to be exposed on the surface of the virus particle (Tang et al., 2007). There are examples from several different shrimp species and one crayfish species where such subunit vaccines have efficiently protected animals against WSSV disease. These subunit vaccines have been shown to protect whether the proteins were administered by injection, immersion or orally. In addition,

successful protection has been reported for both purified protein and protein delivered in inactivated expression bacteria.

The protection seen in the above studies appears to be dependent on the shrimp species and on the time of application of the competing agents (Melena et al., 2006). The durations of protection (maximum seven weeks) and efficacies varied with viral proteins and between studies. An early indication that vaccination of shrimp may be possible came when it was discovered that previous exposure to WSSV could protect shrimp from future challenge with the virus (Venegas et al., 2000). To date, vaccination of shrimp against only a single virus (WSSV) has been reported. Until now, most of the promising results obtained from experimental bioassays have been achieved largely on empirical grounds as all the vaccine related studies in shrimp have used largely, survival as a parameter, mostly after a challenge (van de Braak, 2002). These studies have focused on the practical issues of vaccination, rather than been targeted at understanding the mechanisms that are involved in this immune response.

For a vaccine to be an ideal one, parameters like statistical validity in survival data; finding out the youngest and the most suitable stage of the animal for which the product is recommended; conditions which are to be controlled during administration; minimum amount of antigen or dose that must be in the final product to make it effective with a single dose; precise challenge method; criteria to determine protection; lab and field efficacy studies to establish efficacy of greater than 90%; interference tests to know the effects of binding materials or immunostimulants added; tests for reversion to virulence; risk to environment; testing in three consecutive production batches; potency tests to determine the virus titre; stability test to identify the shelf-life; long lived immunity; effective when given orally; inducing wide range of responses like humoral, cellular and mucosal; low cost and compatibility with local management practices are to be clearly identified. It is far from clear whether the

observed improved survival following 'vaccination' is a result of true protection involving eradication of the pathogen from the host and subsequent defence against reinfection. Alternatively, it may be that prior exposure to viral pathogens or components elicits a response that permits the animal maintain the virus as persistent, but non-lethal infection (Johnson et al., 2008).

A scientific analysis of the underlying mechanisms affecting the efficacy of the stimulant and the constitution of protective defence is required to make effective progress in this field. Obviously, fundamental research on the functioning of the defence system has received less attention than has research from which the results can directly be applied to increase the profit margin, either by expansion of the production or by reduction of the costs. However, for efficient and effective research on defence stimulation, practically applicable parameters are needed. These should be based on scientific data, and they are of major importance to qualify and quantify stimulation of the defence system (van de Braak, 2002). Shrimps were considered to rely solely on an innate immune system characterised by generalized immune responses to conserved molecular structures of invading pathogens such as bacteria and fungi. Some of these pathways are relatively well understood, involving an array of pattern recognition receptors interacting with serine proteases to initiate encapsulation, phagocytosis and an antimicrobial cascade based on the phenoloxidase enzyme system (Soderhall and Cerenius, 1998). Much of the research into shrimp viral vaccination has focussed on delivery by injection (Namikoshi et al., 2004, Witteveldt et al., 2004a, Vaseeharan et al., 2006, Rout et al., 2007, Ha et al., 2008). Whilst most of the studies involving recombinant or subunit vaccines have been conducted over a limited duration, generally less than 21 days, a recent study using DNA vaccine technology has indicated that continual expression of viral protein *in situ* can lead to more sustained protection (Rout et al., 2007). This class of vaccines is only effective if they are introduced into cells. Clearly injection is not feasible for animals as small as marine shrimp.

However, a number of studies have also indicated protective efficacy following oral administration of feed coated with killed bacteria expressing recombinant viral proteins (Witteveldt et al., 2004a, 2004b). It would therefore seem feasible to transfer recombinant killed bacteria into the gut by the same route. The fate of the antigens once in the gut remains uncertain because of the proteolytic enzymes and extremes of pH that tend to digest antigens before they are uptaken by immunocompetent tissues or cells in the gut. This process can be overcome, with varying degrees of success by using a variety of carriers or microparticles, although many of these technologies may be too expensive for use in low margin industries such as shrimp aquaculture. Moreover, if there is a scarcity of knowledge of crustacean immunity *per se*, knowledge of gut immunology is even more scant. Substantial further research is clearly required in this area if such vehicles are to be developed specifically for marine shrimp. If administration of recombinant proteins orally presents some substantial technical and theoretical challenges, the delivery of DNA vaccine vectors by the oral route would appear to be an order of magnitude more complex. Carriers such as microparticles or enriched artemia may potentially protect DNA vaccine vectors from digestion by nucleases, or from acid hydrolysis. It is difficult to envisage how a vehicle may then introduce the vector into cells so that the antigens can be expressed. However, what is becoming more apparent is that the diversity and sophistication of innate responses in invertebrates is far greater than previously assumed (Loker et al., 2004).

Taken together, from studies to date, it would appear that exposure to inactivated virus or viral proteins confers only short-term resistance, in the order of 21–30 days. However, exposure to live virus appears to confer longer-term resistance. This would suggest that the viral persistence or viral tolerance model conceptualized by Flegel should be considered. The updated viral accommodation is stated as follows: ‘‘crustaceans and other arthropods actively accommodate viral pathogens as persistent infections that act as a kind of

memory that functions to specifically reduce the severity of disease and to dampen viral triggered apoptosis'' (Flegel, 2007). However, in experiments using a DNA vaccine expressing only a viral envelope protein, extended resistance to viral disease was reported (Rout et al., 2007) and the DNA expression plasmid was also shown to be persistent in shrimp up to two months after vaccination. This suggests that the continuous presence of specific viral surface proteins may be required for protection (Johnson et al., 2008).

Clearly, the most controllable environment for a shrimp vaccination operation is the hatchery. Here water quality, temperature and biosecurity can be managed relatively easily. Perhaps the next most obvious choice for a point of intervention by vaccination would be in nursery ponds. Nursery ponds are by no means universally adopted throughout the industry but would appear to provide a sensible option for application of a vaccination programme and allow onset of immunity prior to transfer to the rigours and stresses of the growout ponds, where temperature, salinity and water quality can fluctuate quite dramatically (Coman et al., 2003; Corsin et al., 2002).

WSSV still remains the major problem faced by shrimp farming sector. None of the management, chemical or prophylactic interventions attempted has provided a perfect solution for WSD in any part of the world. WSSV has become endemic in many shrimp farming areas. The majority of the wild broodstock are believed to be WSSV positive, adding a new problem to the industry.

In the present study, the following objectives were undertaken to gain knowledge on the immune response of tiger shrimp, *Penaeus monodon* (Fabricius), in response to the administration of inactivated virus preparation (IVP) :

1. Partial protein profiling of *Penaeus monodon* in response to IVP administration.
2. Non-specific immune response of *Penaeus monodon* to IVP administration.
3. Bio-defence genes in immunomodulation of IVP administered *Penaeus monodon* and
4. Viral gene expression of *Penaeus monodon* to IVP administration.

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Chapter 2

PARTIAL PROTEIN PROFILING OF *PENAEUS MONODON* IN RESPONSE TO IVP ADMINISTRATION

CHAPTER - 2

Partial protein profiling of *Penaeus monodon* in response to IVP administration

2.1. Introduction

To understand the pathogenesis of any viral disease, information on the interactions between the virus and the host is critical. Virus–host interactions may result in immune responses against the invader, and may also result in changes in the expression levels of host genes that favor virus replication. To date, virus–host interactions of WSSV have been studied at the transcription level using expressed sequence tags (ESTs), RT-PCR, microarray chips, suppression subtractive hybridization and differential hybridization (Gross et al., 2001; Astrofsky et al., 2002; Bangrak et al., 2002; Rojtinnakorn et al., 2002; Roux et al., 2002; Dhar et al., 2003; He et al., 2005; Pan et al., 2005). These studies have provided good insights for immune-related genes in the biodefense mechanisms in shrimp in specific tissues (Wang et al., 2007c). The differentially expressed cellular proteins in WSSV infected *P. monodon* epithelium have been studied by Wu et al. (2007) using shotgun 2D-LC-MS/MS and cleavable isotope coded affinity tag. Pais et al. (2007) have analyzed the electrophoretic pattern of serum protein in *Penaeus monodon* after WSSV injection challenge. Haemocyte proteins of *P. monodon* to *Vibrio harveyi* infection have been identified by Somboonwiwat et al. (2010). Protein expression profiling of the cellular response in the stomach of *Litopenaeus vannamei* to WSSV infection by intramuscular challenge has been studied (Wang et al., 2007b) by 2D and mass spectrometry. Robalino et al. (2009) used 2D-LC-MS/MS to understand the response in gill tissue in *L. vannamei* to WSSV. However, little is known about the proteins expressed in response to vaccination. Liu et al. (2009) subjected *L. vannamei*, immunized with VP28 DNA vaccine, for protein expression analysis and detected VP28 in different tissues.

Therefore, in the present study, partial comparative proteomics was applied to identify the upregulation or downregulation of proteins after administration of IVP, before and after oral challenge. Comparative proteomics defines the differences in expression of proteins among different biological states (e.g., control vs. treatment or healthy vs. disease), in different time and space, to identify differential proteins or protein groups on the expression amount, expression level, and modification status, and to further investigate the differential proteins and their function. Identifying these proteins is an important first step toward improving our understanding of the cellular pathways that are necessary for WSSV infection and protection.

The protein profile of *P.monodon* after the IVP administration as well as after oral challenge was studied employing 1- Dimensional and 2- Dimensional Electrophoresis. The discontinuous Laemmli system (Laemmli, 1970), a denaturing modification of Ornstein (1964) and Davis (1964), is the most widely used system for research in proteins today. The Laemmli gel provides excellent resolution of proteins because the treated peptides are concentrated in a stacking zone before entering the separating gel. 2-D electrophoresis is a classical, powerful and widely used method for the analysis of complex protein mixtures extracted from cells, tissues, or other biological samples. This method separates individual proteins and polypeptide chains according to two independent properties in two discrete steps - isoelectric point and molecular weight. The 2-D PAGE technology can be used for several applications, including; separation of complex protein mixtures into their individual polypeptide components and comparison of protein expression profiles of sample pairs (normal vs transformed cells, cells at different stages of growth or differentiation, etc.). Two-dimensional electrophoresis was first introduced by O'Farrell (1975).The first-dimension step, isoelectric focusing (IEF), separates proteins according to their isoelectric points (pI); the second-dimension step, sodium dodecyl sulfate

polyacrylamide gel electrophoresis (SDS-PAGE), separates proteins according to their molecular weights (Mr, relative molecular mass). Each spot on the resulting two-dimensional gel potentially corresponds to a single protein species in the sample. Thousands of different proteins can thus be separated, and information such as the protein pI, the apparent molecular weight, and the amount of each protein can be obtained. The first dimension separation which was performed in carrier-ampholyte-containing polyacrylamide gels cast in narrow tubes has been replaced by immobilized pH gradient (IPG) strips. Two-dimensional electrophoresis is used in proteomic studies due to its unparalleled ability to separate thousands of proteins simultaneously and its ability to detect post- and co-translational modifications, which cannot be predicted from the genome sequence. Applications of 2-D electrophoresis include proteome analysis, cell differentiation, detection of disease markers, therapy monitoring, drug discovery, cancer research, purity checks, and microscale protein purification.

In this chapter, the following components have been explained:

1. Preparation of inactivated viral preparation (IVP),
2. Experimental setup, and
3. Partial protein profiling of the experimental samples using 1D SDS-PAGE and 2D SDS-PAGE.

2.2. Materials and Method

2.2.1. Production of Inactivated Virus Preparation (IVP) for administration to *Penaeus monodon*

2.2.1.1. Virus Strain

The White Spot Syndrome Virus (MCCV101) (Singh et al., 2005) strain used for IVP preparation was from the microbial culture collection of National

Centre for Aquatic Animal Health, Cochin University of Science and Technology, Kochi.

2.2.1.2. Generating WSSV for IVP

For generating sufficient quantity of WSSV for IVP, tissues of *Penaeus monodon* infected with the WSSV MCCV101 strain, stored at -35ºC was thawed and fed to a batch of quarantined formalin stressed (100 ppm) batch of *Fennerpenaeus indicus* @ 5% body weight, maintained in a rearing tank fitted with Stringed Bed Suspended Bio-Reactor (SBSBR). The challenged animals in the late moribund stage with prominent white spots were collected and stored at -35ºC.

2.2.1.3. Inactivation of WSSV

Inactivation of WSSV was done as per the protocol of Singh et al. (2005). Briefly, WSSV infected shrimp tissues such as gill and head soft tissues maintained at -35ºC were dissected out. As per the requirement, tissues were homogenized with 15 ppt sterile sea water as the diluent. The ratio of homogenization was 15g WSSV infected tissue: 100 mL of the diluent. It was then passed through a 100µm mesh sieve to ensure uniformity. This preparation was then subjected to two cycles of freezing and thawing to release the virus from the tissues. Immediately after this, in order to confirm the presence of WSSV in the preparation, an aliquot of 10µL suspension was injected into the abdominal segment of WSSV negative shrimps (12g size *Penaeus monodon*). Three days after inoculation, the pleopod samples were subjected to nested PCR analysis following Lo et al. (1996b) and the animals were observed for mortality. At the same time an aliquot of the preparation was also subjected to nested PCR.

Following protocol was adopted for the diagnostic PCR (nested) of WSSV. Briefly, the DNA was extracted by macerating 50mg tissue or 50µL

tissue suspension in 1mL DNAzol® (Molecular Research Center, Inc. USA). The macerate was centrifuged (10,000g, 4ºC, 10 mins.) and the supernatant transferred to a fresh micro-centrifuge tube (MCT) containing 500µL absolute ethanol. After mixing the sample by inverting several times it was incubated at room temperature (28ºC) for 3 mins. and centrifuged (4000g, 20ºC, 5 mins.). The supernatant was removed and the pellet air dried (15 mins.). The pelleted DNA was dissolved in 200µL Milli-Q water and stored in -20ºC till used for amplification. The nested PCR for WSSV detection was done using Lo et al. (1996) primers as adopted by OIE for WSSV detection (5' - ACTACTAACTTCAGCCTATCTAG - 3' and 5' - TAATGCGGGTGTAATGTTCTTACGA - 3' for Ist step ; 5' - TAACTGCCCCTTCCATCTCCA - 3' and 5' - TACGGCAGCTGCTGCACCTTGT - 3' for IInd step) to yield amplicons of 1447bp and 941bp. The 25µL reaction mixture contained 200µM dNTP mix, 10pmol of forward and reverse primers, 1X PCR Buffer (Thermopol), 0.5U of Taq DNA polymerase and 1µL of solubilized DNA. The hot start PCR programme used for amplification on the Thermal cycler (Master Cycler personal, Eppendorf) was 94ºC for 4 mins. followed by 35 cycles of 94ºC for 1 min. (denaturation), 55ºC for 1 min. (annealing), 72ºC for 2 mins. (extension) and final extension at 68ºC for 5 mins. The PCR products were analyzed on 1% agarose run with Tris Acetate EDTA (TAE) buffer (1X) (40mM Tris-acetate, 1mM EDTA) and stained with ethidium bromide. The gel documentation under UV light was done with Molecular Imager® Gel Doc™ XR+ Imaging System (Bio-Rad).

To determine the bacterial and fungal loads, a loopful of preparation was streaked on to ZoBell's Marine agar and Sabouraud Dextrose agar plates (prepared in sea water) and incubated for 7 days.

Inactivation of the preparation was achieved by adding 0.3% (v/v) formaldehyde solution (Qualigens, India) and incubating at room temperature (28 ± 1ºC) for 48 hrs. Subsequently, the preparation was stored at -35ºC for lyophilization. As control (placebo), gill and head soft tissues of apparently healthy animals (tested negative by nested PCR) were treated as above for lyophilization.

2.2.1.4. In vitro titration of WSSV infected tissue used for IVP

WSSV infected tissues, tested first step positive and which belonged to the batch of infected tissue used for preparing IVP, were used for determining the viral titre of IVP. The viral titration was done in haemocyte primary cell culture, and measured by the MTT assay.

The development of haemocyte primary cell culture was done as described by Jose (2009) and Jose et al. (2010). The haemolymph collection was done from *P. monodon* reared in Recirculation Aquaculture System (RAS). The animals were sacrificed by immersing in crushed ice and surface sterilized by washing the animals with 800mg/L sodium hypochlorite in ice-cold 15ppt sea water for 10 mins. Subsequently, the animals were washed 5 times in sterile ice-cold sea water and dipped in 70% ethanol and again rinsed with the ice-cold sea water. Haemolymph was drawn by using capillary tubes containing anticoagulant (Tris HCl 0.01M, Sucrose 0.025M, Tri sodium citrate 0.1M) from the rostral sinus and the haemocyte count was enumerated using a haemocytometer. The haemolymph was then diluted with Modified L-15(2X) medium (Leibovitz's L-15 Sigma 2X supplemented with 2% glucose, 20% FBS, 2.295g/L Tryptose phosphate broth, 10mM N-phenylethemourea, 1X MEM vitamins, 100µg/mL streptomysin, 1001U/mL penicillin, 0.06µg/mL chloramphenicol and MQ water to make upto 100mL with a final osmolality of 720 mOsmol) to obtain a concentration of 10^5 cells/mL. It was then seeded onto

96 well plates as 200µL aliquots and incubated at 25 ºC for 5 hrs. for monolayer formation.

Determination of WSSV titre was done as soon as the monolayer was formed. The suspension of WSSV lysate to be used for the viral titration was prepared by macerating the infected tissue with mortar and pestle in an ice bath with 10mL culture medium and glass fiber wool. The extract was then centrifuged at 10,000g for 10 mins. at 4 ºC and the supernatant passed through 0.22µm polyvinylidene fluoride (PVDF) membrane. A double dilution series of this WSSV containing filtrate was prepared in a deep well plate (Axygen) and was then used for titration by the method of MTT_{50}.

The MTT_{50} is a colorimetric method based on the determination of cell viability utilizing the reaction of a tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, MTT) with the mitochondria of metabolically active cells. The reduction of the tetrazolium salt by nicotinamide adenine dinucleotide dehydrogenase (NADH) and nicotinamide adenine dinucleotide phosphate dehydrogenase (NADPH) within the cells produces insoluble purple formazan crystals, which are later solubilized yielding a purple coloured solution (Mosmann, 1983).

For the MTT_{50} assay, the medium was removed from the monolayer formed wells and 200µL WSSV inoculum was added to each well maintaining quadruplicate for each dilution. For comparison, uninfected controls were also maintained. The cells were observed under phase contrast microscope (Carl Ziess, Germany) for CPE. The extract from apparently healthy *P.monodon* was applied as controls to haemocyte culture to check the cytotoxicity. After replacing the medium, a sample of 50µL of MTT (Sigma) solution (5mg mL^{-1} in PBS; 720 mOsmol) was added to each well and incubated for 5 hrs. in dark. Control consisted of medium alone with MTT added. After incubation, the medium was removed and MTT-formazan crystals were dissolved in 200µL

dimethyl sulphoxide. Absorbance was recorded immediately at 570 nm in a microplate reader (TECAN Infinite Tm, Austria).

The 50% infectious dose, termed MTT_{50} of the virus is defined as the titre of the virus at which the average well absorbance was 50% that of the uninfected cells. This was determined by plotting the optical density of each well versus the $-\log_{10}$ of the dilution factor. The optical density was defined as:

Optical density = (Blank absorbance – Well absorbance / Blank absorbance) x 100%

The blank absorbance was the average absorbance of 12 – 48 uninfected wells and the well absorbance was the average absorbance of infected wells. From the plot of optical density versus $-\log_{10}$ of dilution factor, the 50% optical density was determined. This value was converted per milliliter basis and stated as the MTT_{50} titre (Heldt et al., 2006).

2.2.1.5. Lyophilisation of IVP and Coating onto feed

IVP and the placebo were lyophilized (Lyolab, India) to yield ~50g of dry mass from 500ml of tissue suspension prepared from 75g of infected tissue.

From the titre determined by MTT_{50}, the binding of lyophilized IVP to the feed was done by using 4% aqueous gelatin in distilled water and egg white. Briefly, 1.6g of IVP and 10g feed were mixed and 4% gelatin (~9ml) was added to the mixture and mixed thoroughly until the IVP in powder form was completely attached to the feed. After placing in RT for 30min., it was mixed with egg white in the ratio 10g (IVP coated feed): 1 mL (egg white). The IVP coated feed was then placed in a vaccum desiccator at 4ºC until the feed got dried. Normal pelleted feed was used as the control.

2.2.2. IVP administration experiment

2.2.2.1. Animal maintenance

P.monodon larvae tested negative for Monodon Baculovirus (MBV) and WSSV (Lo et al., 1996b) obtained from a local hatchery were stocked and reared in a RAS for shrimp integrated with nitrifying bioreactors (Kumar et al., 2009) in 15gL^{-1} salinity sea water. Water quality was maintained by addition of a probiotic Detrodigest ^{TM} (NCAAH, India) to manage detritus and Enterotrophotic ^{TM} (NCAAH, India) to control *Vibrio*. The larvae were fed with commercial pelleted feed (Higashimaru, India) and maintained for three months for obtaining the experimental sized animals.

2.2.2.2. Experimental treatment groups

Animals of 11 ± 2g size (10 nos. per treatment) were transferred to experimental tanks of 200-L capacity filled with 15gL^{-1} salinity sea water and maintained with SBSBRs (Stringed Bed Suspended Bioreactors) (Singh et al., 2000) to manage the ammonia concentration below toxic level during the experimental period. Water quality was monitored on all days and was found to be in the pH range of: 7.6 to 8.25, Salinity: 15ppt, Ammonia: <0.001 to 0.2ppm, NO_2: 0.1 to 0.3ppm and dissolved oxygen: 6-7 mg L^{-1}.

Based on the findings of Singh et al. (2005) on the effect of administration of IVP to *P. indicus*, the following different sets of treatments were considered for sampling.

1. Normal (fed with normal feed, 1st day after 7 days of administration)
2. IVP (fed with IVP coated feed, 1st day after 7 days of administration)
3. Normal (fed with normal feed, challenged 1st day after 7 days of administration and samples collected on 4th day post challenge)
4. IVP (fed with IVP coated feed, challenged 1st day after 7 days of administration and samples collected on 4th day post challenge)
5. Normal (fed with normal feed, 5th day after 7 days of administration)

6. IVP (fed with IVP coated feed, 5th day after 7 days of administration)
7. Normal (fed with normal feed, challenged 5th day after 7 days of administration and samples collected on 4th day post challenge)
8. IVP (fed with IVP coated feed, challenged 5th day after 7 days of administration and samples collected on 4th day post challenge)
9. Normal (fed with normal feed, 10th day after 7 days of administration)
10. IVP (fed with IVP coated feed, 5th day after 7 days of administration)
11. Normal (fed with normal feed, challenged 10th day after 7 days of administration and samples collected on 4th day post challenge)
12. IVP (fed with IVP coated feed, challenged 10th day after 7 days of administration and samples collected on 4th day post challenge)

2.2.2.3. IVP administration

The experimental animals were fed *ad libitum* for 7 consecutive days with the calculated quantity of appropriate feed type (IVP coated / Normal).

2.2.2.4. Oral challenge with WSSV

The quantity of WSSV infected tissue, for oral challenge, was determined by choosing the lowest quantity of WSSV infected head soft tissues required for causing 100% mortality in *P. monodon* within 7 days of administration. The experimental animals were challenged with WSSV infected head soft tissues (@ 2% of body weight of experimental animals) on 1st day, 5th day and 10th day post- administration of the appropriate feed type.

2.2.2.5. Collection of samples and storage for analysis

The samples were collected on 1st day, 5th day and 10th day after 7 days of administration of the appropriate feed type (IVP coated/ Normal) as control to the challenged set of animals. Appropriate samples were also collected from all the groups of animals on 4th day post WSSV oral challenge.

The collection of samples for analysis from the above groups of animals included: haemolymph (for protein profiling, immune gene and viral gene expression), gills (for protein profiling, immune gene and viral gene expression and histopathology), hepatopancreas (immune gene and viral gene expression), and pleopods (for diagnostic PCR).

Briefly, samples (haemolymph, gills, hepatopancreas) for analyzing the immune gene expression by RT-PCR were immediately stored in TRI® Reagent (Sigma) at -80ºC for RNA extraction. Gills and the haemocyte lysate were also stored in -80ºC for protein profiling. Pleopods for PCR were fixed in 70% ethanol and stored in 4ºC. The experimental animals were also fixed by injecting and immersing in Davidson's fixative for histopathology. The detailed procedures for the collection and treatment of samples for each of the above mentioned analysis have been dealt with in the respective areas in the following chapters.

2.2.3. Protein profiling

2.2.3.1. Samples for 1Dimensional Sodium Dodecyl Sulphate – Poly Acrylamide Gel Electrophoresis (1D SDS - PAGE) and 2 Dimensional (D) Electrophoresis

For the collection of haemolymph, 6 shrimps from each treatment were taken with minimum stress. The haemolymph was drawn from the rostral sinus of shrimps by inserting sterile capillary tubes rinsed with and containing 100µL anticoagulant (10% sodium citrate with 40µg Aprotinin/mL as a protease inhibitor). The collected haemolymph was transferred into microcentrifuge tubes (MCTs) containing 100µL of the anticoagulant and was mixed by gentle tapping. For separating the haemocyte, the haemolymph was subjected to centrifugation at 3000rpm (4ºC, 15 mins). The resulting supernatant was stored as plasma. The haemocyte pellet thus obtained was washed with Phosphate Buffered Saline (PBS) (720mOsmol, pH-7.5 with 40µg Aprotinin/mL) by

centrifuging at 3500rpm (4ºC, 5 mins). Finally, haemocytes were subjected to hypotonic lysis by adding 50µL of Milli-Q water, and repeatedly pipetted and stored at -80ºC. The samples in each treatment were then pooled and stored at -80ºC for the electrophoretic analysis.

Gill from the experimental animals (6 shrimps from each treatment) were dissected out using sterile dissectors and placed in MCTs containing PBS (NaCl-0.8%, KCl-0.02%, Na_2HPO_4-0.144%, KH_2PO_4-0.024%, pH-7.4) with 40µg Aprotinin/mL. The samples of each treatment were then stored at -80ºC. Later, the samples in each treatment were pooled and homogenized in glass homogenizer. The homogenized samples were then kept in ice at 4ºC for 30 mins. It was then centrifuged at 8,000g (4ºC, 20 mins). The supernatant was then stored at -80ºC for the electrophoretic analysis.

2.2.3.2. Protein estimation

Total proteins in the haemocyte, gill and hepatopancres lysate of the experimental animals were determined following the method of Bradford (1976) with Bovine serum albumin (BSA) as the standard.

2.2.3.3. 1Dimensional Electrophoresis

One dimensional electrophoresis protein analysis by Sodium Dodecyl Sulphate - Poly Acylamide Gel Electrophoresis (SDS-PAGE) of gill and hepatopancreas lysates was carried out by employing the discontinuous Laemmli system (Laemmli, 1970).

Fractions of the experimental protein samples were electrophoresed in discontinuous PAGE under denaturing conditions in a 4% stacking gel (pH-6.8) and 10% resolving gel in Tris-glycine buffer (pH- 8.8) system at 12mA.

Briefly, 50-60 µg protein of the samples and the broad range molecular weight markers (Genei, India) were treated by adding treatment buffer (0.125M Tris-Cl, 2% SDS, 10% gycerol, 5% β-mercaptoethanol, 0.02% bromophenol blue, pH-6.8) in 1:1 ratio and 1:3, respectively. They were then immediately kept in a boiling water bath for 1½ min. and cooled to room temperature for loading on to the gel. An aliquot of 10µL of the treated sample containing 50-60 µg of the protein were loaded onto a slab gel of size 10×10cm and electrophoresis was carried out on a Hoefer™ miniVE vertical electrophoresis system (Amersham BioSciences, Sweden) in room temperature (28ºC) until the dye front reached the bottom of the gel. The gels were stained overnight with Coomassie Brilliant Blue R-250 (methanol 40%, acetic acid 7%, Coomassie Brilliant Blue, 0.025%). The gels were fixed and destained with Solution-1 (40% methanol, 7% acetic acid) for 30 mins. and again destained with Solution-2 (5% methanol, 7% acetic acid) for 5-6 hrs. Documentation and analysis of the gels were done by using Molecular Imager® Gel Doc™ XR+ Imaging System (Bio-Rad) and The Discovery Series™ Quantity One® 1-D Analysis Software (Bio-Rad) respectively.

2.2.3.4. 2D Electrophoresis

The two-dimensional electrophoresis was carried out based on the method developed by O'Farrell (1975) with modifications.

2.2.3.4.1. Sample Preparation for First-dimension Isoelectric Focusing (IEF)

The sample preparation for IEF was done using the 2-D Clean-Up Kit (GE Healthcare) by following the procedure supplied by the manufacturer. Briefly, 400µg of the protein samples (haemocyte lysate and gill extract) were mixed with 300µL of precipitant by vortexing. After incubation on ice for 15 mins, 300µL of the co-precipitant were added and vortexed briefly. The mixture

was centrifuged (12,000g, 4ºC, 5 mins.) and the resultant supernatant was completely removed immediately without causing any disturbance to the pellet. An aliquot of 40µL of the co-precipitant was layered on top of the pellet and incubated for 5 mins. in ice. It was then centrifuged (12,000g, 4ºC, 5 mins.) and the supernatant removed. Autoclaved double distilled water (25 µL) was added to the pellet and vortexed for 5-10 s. Following the addition of 1mL of wash buffer (pre-chilled for at least 1 h at -20 ºC) and 5 µL of wash additive, the pellet was fully dispersed by vortexing. The samples were then incubated at -20ºC for 30 mins. and vortexed in every 10 mins. Subsequently, the samples were subjected to centrifugation (12,000g at 4ºC for 5 mins.) and the supernatants were discarded and the pellet air dried (<5min.). The pellet was resuspended in 100µL of DeStreak Rehydration buffer (equilibrated at room temperature for 30 mins.), vortexed (30 s) and incubated at room temperature (RT) (<25ºC) overnight to allow complete solubilisation. Immobilized pH gradient (IPG) buffer of appropriate pH (3-10) were added to the DeStreak Rehydration buffer at 0.5% and the total volume was made upto 125µL for Immobiline^{TM} DryStrip of 7cm length. The sample was again incubated for 1.5 hrs. at 25ºC and centrifuged (13,000g, 20ºC, 5 mins.) and the supernatant was used for strip rehydration.

2.2.3.4.2. Rehydration of Immobiline DryStrip

Rehydration of Immobiline DryStrip (GE Healthcare) or the IPG strip was done in the Reswelling tray placed inside the IPG Box (GE Healthcare). The sample prepared in the DeStreak Rehydration solution (125µL) was pipetted and evenly distributed into the wells in the Re-swelling tray and the Immobiline^{TM} DryStrip (pH 3-10 for haemocyte lysate and pH 4-7 for gill extract) was carefully placed onto the sample with the positive end towards the anode of the strip holder. The strip was then overlayed with Immobiline DryStrip Cover Fluid (GE Healthcare), covered and subjected to overnight rehydration.

2.2.3.4.3. Isoelectric focusing

Isoelectric focusing of the samples was done on Ettan™ IPGphor™ 3 Isoelectric Focusing System (GE Healthcare). The strip holders (2 nos.) with the rehydrated Immobiline DryStrip were placed on the platform of the system with the pointed end (anodic) on anode and blunt end over to the cathode. After closing the safety lid, the strips were subjected to an IPGphor software controlled rehydration at 20ºC for 2 hrs. The automatic IEF was performed at 20ºC using the following voltage focusing protocol: step 150V for 4hrs., step 300V for 1h, gradient 1000V for 3hrs., gradient 5000V for 6hrs. and the final step and hold at 5000V for 5hrs.

2.2.3.4.4. Equilibration of Immobiline DrStrips subjected to IEF

After the first dimensional IEF, the overlayed coverfluid was completely removed and the IPG strips were equilibrated in 5ml of SDS equilibration buffer (6M urea, 50mM Tris-HCl, pH 8.8, 29.3% glycerol, 2% SDS, 0.002% bromophenol blue) containing 60mM DTT (Dithiothreitol) for 15 mins. The IPG strips were then subjected to a second equilibration with equilibration buffer containing 50mM Idoacetamide for a further 15 mins.

2.2.3.4.5. Second Dimensional SDS-PAGE

The equilibrated IPG strips were placed onto SDS-polyacrylamide gel that consisted of 12.5% acrylamide, pH-8.8, for the resolving gel and 4% acrylamide, pH-6.8, for the stacking gel. Broad range marker (Genei), was loaded towards the right end of the gel. The IPG strip and marker were then sealed with sealing solution (0.5% Agarose, 0.002% Bromophenol Blue). Electrophoresis was carried out on a Hoefer™ miniVE vertical electrophoresis system (Amersham BioSciences, Sweden) at 15mA per gel in room temperature until the dye front reached the bottom of the gel. The gels were subjected to Coomassie staining (methanol 40%, acetic acid 7%, Coomassie Brilliant Blue R-250, 0.025%) for overnight. The gels were then fixed and destained with

Solution-1 (40% methanol, 7% acetic acid) for 30 mins. and again destained with Solution-2 (5% methanol, 7% acetic acid) for 5-6 hrs. Documentation of the gels were done using ImageScanner III (GE Healthcare) scanner with the software LabScan 6.0 (GE Healthcare) and the gel images were analysed.

2.3. Results

2.3.1. Inactivation of WSSV

The WSSV preparation meant for inactivation was found to be first step PCR positive for WSSV. It was inactivated using 0.3% formalin for 48hrs which on subsequent bioassay was found to be non infectious; on administering to *P. monodon* the animals did not show any sign of the disease and no mortality was recorded.

2.3.2. In vitro titration of WSSV infected tissue used for IVP

The haemocyte culture which was inoculated with the gill extract (diluted 20 times) was found to show CPE (cytopathic effect) including shrinkage and necrosis. On performing MTT assay, sigmoid curve was obtained on plotting optical density versus $-\log_{10}$ of the dilution factor, and 50% optical density was extrapolated from the curve and converted to per milliliter. The MTT titre of the infected tissue was 2 $\log_{10}$ (MTT_{50}/mL) per milligram wet tissue (Fig.1).

2.3.3. Lyophilisation of IVP and coating onto feed

The IVP prepared was lyophilized to obtain it in a powder form. For coating the lyophilized IVP on to the feed, the preparation having the strength of MTT_{100}/mL was worked out from MTT_{50}/mL. Accordingly, 2mg wet weight of tissue was calculated to be required for MTT_{100}/mL. One mg wet weight of the tissue was found to be equivalent to 0.16mg dry weight and 2mg wet weight to 0.32mg dry weight of tissue which would give 4 $\log_{10}$ MTT_{50}/mL. To arrive at the quantity of IVP required for administering per gram animal, an aliquot of

10µL WSSV inoculum used for preparing IVP was administered into a 200µL cell (shrimp haemocyte) suspension. Accordingly, 4 $\log_{10}$ (MTT_{50}/mL) in 200µL cell suspension was calculated to be 0.32mg dry weight of tissue. Considering 1000µL cell suspension equivalent to 1g tissue, the amount of dry weight of infected tissue for MTT_{100}/mL was calculated to be 1.6mg dry weight. Five times the calculated dry weight (8mg of IVP/g of the animal) was used per gram animal for administration. Accordingly, the IVP (4% gelatin-egg white coated dried feed) was prepared for feeding.

2.3.4. IVP administration, challenge and sample collection

The coated appropriate feed type (IVP coated / Normal) were fed ad libitum to *P. monodon* for 7 consecutive days. On 1st, 5th and 10th day after completion of administration of the above diet and on 4th day post WSSV oral challenge, the samples such as haemolymph, gill and hepatopancreas were collected for the protein analysis.

2.3.5. Protein profiling

1D SDS-PAGE was carried out with tissue extracts of gill and hepatopancreas of experimental animals (Fig. 2a, 2b). It was observed that there was no significant variation in the 1D protein profile of the gill extracts of normal feed and IVP administered animals before and after challenge (Fg.2a). However, in the extracts of hepatopancreas of IVP administered and challenged group of animals on 1DPA, the 1D SDS-PAGE revealed two bands of ~75KDa protein, which could be considered as a significant upregulation of a specific protein subsequent to challenge after IVP administration. However, the same protein was apparent in normal control animals also with out a challenge, but, not in any other situations (Fig. 2b).

2D SDS-PAGE was done for haemocyte lysate and gill extract of the experimental animals (Fig.3 – Fig.8). The 2D expression pattern in the haemocyte lysate and gill tissue extract differed tissue wise and the spots could

be identified as upregulated /down regulated/ without variation. Accordingly in the haemocyte lysate of normal feed administered *P. monodon* (1DPA, 5DPA, 10DPA) after WSSV challenge, an upregulation (increase in intensity of protein spots) and increase in the number of proteins were observed (Fig.3a,4a,5a). Meanwhile, in the haemocyte lysates of IVP administered animals after WSSV challenge, downregulation (decrease in intensity of protein spots) was observed (Fig.3b, 4b, 5b). However, in the 2D SDS-PAGE analysis of the gill extracts of experimental animals after WSSV challenge, an upregulation in protein spots was observed in normal feed administered and challenged on 1DPA and 5DPA) (Fig.6a, 7a) and in IVP administered and challenged animals only on 10DPA (Fig.8b). However, down regulation was observed in the gill extract of normal feed administered and challenged on 10DPA (Fig.8a) and in IVP administered and challenged on 1DPA and 5DPA (Fig.6b, 7b).

2.4. Discussion

The application of IVP (inactivated virus preparation) and its efficacy as an inducer of protection against WSSV in *Fenneropenaeus indicus* have been reported by Singh et al. (2005). The viral titre (LD_{50}) of WSSV used for the preparation of IVP was determined by Singh et al. (2005) as per Reed and Muench (1938), while in the present study the WSSV titre (MTT_{50}) was quantified using the primary haemocyte culture using the MTT assay (Jose et al., 2010) in which the metabolic activity of the cell was assessed.

The present study also revealed the efficacy of IVP as feed additive, as 7 day administration did not cause mortality in *P. monodon*. The RPS (relative percentage survival) obtained in IVP administered animals after a WSSV challenge in the preliminary experiments over a period of four years in *P. monodon* was found to vary between 50-80% with higher value in the initial years and lower value in the final years (data not shown). However, the present

experimental study is focused to elucidate the mechanism involved in providing protection from WSSV by oral administration of IVP in *P. monodon*.

The absence of variation observed in the 1D SDS-PAGE analysis of the gill extracts of IVP administered animals might be due to the absence of a dominant protein related mechanism in the gill tissue in combating the WSSV on 4th day post challenge. It might also be the limitation of 1D electrophoresis technique in analyzing the gill extract of *P. monodon,* where the expression of a particular protein is not prominent to determine its level through ID electrophoresis. However, the 1D analysis of hepatopancreas revealed the upregulation of two bands of ~75KDa protein in the IVP administered animals challenged on 1DPA. The upregulation observed could not be related to the exclusive specific difference between the groups, as unchallenged group also were showing upregulation of these bands. The 2D protein profile of haemocyte lysate and gill extract of *P. monodon* in the present study were done for resolving and understanding the hidden difference in 1D analysis in the protein expression pattern that might be present in the test (IVP administered/ challenged groups) and control groups (normal feed administered/ unchallenged groups). This has provided information that nearly parallels to the 2D findings of Somboonwiwat et al. (2010) in which protein spots of haemocytes were upregulated/ downregulated/ 'constant' in the test (*V. harveyi* infected *P. monodon*) relative to the control. However, in the present study the pH range used for IEF was 3-10 unlike the 4-7 used by Somboonwiwat et al. (2010). Hence, in the present study the protein spots were observed in a narrow region (Fig. 4 – Fig.6) but clear differences could be observed between the unchallenged and challenged groups. In the haemocyte lysate of normal feed administered group (before a WSSV challenge) protein spots were lower when compared to IVP administered groups. The increase in number of protein spots in IVP administered group than in normal feed administered group shows that the supplementation of formalin inactivated material in shrimp diet is causing a

shift in the protein expression in *P. monodon* which in turn is proving that the haemocytes can specifically elicit a protein related response towards the orally ingested formalin inactivated material. 2D spots of normal challenged is comparable to the IVP administered unchallenged. Montano-Perez et al. (2005) have reported that haemocytes from *L. vannamei* are capable of recognizing and distinguishing different foreign materials (abiotic particle – DEAE-Sephadex or potential pathogen – Heat inactivated *V. alginolyticus*) when administered by injection route, indicating that there exists phagocytic response to foreign materials which might be active in the alimentary canal epithelial lining of shrimp. However, the present study has observed the elicitation of specific kind of response in *P. monodon* haemocyte lysate to the formalin inactivated viral preparation which was administered orally. The increase in intensity of protein spots in the haemocyte lysate of IVP administered animals might well be due to the inactivated WSSV proteins. When analyzing the haemocyte lysate of normal feed administered animals, it was observed that on WSSV challenge the haemocyte lysate showed an increase in protein spots which might be the viral proteins or the haemocyte proteins involved in the immune response. In contrast, in IVP administered haemocyte lysate, there was decrease in the number and intensity of protein spots than in normal animals challenged with WSSV after the 1DPA. This observation shows a unique form of response by *P. monodon* haemocytes which are exposed to IVP and subsequently on encountering with active WSSV a decrease in the presence of proteins happens. This decrease in number and intensity of protein spots shows that *P. monodon* haemocytes can respond to the oral administration of IVP subsequent to WSSV challenge through a protein related mechanism.

Protein profiles of gill extracts of experimental animals by 2D SDS-PAGE were different from those observed in haemocyte lysate of the experimental animals. This difference in protein profile might be due to the different immune related mechanism adopted by the haemocyte and gill tissue in

P. monodon. Difference could be observed in the gill extracts of unchallenged and challenged animals which was not visible in 1D SDS-PAGE analysis. Upregulation of protein spots was evident in normal feed administered animals challenged on 1DPA and 5DPA. The absence of upregulation of protein spots in normal feed administered animals challenged on 10DPA might be due to the lower rate of WSSV infection in response to the challenge in this group of animals. However, the upregulation of protein spots was observed in IVP administered animals only on 10DPA WSSV challenge and down regulation was observed in IVP administered animals challenged on 1DPA and 5DPA. In gill extract, the expression seems to be dependent on time elapsed post administration. This observation however points that in gills of IVP administered animals the protein expression related response to the WSSV challenge is active only on 10DPA. The down regulation in protein expression of gill extracts of IVP administered animals after WSSV challenge sheds light on some unique mechanism/s that *P. monodon* adopts as an immune response to WSSV after being exposed to an oral administration of inactivated virus.

The 2D protein expression pattern observed in IVP administered animals before and after a WSSV challenge sheds some light on the protein related antiviral mechanism involved. The partial protein profile using 2D SDS-PAGE has revealed that *P. monodon* haemocyte and gill tissue (exposed to a particular dose of formalin inactivated virus preparation through oral route) can respond to a WSSV challenge. Down regulation of proteins which was upregulated in normal feed administered group on WSSV challenge, was observed in IVP administered animals. Further investigation of 2DE in conjuction with mass spectrometry for protein identification is needed to untie the underlying mechanism/s (eg: phagocytosis/ enzyme activations/ gene regulation).

Fig 2a. 1D SDS-PAGE of Gill

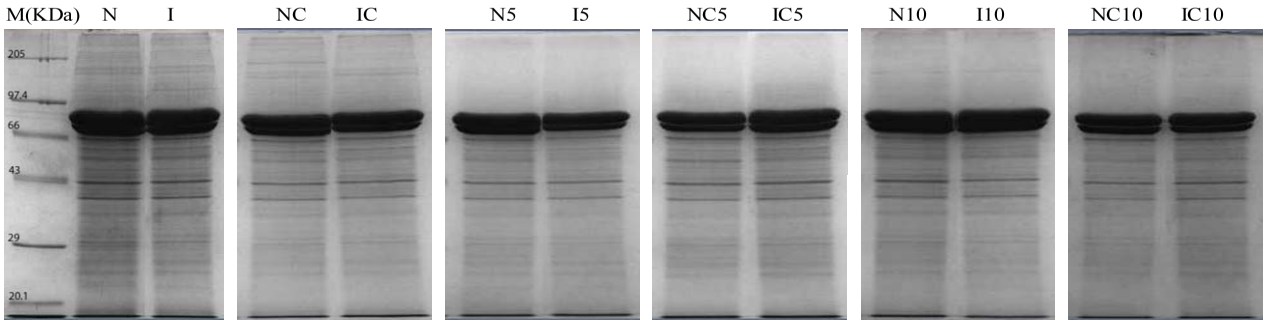
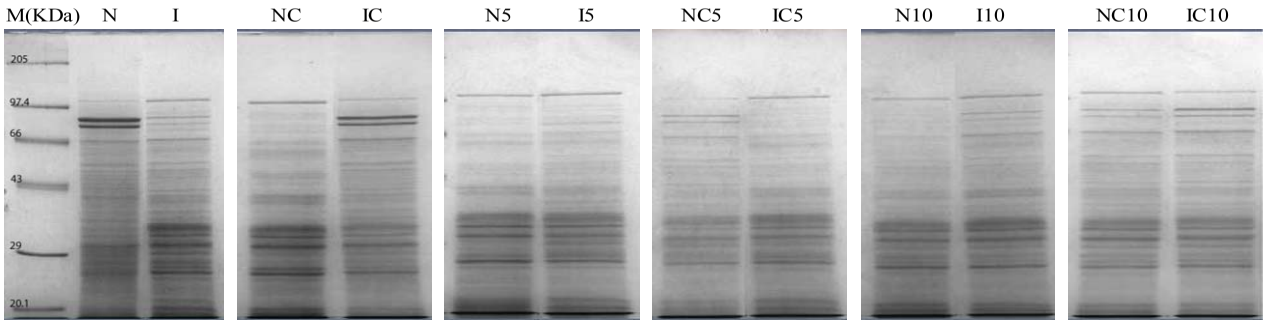


Fig 2b. 1D SDS-PAGE of Hepatopancreas



N, N5, N10 – Normal feed administered on 1DPA, 5DPA and 10DPA
I, I5, I10 – IVP administered on 1DPA, 5DPA and 10DPA
NC, NC5, NC10 – Normal feed administered challenged on 1DPA, 5DPA and 10DPA
IC, IC5, IC10 – IVP administered challenged on 1DPA, 5DPA and 10DPA

Fig.3. Haemolymph 1DPA (pH 3-10)

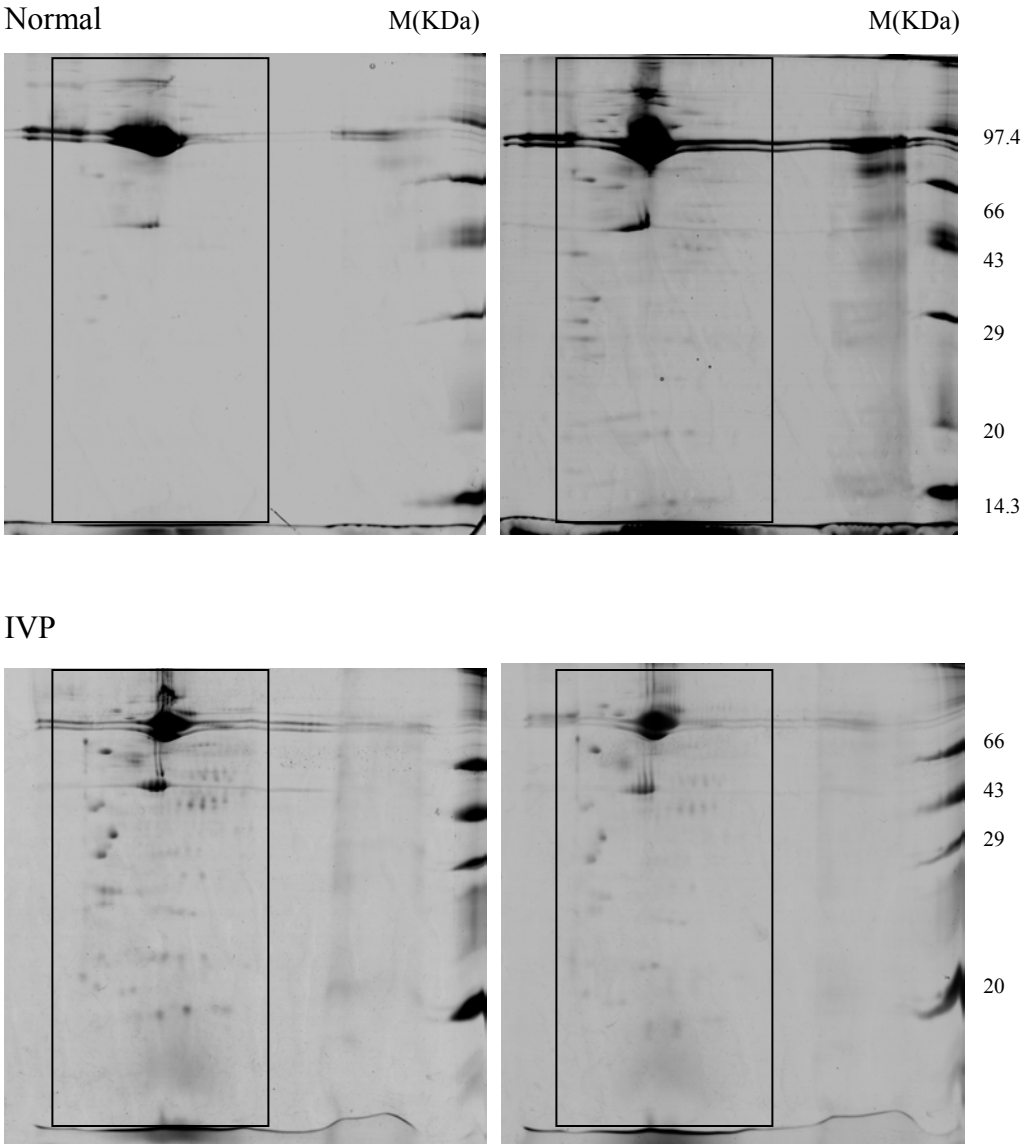


Fig.4. Haemolymph 5DPA (pH 3-10)

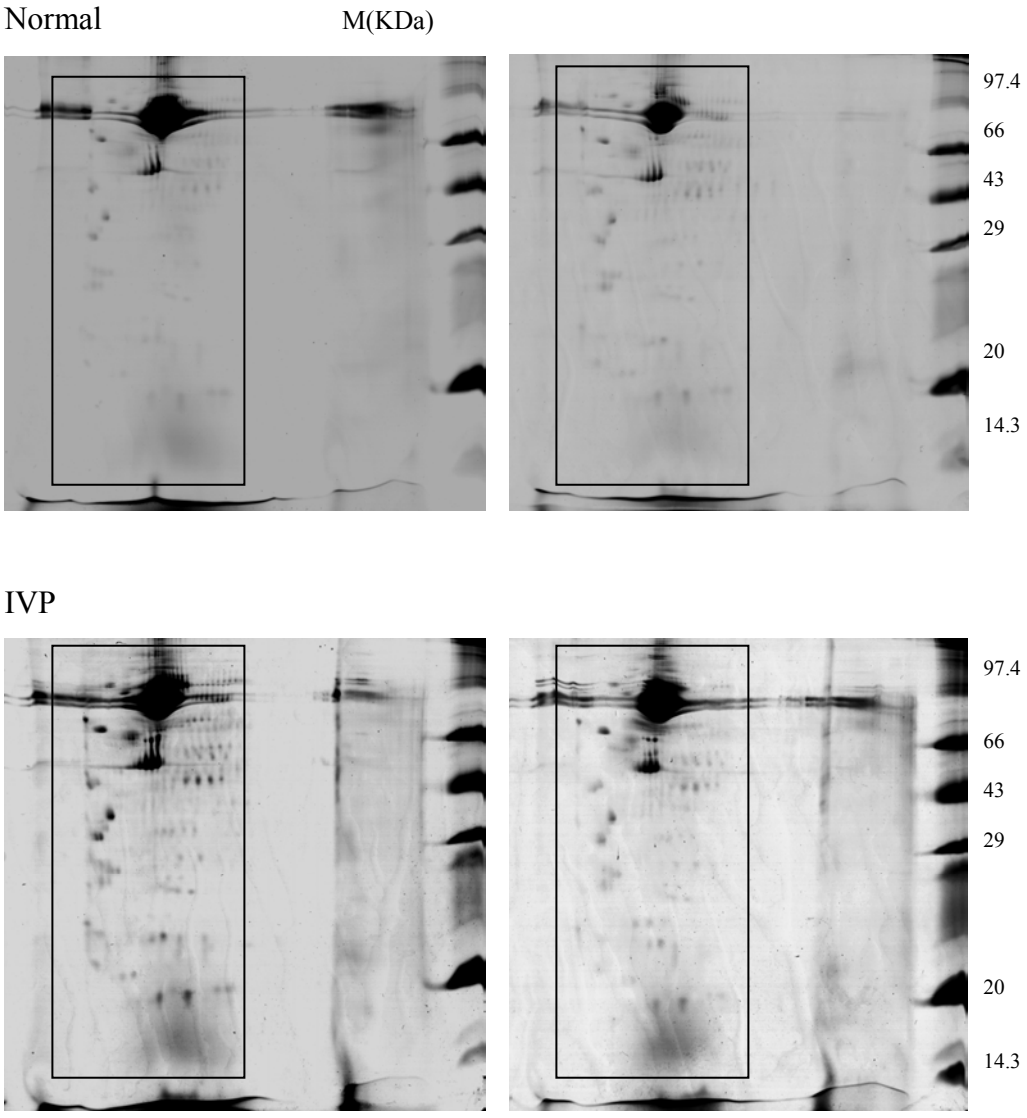


Fig.5. Haemolymph 10DPA (pH 3-10)

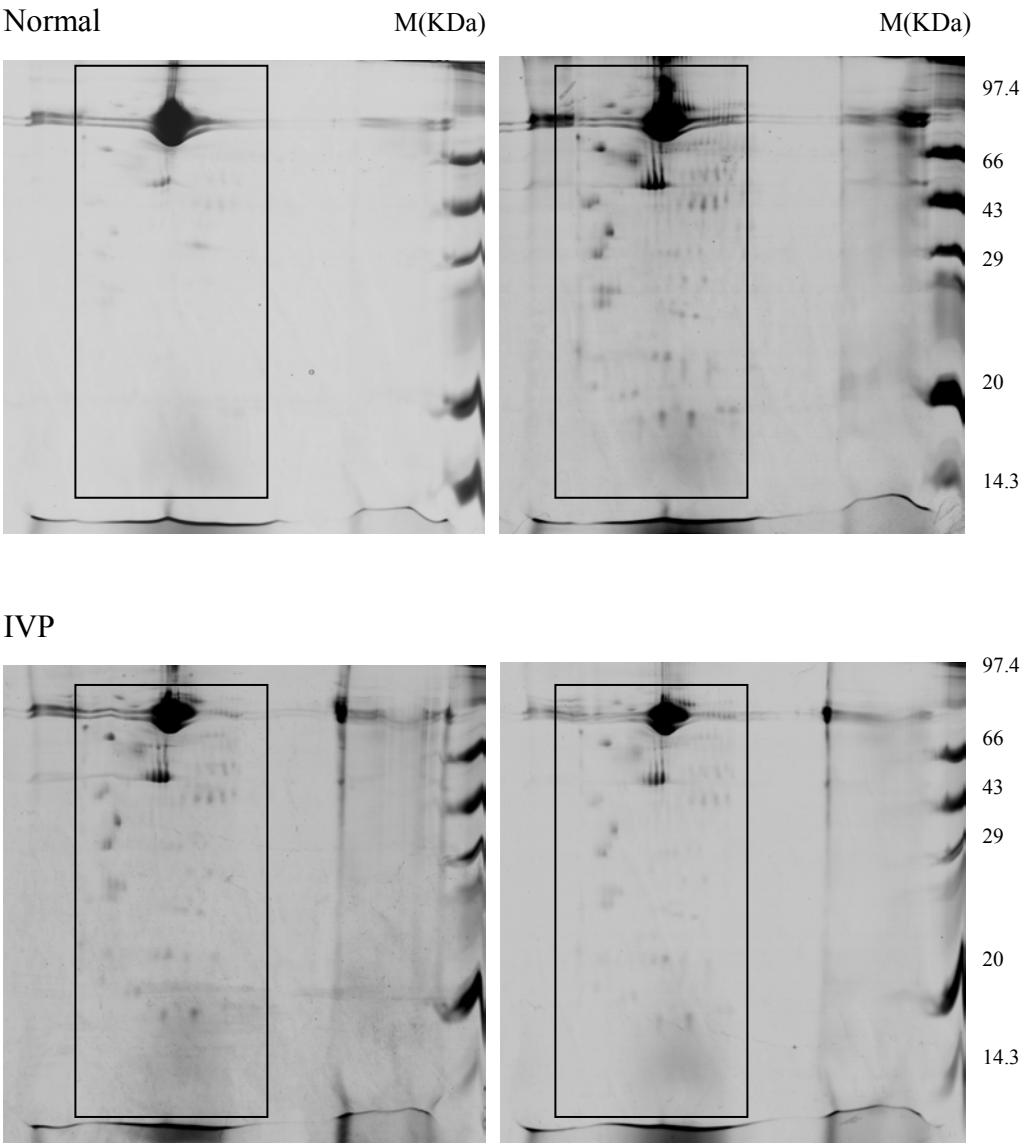


Fig.6. Gill 1DPA (pH 4-7)

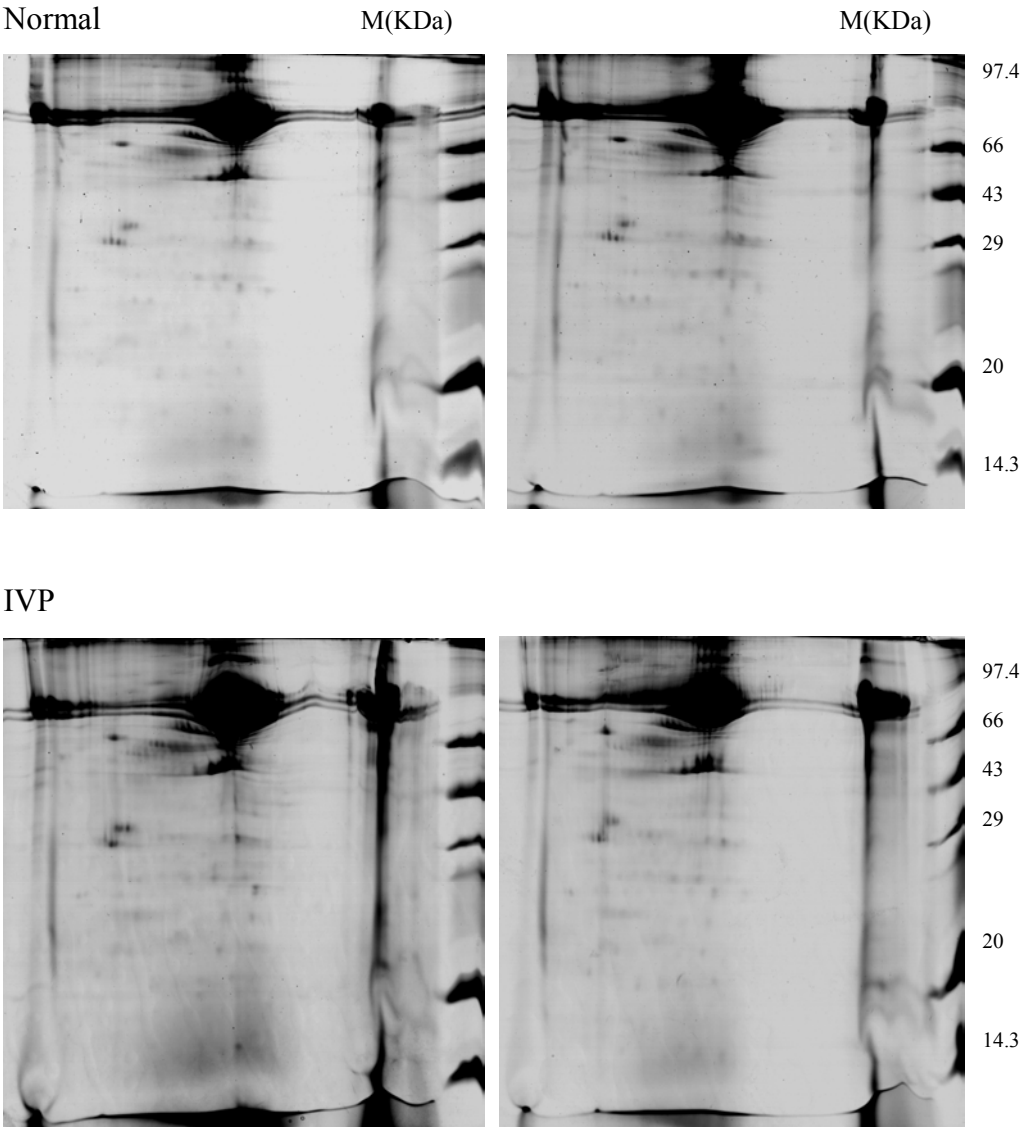


Fig.7. Gill 5DPA (pH 4-7)

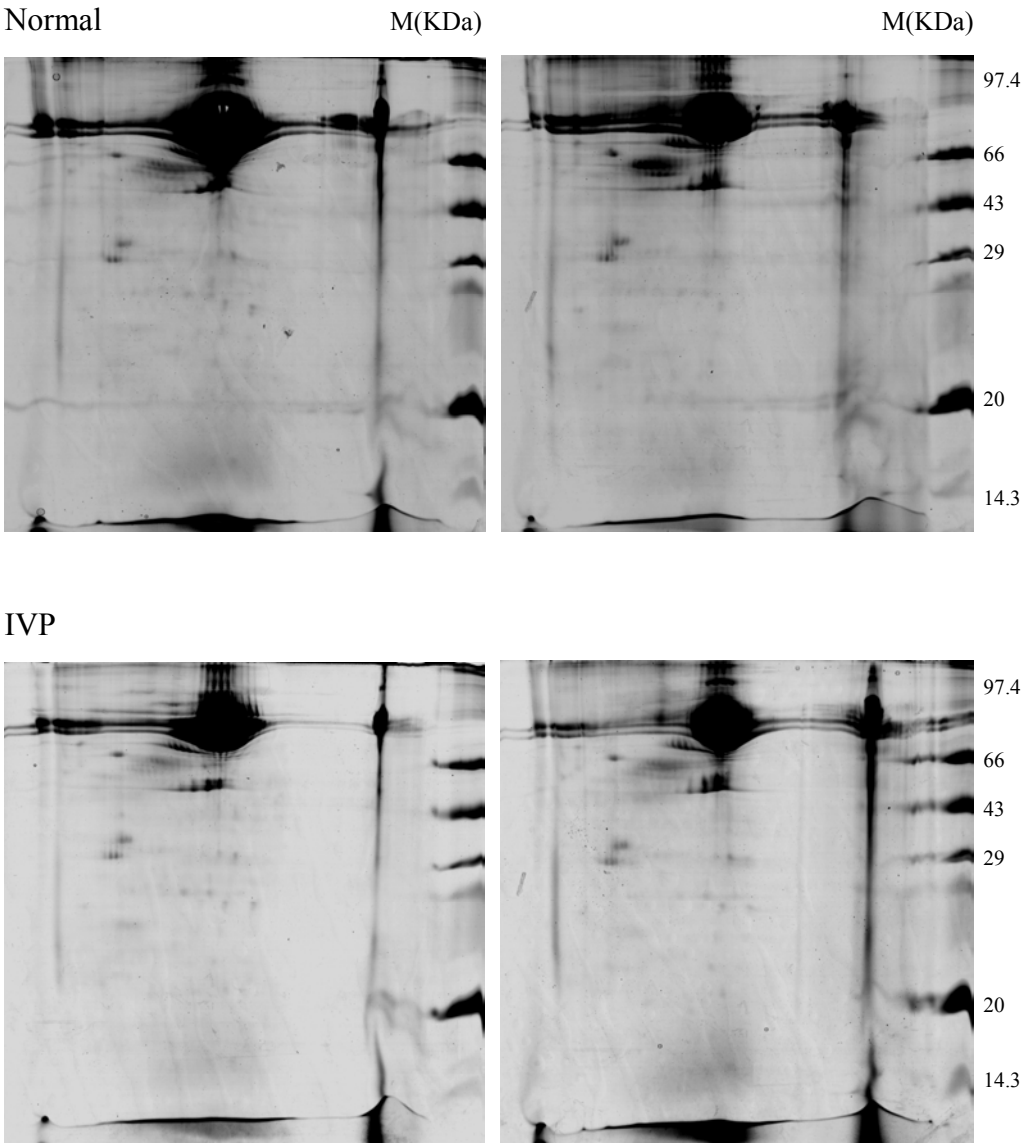
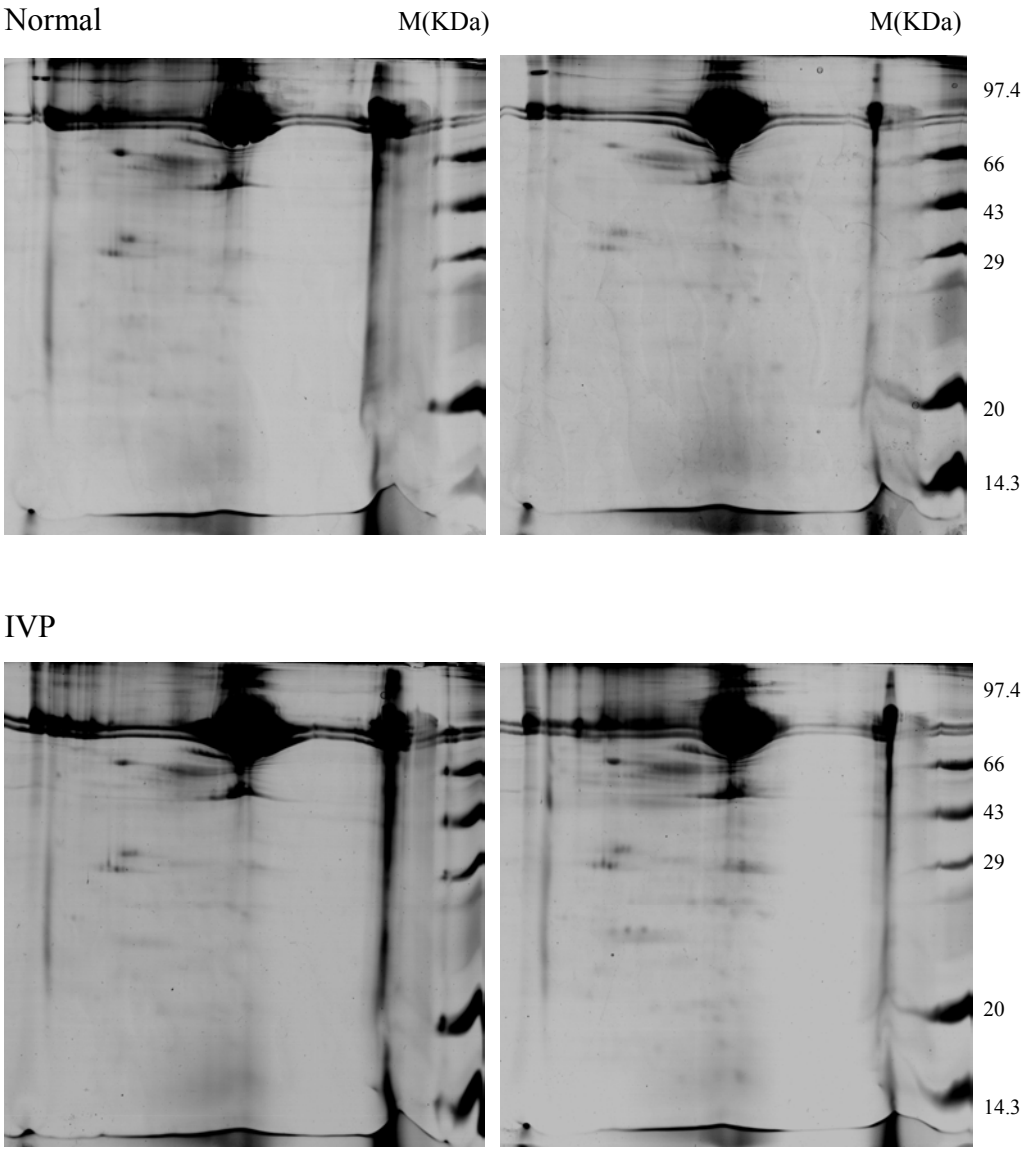


Fig.8. Gill 10DPA (pH 4-7)



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Chapter 3

NON-SPECIFIC IMMUNE RESPONSE OF *PENAEUS MONODON* TO IVP ADMINISTRATION

CHAPTER - 3

Non-specific immune response of *Penaeus monodon* to IVP administration

3.1. Introduction

Successful shrimp production requires the use of effective disease prevention strategies and a good understanding of the basic immune functions. Several factors such as water quality, disease, toxins, trace nutrients (astaxanthin, vitamins and minerals), probiotics, immune stimulants (β-glucan, peptidoglycan and lipopolysaccharide) and genetical make up have been shown to influence the immune mechanisms in *Penaeus monodon* (Supamattaya et al., 2006). Shrimp immune system involves actions and reactions against its pathogens by shrimps. The state of protection from infectious disease in shrimps includes the non-specific as well as the specific component which cannot be equated to a vertebrate immune mechanism.

It is observed that unlike vertebrate immunity which is composed of innate and adaptive responses, invertebrates rely solely on an efficient multiple innate defense reaction mechanism to combat infections. The innate immune system in shrimp, characterized by the lack of immunoglobulin, is efficient to protect and preserve them from all intruding pathogens or environmental antigens. The target recognition of innate immunity is the so-called pattern recognition molecules (PRMs) shared among groups of pathogens. Host organisms have developed the response to these PRMs by a set of receptors referred to as pattern recognition proteins or receptors (PRPs or PRRs) (Janeway, 1989). These patterns include the lipopolysaccharides (LPS) of Gram negative bacteria, the glycolipids of mycobacteria, the lipoteichoic acids of Gram positive bacteria, the mannans of yeasts, the β-1, 3-glucan of fungi and double-stranded RNAs of viruses (Hoffmann et al., 1999). Non-specific or the innate immune mechanisms in *P. monodon* involves the fixed and the mobile

defence mechanisms. The fixed non-specific immune mechanism involves the structural barriers which act as the first line of defence against pathogens. These include: hard cuticle, tegumental glands, epithelial immunity, branchial podocytes, autotomy of appendages, regeneration of appendages and rapid wound healing to prevent loss of haemolymph.

The mobile non-specific immune system has two main components, the humoral and cellular systems both of which are activated upon immune challenge (Liu., 2009). The cellular component involves those mediated by haemocytes and the humoral component involves those mediated by cell free haemolymph. The cellular and humoral immune mechanisms of the shrimp function synergistically to protect the shrimp and eliminate foreign particles and pathogens.

The cellular immune response in *Penaeus monodon* involves a number of different cell types including haemocytes and the fixed phagocytes (Supamattaya et al, 2006). *Penaeus monodon* possesses three different types of blood cells. According to ultrastructural features seen with electron microscopy (EM), *P. monodon* haemocytes can be divided into granular, semigranular and hyaline (or agranular) haemocytes (van de Braak, 2002).

The various cellular immune response include phagocytosis (Bachere et al., 1995; Itami et al., 1998; van de Braak et al., 2002b; He et al., 2004), apoptosis (Sahtout et al., 2001; Wang et al., 2008b), encapsulation (Sung et al., 2003; Bian and Egusa, 1981), nodule formation (Bian and Egusa, 1981; Nash et al., 1988), melanisation (Bian and Egusa, 1981; Nash et al., 1988) and cell adhesion (Sritunyalucksana et al., 2001; Lin et al., 2006).

The humoral response in shrimp is favoured by the different biological compounds in the haemolymph that inhibit or eliminate foreign bodies and pathogens. These include the pro Phenol Oxidase (pro PO) activating system

(Hernandez-Lopez et al., 1996; Vargas-Albores et al., 1996); the clotting cascade (Yeh et al., 1998; 1999); soluble pattern recognition proteins (PRPs) (He et al., 2004; Vargas-Albores and Yepiz-Plascencia, 2000) lectins (Luo et al., 2006; Rittidach et al., 2007; Yang et al., 2007; Ma et al., 2008), anti LPS factors (ALF) (Somboonwiwat et al., 2005; de la Vega et al., 2008), peptidoglycan-binding proteins, lipopolysaccharide (LPS)-binding proteins, beta 1,3-glucan-binding proteins (Romo-Figueroa et al., 2004; Jimenez-Vega et al., 2002; Cheng et al., 2005) etc.; the enzymes involved in the antioxidant defense mechanism - superoxide dismutase, peroxidase, catalase, nitric oxide synthase (Rameshthangam and Ramasamy, 2006;; Jiang et al., 2006., Mathew et al., 2007); defensive enzymes like lysozyme (Sotelo-Mundo et al., 2003), acid phosphatase (Chen et al., 1999), alkaline phosphatase (Jiang et al., 2004; Joseph and Philip, 2007), other molecules like haemocyanin (Adachi et al., 2003); reactive oxygen intermediates (ROI) (Munoz et al., 2000; Wang et al., 2006b); reactive nitrogen intermediates (RNI) (Jiang et al., 2006); alpha 2 macroglobulin (Gollas-Galvan et al., 2003; Rattanachai et al., 2004) and antimicrobial peptides (Destoumieux et al., 1997; Bachere et al., 2000; Munoz et al., 2002; Hu et al., 2006). Studies on the response of shrimps to pathogens at the gene and molecular level reveal that more immune related mechanisms are involved in pathogen exclusion.

Studies on the immunological responses of *Penaeus monodon* to DNA vaccine by analyzing the immune indices such as proPO, SOD, Respiratory burst, lysozyme and alkaline phosphatase activity showed a high level of proPO, SOD, lysozyme and alkaline phosphatase activity in the haemolymph of vaccinated group (without WSSV challenge) (Rajeshkumar et al., 2008; Li et al., 2010).

Various non-specific humoral immune mechanisms present in the hemolymph of experimental animals (*Penaeus monodon)* were analysed by

employing the following non-specific immune response indices, and the results are presented in this Chapter.

1. Total Haemocyte Count
2. Respiratory burst / ROI
3. pro PO activity
4. Lysozyme activity
5. α-2 macroglobulin activity
6. Transglutaminase activity
7. Superoxide dismutase
8. Catalase
9. Glutathione peroxidase
10. Glutathione s transferase
11. Acid phosphatase
12. Alkaline phosphatase
13. Lipid peroxide
14. Aspartate aminotransferase
15. Alanine aminotransferase
16. Nitric oxide synthase

3.2. Materials and Methods

3.2.1. Sample collection for non-specific immune assays

Haemolymph sample was collected using capillary tube rinsed with the anticoagulant (0.01M Tris-HCl, 0.25M Sucrose, 0.1M Tri Sodium Citrate, pH-7) from a set of experimental animals (6 animals of 10±1g) administered with the feed types (normal feed/ IVP) as described in Chapter 1. The samples were collected on the 3rd day post challenge after the 7 day administration of the test and control feeds, and the non-specific immune parameters such as total haemocyte count, phenol oxidase activity and reactive oxygen intermediate production / respiratory burst activity were determined within 8hrs. Aliquots of haemolymph were also stored at -80ºC for analyzing the remaining non-specific immune response parameters.

3.2.2. Non-Specific Immune Assays

3.2.2.1. Total Haemocyte Count

Total haemocyte count (THC) was determined by using a Neubaeur's haemocytometer. A drop of anticoagulant-haemolymph mixture was placed on

the haemocytometer and haemocytes were counted by observing under a bright field microscope and expressed as cells mL^{-1} haemolymph.

3.2.2.2. Phenoloxidase (PO) activity

Phenol oxidase activity was determined by incubating 100µL of haemolymph with 100µL of 10% SDS for 30 min. at 25°C and by the addition of 2.0 mL of substrate (0.19% L-DOPA in Tris-HCl buffer). The dopachrome formed was measured in a UV-Visible spectrophotometer at 490 nm, at every 30s for 3 mins. and the activity expressed as increase in absorbance $minute^{-1}$ 100 μL^{-1} haemolymph (Soderhall et al., 1981).

3.2.2.3. Respiratory Burst Activity

Intracellular superoxide anion (O_2^{-}) or the reactive oxygen intermediates (ROI) or the respiratory burst activity was measured by the reduction of Nitro blue tetrazolium (NBT) (Song and Hsieh, 1994) as described by Chang et al. (2000). For this assay, a sample of 100µL haemolymph was incubated with 100µL 0.2% NBT for 30 mins. at 10°C. The cells were separated by centrifugation (1200rpm, 4ºC, 10 mins.) and fixed in 100% methanol. It was then incubated for 10mins. at room temperature (RT, 28ºC) and subjected to centrifugation (300rpm, 4ºC, 10 mins.). The supernatant was removed after centrifugation and the cells were dried and then rinsed in 50% methanol and solubilised in 140µL DMSO and 120µL 2M KOH. The absorbance at 620nm was recorded and the activity expressed as O.D. 100μL^{-1} haemolymph.

3.2.2.4. Lysozyme activity

Lysozyme activity was determined by a turbidometric assay (Acharya et al., 2004) where in the bacterium - *Micrococcus luteus (lysodeikticus)*- exquisitely sensitive to lysozyme, when exposed to the enzyme, lyses it rapidly. Lysozyme activity was monitored by a decrease in absorbance at 450nm, as organisms lyse in the cuvette and the transmitted light is directly proportional to

the lysozyme concentration. Briefly, a 50µL haemolymph sample was mixed with 150µL suspension of *Micrococcus lysodeikticus* (0.2mg/mL) in a microplate and absorbance measured at 450nm, every 2 min. for 30 min. and the activity expressed as difference of absorbance at initial and at 10min. $100\mu L^{-1}$ haemolymph.

3.2.2.5. α-2 macroglobulin activity

The α-2 macroglobulin activity was determined (Gollas-Galvan et al., 2003) based on its ability to inhibit trypsin's ability to hydrolyse proteins but allowing trypsin's ability to hydrolyze low molecular mass 'amide substrates' such as BAPNA (N-benzoyl-DL-arginine-p-nitroanilide). For the assay, a 50µL sample was mixed with 10µL trypsin (1mg/mL) and incubated for 15 min. at 37°C. It was then mixed with 10µL soyabean trypsin inhibitor and again incubated for 10 min. at 37°C, and 100µL BAPNA (N-benzoyl-DL-arginine-p-nitroanilide) (1mg/mL) in 50mM Tris having pH8 was added to the reaction mixture and incubated for 2hr at 37°C. The absorbance was measured at 415nm in a microplate reader (TECAN Infinite^{TM}, Austria) and expressed as OD $100\mu L^{-1}$ haemolymph.

3.2.2.6. Transglutaminase activity

Transglutaminase activity was determined by the colorimetric hydroxamate assay according to Folk and Cole (1965) method as described in Montero et al. (2005). Briefly, a 100µL haemolymph sample was mixed with a solution containing 100µL 1M Tris-acetate buffer (pH-6), 100µL 0.1M CBZ-L-glutaminyl glycine, 25µL 0.1M $CaCl_2$, 25µL 2M hydroxylamine and 25µL 0.02M Na_2EDTA. The mixture was then incubated at 37°C for 10min. and 0.75mL of ferric chloride – trichloroacetic acid added to stop the reaction. It was then centrifuged at 4000g for 15 min. and the absorbance of the supernatant taken at 525nm and expressed as O.D. $100\mu L^{-1}$ haemolymph.

3.2.2.7. Superoxide dismutase

Superoxide dismutase activity was determined according to Marklund and Marklund (1974) as described in Rajeshwari et al. (2007) where the rate of autooxidation of pyrogallol (benze 1-2-3 –triol) is inhibited by the presence of superoxide dismutase. Briefly, a 0.1mL haemolymph sample was mixed with 0.05mL ethanol and 0.03mL chloroform and shaken for 15 min. Centrifugation was carried out at 12,000xg for 15 mins. and the supernatant taken for the assay. The absorbance of a mixture of 25µL 50mM Tris –HCl (pH 8.2), 25µL distilled water, 75µL supernatant and 2mM pyrogallol was taken at 470nm at 1 min interval for 3 min. in a microplate reader (TECAN Infinite^{TM}, Austria) and expressed as change in absorbance/min. 100µL^{-1} haemolymph.

3.2.2.8. Catalase

The absorbance of hydrogen peroxide at 240nm is measured directly to calculate the reaction rate of catalase (Rajeshwari et al., 2007). In the presence of catalase, the reaction rate is proportionally (linearly) enhanced. Briefly, 125µL potassium phosphate buffer was mixed with 75µL 15mM H_2O_2 and 10µL haemolymph and the absorbance was taken at 240nm in 30sec. interval for 3mins. and expressed as change in absorbance/min. 100µL^{-1} haemolymph.

3.2.2.9. Glutathione peroxidase

Glutathione peroxidase activity was determined by the amount of enzyme that converted reduced glutathione to oxidized glutathione in the presence of hydrogen peroxide which was detected by the reduction of 5,5 dithiobis 2 nitro benzoic acid (DTNB). The assay was done as described in Rajeshwari et al. (2007). Briefly, 100µL sodium phosphate buffer (pH 7), 20µL 10mM Sodium azide, 200µL 4mM reduced glutathione, 20 µL 2.5mM H_2O_2, 100 µL haemolymph and 280 µL distilled water were mixed and incubated at 37ºC for 3min. One hundred µL 10% TCA was added to terminate the reaction and the mixture was centrifuged at 8000rpm for 10mins. One hundred µL of the

supernatant was taken in a microplate and mixed with 75µL 0.3M disodium hydrogen phosphate and 25µL 10mM DTNB and the absorbance was taken at 412nm at 30s interval for 3mins. and expressed as change in absorbance min^{-1} 100 μL^{-1} haemolymph.

3.2.2.10. Glutathione-s-transferase

Glutathione-s-transferase (GST) activity was determined by following the method described in Rajeshwari et al. (2007). 1-chloro-2, 4-dinitrobenzene (CDNB) is a synthetic GST substrate and the GST assay measures the conjugation of CDNB with reduced glutathione. This conjugation increases the wavelength of maximum absorption to 340nm and the increase in absorbance correlates to the amount of glutathione in the sample. 100µL potassium phosphate buffer (pH-7) was mixed with 50µL haemolymph, 20µL distilled water, 25µL CDNB and incubated at 37ºC for 10mins 25µl of 30mM reduced glutathione was added to the reaction mixture and the absorbance read at 340nm in 30sec interval for 3mins. and expressed as change in absorbance/min 100μL^{-1} haemolymph.

3.2.2.11. Acid phosphatase

Acid phosphatase activity of the haemolymph was determined as described by Bisswanger (2004) with o-carboxyphenyl phosphate, under acid conditions. Briefly, a 20µL haemolymph sample was mixed with 140µL 0.15M Acetate (pH-5) and 40µL 3.65mM o-carboxyphenyl phosphate in a UV microplate and incubated at 25ºC for 3 min. The absorbance was taken at 300nm at 1min interval for 6min. and expressed as change in absorbance/min 100μL^{-1} haemolymph.

3.2.2.12. Alkaline phosphatase

Alkaline phosphatase activity was determined by the hydrolysis of the chromogenic substrate p-nitrophenyl phosphate to p-nitrophenol by the enzyme as described by Bisswanger (2004). Briefly, 170 µL 0.1M Glycine-KOH (pH-

10.5) was mixed with 10µL 0.5M p-nitrophenyl phosphate and 2µL haemolymph in a microplate and the absorbance taken at 405 nm at 1min interval for 6 min and expressed as change in absorbance/min. $100\mu L^{-1}$ haemolymph.

3.2.2.13. Lipid peroxide

Lipid peroxide in haemolymph was determined as per Ohkawa et al. (1979) in which the lipoprotein was precipitated by trichloroacetic acid, and only the water-soluble malondialdehyde present in the supernatant estimated by its reaction with thiobarbituric acid (TBA) and the resultant thiobarbituric acid reactive substances (TBARS) were measured. Briefly, a 100µL haemolymph sample was mixed with 1mL 20%TCA and incubated at 25°C for 20 min. The mixture was centrifuged at 500xg for 10min. An aliquot of 0.75mL of the supernatant was mixed with 0.25mL 0.8% Thiobarbituric acid (TBA) and heated in a boiling water bath for 1 hr. 1mL n-butanol was added to the above mixture and centrifuged at 4000rpm for 10 min. 100µL of the organic layer was taken in a microplate and the absorbance was measured at 532nm and expressed as absorbance $100\mu L^{-1}$ haemolymph.

3.2.2.14. Aspartate aminotransferase and alanine aminotransferase

Haemolymph aspartate aminotransferase and alanine aminotransferase activity levels were determined as per Reitman and Frankel (1957). The reaction mixture containing 93µL 0.25M α-ketoglutarate, 890µL 0.1M L-Aspartic acid / 0.1M DL-Alanine (for aspartate aminotransferase and alanine aminotransferase respectively), 55.4µL 0.277mM pyridoxal, 44µL 0.01M Arsenate, 106.6µL potassium phosphate buffer (pH-7.4) and 30µL haemolymph were incubated for 30mins. The reaction was stopped by adding 2µL of 0.1% 2, 4 dinitrophenyl hydrazine and centrifuged at 5000xg for 3mins. Fifty µL of the supernatant was mixed with 150µL 1.3M NaOH and the absorbance measured at 440nm and expressed as O.D. $100\mu L^{-1}$ haemolymph.

3.2.2.15. Nitric oxide synthase activity

As nitric oxide synthase (NOS) causes the conversion of arginine to L-citrulline and NO, NOS activity was examined by determining the L-citrulline and total nitrite in the sample. L-citrulline was determined according to Jiang et al. (2006). Breifly, a 100µL haemolymph sample was mixed with 50µL 165 U mL^{-1} urease and incubated at 37ºC for 30mins. The reaction was stopped by adding 50µL 2.45M TCA and centrifuged at 13,000xg for 15mins. at room temperature. 50µL of the supernatant was mixed with 150µL ADMS reagent, incubated in boiling water bath for 25mins, cooled to room temperature (28ºC) in dark, centrifuged at 13,000xg for 5 mins. and the absorbance was read at 450nm and expressed as O.D. $100\mu L^{-1}$ haemolymph.

Nitrite was determined by formation of reddish purple azo dye produced at pH 2 to pH 2.5 by coupling diazotized sulphanilamide and N-(1-naphthyl)-ethylene diamine dihydrochloride (NED). For the reaction, a 100µL haemolymph sample was mixed with 900µL Milli Q water, 20µL sulphanilamide and 20µL NED and incubated for 10mins. The absorbance was taken at 543nm and expressed as O.D. $100\mu L^{-1}$ haemolymph.

3.2.3. Statistical Analysis

The data collected were analyzed statistically by one way and two way ANOVA. Wherever the treatment mean was found to be significant at Least Significant Difference (LSD) at 5% level, significant difference between the groups were calculated using the formula,

$$LSD = \sqrt{2} \times Ve / r \times 5\% \text{ table t value}$$

where, r = no. of samples / treatment

Ve = Mean Square within the group

Differences were considered statistically significant at $p < 0.05$.

3.3. Results

Level of significance in the variation of non-specific immune parameters of *Penaeus monodon* with in the groups (unchallenged and challenged) was obtained by one-way ANOVA and between the groups (unchallenged and challenged on 5th and 10th day post administration) was obtained by two way ANOVA as tabulated in Table 1 and Table 2, respectively.

3.3.1. Within the groups - Unchallenged group comprising normal and IVP (C0); normal and IVP challenged on 5th day post administration (C5) and normal and IVP challenged on 10th day post administration (C10)

3.3.1.1. Unchallenged group (Normal- N0, IVP- I0)

Non-specific immune parameters such as reactive oxygen intermediates (ROI) and lipid peroxide showed significant differences within the group (one way ANOVA at $p<0.05$). ROI, and lipid peroxide levels were higher in the IVP administered group with out challenge than in the group of animals fed on normal diet, (Table 1 and 3)

3.3.1.2. Challenged group

3.3.1.2.1. Challenged on 5th day post administration (Normal- NC5, IVP- IC5)

In the group which was challenged on 5th day post administration and sampled on 3rd day post challenge (Normal- NC5, IVP- IC5), total haemocyte count and phenol oxidase activity showed significant differences within the group (one way ANOVA at $p<0.05$). In this case total haemocyte count and phenol oxidase activity were higher in the IVP administered and challenged group on 5th day than that of the normal (Table 1 and 3)

3.3.1.2.2. Challenged on 10th day post administration (Normal- NC10, IVP-IC10)

In the group which was challenged on 10th day post administration and sampled on 3rd day post challenge (Normal- NC10, IC10), no significant differences in non-specific immune parameters were observed between the treatments at $p<0.05$ (Table 1 and 3).

3.3.2. Between the groups, unchallenged group comprising normal and IVP (C0); normal and IVP challenged on 5th day post administration (C5) and normal and IVP challenged on 10th day post administration (C10)

The analysis of non-specific immune parameters between the groups (C0, C5, C10), by two way ANOVA showed significant difference ($p<0.05$) only in total haemocyte count (THC). The THC was higher in the group C0, unchallenged, followed by 5th post administration challenged and least on 10th day post administration challenged. The THC did not show significant difference between the unchallenged group (C0) and the group challenged on 5th day post administration (C5). Same is the situation between the THC in the unchallenged group (C0) and the group challenged on 10th day post administration (C10) (Table 2 and 3).

3.3.3. Between the groups (Normal (N) and IVP (I) each comprising unchallenged group and challenged group on 5th day and 10th day post administration)

Non-specific immune parameters like ROI and transglutaminase showed significant difference ($p<0.05$) between the groups N and I in statistical two way ANOVA. IVP administered group showed significantly higher ROI and transglutaminase than the normal feed administered group.

3.3.4. Non-specific immune parameters which do not show significant difference ($p<0.05$) within the group and between the groups.

Non-specific immune parameters like superoxide dismutase, catalase activity, glutathione s transferase, nitric oxide synthase, glutathione peroxidase activity, alpha 2 macroglobulin activity, lysozyme activity, acid phosphatase activity, alkaline phosphatase activity, aspartate aminotransferase activity and alanine aminotransferase activity did not show significant difference in the different groups (N0, I0; NC5, IC5; NC10, IC10; C0, C5, C10; N, I) analyzed.

3.4. Discussion

Research on shrimps has shown that the basic mechanisms such as direct sequestration and killing of infectious agents which involve synthesis and exocytosis of a battery of bioactive molecules operate through the open circulating system (circulating haemolymph comprising different types of haemocytes) play extremely important roles in the immune defence mechanism. Essentially, the haemocytes execute inflammatory-type reactions such as phagocytosis, haemocyte clumping, production of reactive oxygen metabolites and the release of microbicidal proteins (Smith et al., 2003). Hence, haemolymph of the experimental animals were analyzed for the non-specific immune response in *P. monodon* IVP administration and challenge with WSSV.

3.4.1. Within the groups of animals unchallenged under normal diet and IVP administered (N0, I0); challenged on 5th day post administration (NC5, IC5); challenged on 10th day post administration (NC10, IC10)

3.4.1.1. Unchallenged under normal diet and IVP administered (N0, I0)

In the present study, the reactive oxygen intermediates (ROI) and lipid peroxide in the experimental animals (*P.monodon*) after the administration of

IVP (I0) (before challenge) was significantly higher than in the normal feed administered animals.

Song and Hsieh (1994) demonstrated in vitro the phenomenon known as respiratory burst which could elicit a respiratory burst in *P. monodon* haemocytes, thought to be related to phagocytosis, when shrimp responded to immunostimulants such as PMA, zymosan and β-glucan. The amount of microbicidal substances generated by shrimp haemocytes were similar to those observed in fish macrophages (Lee and Shiau 2004). The stimulation of the phagocytic cell membrane leads to increased consumption of oxygen, the reduction of which, catalysed by a membrane-bound enzyme, NAD(P)H oxidase, gives rise to $O2^{-}$ (Lee and Shiau, 2004). Since $O2^{-}$ is the first product to be released from the respiratory burst, the measurement of $O2^{-}$ has been accepted as a direct and accurate way of measuring respiratory burst activity (Citarasu et al., 2006). An increase in ROI has been observed in *P.monodon* administered with specific amount (lesser / higher amount caused immune defect) of feed incorporated with β 1,3 glucan, certain Indian herbs, Vitamin C with its derivatives, copper and Vitamin E (Chang et al., 2000, 2003; Citarasu et al., 2006; Lee and Shiau, 2002a, 2002b, 2003, 2004). Liu et al. (2006c) has observed that the administration of sodium alginate has decreased its ROI.

Lipid peroxidation has been reported to inactivate membrane bound enzymes because oxidation of 'SH' groups present in the active sites leads to conformational alteration in the enzymes (Kako et.al., 1988). Lipid peroxidation and consequent tissue damage are the major problems associated with failure of the antioxidant system. Lipid peroxides are themselves free radicals with large reaction constants and will therefore lead to cell death. Kidd (1991) indicate that peroxides, hydroxyradicals etc. can attack molecules like DNA, RNA, enzyme, protein, phospholipids etc. and then damage membrane integrity. Increased lipid peroxidation can lead to production of malondialdehyde (MDA) that enhances

the formation of free radicals from polyunsaturated fatty acids in cell membranes. Viral infection seems to result in an alteration in the cellular activity, which leads to the dysfunction of the complex antioxidant system. Pathogenic stress such as bacterial, fungal and viral infection can induce peroxidation of membrane lipids (Chih-Hong et.al., 2003). WSSV infection in *P.monodon* caused a significant increase in the activity of lipid peroxidation in all tissues and a substantial decrease in the activity of antioxidants (superoxide dismutase (SOD); catalase (CAT); glutathione peroxidase (GPx) and glutathione-s-transferase (GST)) (Mohankumar and Ramasamy, 2006a, 2006b; Mathew et.al., 2007). Enhanced levels of lipid peroxidation in WSSV infected animals indicate that they are under high oxidative stress. Mathew et al. (2007) has found a sharp increase in the concentration of lipid peroxides in hemolymph of WSSV infected *P.monodon*.

The significant difference observed in *P. monodon* administered with test feed IVP and those studies where the animals were administered with other feed additives, is the activation of non-specific immune parameters like THS, PO activity and SOD activity along with the ROI. Though the lipid peroxide was significantly higher in IVP administered group, the antioxidant activity (involving SOD, catalase, glutathione peroxidase, and glutathione-s-transferase) of the same did not show significant differences from the normal feed administered group. However, the significant activation of ROI and lipid peroxide in IVP administered group (unchallenged) than in control, unlike in other feed additive studies, shows that the IVP administered had a different mechanism of immune system activation in these animals.

3.4.1.2. Challenged on 5th day post administration (NC5, IC5)

In the group which was challenged on 5th day post administration of different feeds (normal-NC5, IVP-IC5) and sampled on 3rd day after challenge, THC and phenol oxidase activity showed significant difference between NC5 and IC5.

The THC and phenol oxidase activity in the IVP administered group (IC5) was significantly higher than in the normal feed administered (NC5) group.

Total Haemocyte Count (THC) refers to the generally accepted three major categories of blood cells (haemocytes) in decapod crustaceans such as hyaline cells, semi-granular cells and granular cells. Each has distinctive morphological features and physiological functions (Johannson et al., 2000). Haemocytes are responsible for clotting, exoskeleton hardening and elimination of foreign materials (Song and Hsieh, 1994). Mean THCs of healthy penaeid shrimps ranged from 20 to 40 x 10^6 cells $ml^{-}1$ (Chang et al., 1999). Molting, development of organs, reproductive status, nutritional condition and disease have been shown to influence haemocyte abundance (Cheng and Chen, 2001). A low circulating haemocyte count is strongly correlated with a greater sensitivity to pathogens (Persson et al., 1987). It has been reported by Chang et al. (2003) and Citarasu et al. (2006) that the WSSV infection by injection has caused a decrease in the THC.

The phenol oxidase system is the prime immune defence of invertebrates and any reductions in the activity of phenol oxidase enzyme might lead to the failure of phagocytosis, which is the common process in the cellular defence of crustaceans. The prophenoloxidase activating system (proPO system) has an important role as a non-self recognition system and participates in the innate immune response through melanization, cytotoxic reactions, cell adhesion, encapsulation, nodule and capsule formation, hemocyte locomotion and phagocytosis (Soderhall and Cerenius, 1998; Lee and Soderhall, 2002; Cerenius and Soderhall, 2004; Jiravanichpaisal et. al. 2006; Rajeshkumar, 2008). Phenol oxidase (PO), the key enzyme in the synthesis of melanin, occurs in haemolymph as an inactive pro-enzyme pro phenol oxidase (proPO). proPO is activated to form PO when it reacts with zymosan (carbohydrates from yeast cell walls), bacterial lipopolysaccharide (LPS), urea, calcium ion, trypsin or heat

(Chang et al., 2003). Though the exact mechanism by which WSSV affects phenol oxidase enzyme system in *P.monodon* is still unknown, studies in other crustaceans suggest that viral infection mediated oxidative stress is a major factor responsible for the reduction of phenol oxidase activity (Le Moullac et.al., 1998; Cheng et.al., 2002). A drastic reduction in phenol oxidase activity was noticed in WSSV infected animals by Mathew et.al. (2007). Increase in PO activity has been reported in *P.monodon* (after challenge) after the administration of beta glucan (Chang et al., 2003) immunostimulant herbs (Citarasu et al., 2006) and 'DNA vaccination' (Rajeshkumar et al., 2008).

From the activation of phenol oxidases generating highly cytotoxic quinines that might inactivate viral pathogens (Ourth and Renis, 1993), and the significant high THC observed on 5th day post administration challenge group, it could be inferred that the IVP administered animals showed lower WSSV infection rate as compared to the infection rate in the normal feed administered group.

3.4.1.3. Challenged on 10th day post administration (NC10, IC10)

In the group which was challenged on 10th day post administration and sampled on 3rd day post challenge (Normal- NC10, IVP- IC10), the absence of significant difference in the non-specific immune parameters showed that there was no significant variation between the treatments (normal feed administered and IVP administered) on the 10th day. This suggests the subsiding efficacy of IVP over a period of time.

3.4.2. Between the unchallenged (C0) and challenged groups on 5th day post administration (C5) and 10th day post administration C10)

Significant difference was observed in *P. monodon* (comprising IVP and normal feed administered) before and after WSSV challenge only in total haemocyte count.

Among them, the total haemocyte count (THC) was the highest in unchallenged group (C0) followed by the 5th day post administration challenge (C5) group and the 10th day post administration challenge (C10) group. Significant higher THC was observed in the unchallenged group than in 10th day post administration challenge (C10) group but with 5th day post administration challenge (C5) the difference was not significant. This observation parallel to the findings of Chang et al. (2003) and Citarasu et al. (2006) that a decrease in the THC could be caused by WSSV infection. The lack of significant difference between the unchallenged group and the group challenged on 5th day post administration may be because of the oral route of challenge adopted in the present study which in-turn might have taken a prolonged time for infection compared to injection challenge. However, the 5th day post administration challenged group did not show significant difference from 10th day post administration group. Possibly, a reduction in THC subsequent to challenge both in normal and IVP administered group suggests that WSSV may be acting on the heamocytes inspite of IVP administration reducing their count.

3.4.3. Between the normal feed (N) and IVP (I) administered

Between the groups, IVP (I) and normal (N) feed administered (comprising unchallenged and challenged), ROI and transglutaminase showed significant difference. ROI and transglutaminase was significantly higher in IVP administered *P. monodon* than in the normal feed administered group which was similar to the response shown to feed additives (Chang et al., 2000; Chang et al., 2003; Lee and Shiau, 2002a; 2002b; 2003; 2004; Citarasu et al., 2006).

Coagulation of haemolymph is part of the innate immune response of crustaceans which prevents loss of body fluids and entry of opportunistic pathogens. In shrimps, coagulation is initiated by activation of hyaline cells which releases its contents including the clotting enzymes. Transglutaminase (TGase) in the haemocytes effectively polymerizes shrimp clottable proteins to

form stabilized gel (Yeh et al., 2006). A decrease in the transglutaminase activity in WSSV infected *Fenneropenaeus chinensis* by RT-PCR analysis (Liu et al., 2007b) and delay in clotting time in *F. indicus* has been reported (Yoganadhan et al., 2003b; Sarathi et al., 2007). Song et.al. (2003) has observed that the decrease in TGase activity coincided with poor hemolymph coagulation in *Litopenaeus vannamei* infected with Taura syndrome virus. The present study shows that in *P. monodon*, administered with IVP, there was significantly higher transglutaminase activity there by causing an effect on the coagulation mechanism.

The non-specific immune response of *P.monodon* after the challenge cannot be compared to previous studies related to feed additives followed by a WSSV challenge. This is due to the fact that in these studies, the route of challenge has been different i.e., injection (Chang et al., 2003; Citarasu et al., 2006). Moreover, a single defined nature of WSSV challenge (eg: virus titre / strain, type of infected tissue, dose of infected tissue used, nature / frequency / time / route of challenge etc.) has not been used in studies analyzing the non-speific immune parameters in response to WSSV infection in *P. monodon* (Chang et al., 2003; Citarasu et al., 2006; Rameshthangam and Ramasamy, 2006; Mathew et al., 2007). Rajeshkumar et al. (2008) has not mentioned a defined nature of challenge in their report of non-specific immune parameters in 'DNA vaccinated' *P. monodon*. In these experiments using *P. monodon,* another factor which varies is the day of sampling after the challenge.

However, in the present study significant difference was not observed in the non-specific immune parameters like SOD activity, catalase activity, glutathione-s-transferase, glutathione peroxidase activity, nitric oxide synthase, alpha 2 macroglobulin activity, acid phosphatase activity, alkaline phosphatase activity, lysozyme activity, aspartate aminotransferase activity and alanine aminotransferase activity. These immune indices which are reported to be active

in their respective studies may not be functional in a significant way for the immune response of *P. monodon* against WSSV as seen in the present study.

All together, it is inferred that *P. monodon* when administered with IVP, adopts a unique immune mechanism with an inter play of various pathways which has to be explored in a deeper and wider perspective.

Table 1. Level of significance of non-specific immune parameters of *Penaeus monodon* within the groups determined by one-way ANOVA (p-value < 0.05)

Sl. No.	Phenotype	Unchallenged (N0, I0)	Challenged on	
			5DPA (NC5, IC5)	10DPA (NC10, IC10)
1.	Total Haemocyte Count	0.077697	*0.047688	0.436918
2.	Phenol oxidase activity	0.900577	*0.025084	0.330355
3.	Reactive oxygen intermediates (ROI)	*0.010231	0.324749	0.953653
4.	Transglutaminase	0.112157	0.591145	0.346203
5.	Superoxide dismutase	0.373255	0.355232	0.771850
6.	Glutathione-s-transferase	0.366706	0.928043	0.315266
7.	Nitric oxide synthase	0.196407	0.288193	0.526846
8.	Catalase	0.473069	0.099648	0.511446
9.	Glutathione peroxidase	0.772011	0.227407	0.888205
10.	Lysozyme activity	0.821316	0.358730	0.256660
11.	Alpha 2 macroglobulin	0.745041	0.078217	0.481408
12.	Acid phosphatase	0.635311	0.100192	0.390267
13.	Alkaline phosphatase	0.537119	0.057566	0.127934
14.	Lipid peroxide	*0.041367	0.988517	0.584691
15.	Aspartate aminotransferase	0.668668	0.172116	0.393692
16.	Alanine aminotransferase	0.278359	0.371495	0.326886

* - significant difference (p-value < 0.05)

Table 2. *p*-value of non-specific immune parameters of *Penaeus monodon* (between the groups) determined by two-way ANOVA

Sl. No.	Phenotype	Unchallenged (C0) and Challenged (C5, C10)	N, I
1.	Total Haemocyte Count	*0.023990	0.745891
2.	Phenol oxidase activity	0.142214	0.064373
3.	Reactive oxygen intermediates (ROI)	0.056990	*0.017629
4.	Transglutaminase	0.078330	*0.048097
5.	Superoxide dismutase	0.312304	0.337821
6.	Glutathione-s-transferase	0.425096	0.283200
7.	Nitric oxide synthase	0.364233	0.359184
8.	Catalase	0.340420	0.349084
9.	Glutathione peroxidase	0.687518	0.287790
10.	Lysozyme activity	0.399615	0.298218
11.	Alpha 2 macroglobulin	0.787786	0.306189
12.	Acid phosphatase	0.546694	0.191602
13.	Alkaline phosphatase	0.237333	0.392147
14.	Lipid peroxide	0.491364	0.225949
15.	Aspartate aminotransferase	0.145564	0.097107
16.	Alanine aminotransferase	0.290019	0.076295

* - significant difference (*p*-value < 0.05)

Table 3. The order of significance in the experimental groups

Sl. No.	Phenotype	Order of Significance (as obtained from Least Significant Difference – LSD at 5% level)
Within the groups (N0, I0)		
1.	Reactive Oxygen Intermediates (ROI)	I0 > N0
2.	Lipid peroxide	I0 >N0
(NC5, IC5)		
3.	Total Haemocyte Count (THC)	IC5 > NC5
4.	Phenol oxidase activity	IC5 > NC5
Between the groups - (C0, C5, C10)		
1.	Total Haemocyte Count (THC)	C0 > C10, {C0 = C5, C5 = C10}
Between the groups - (N, I)		
1.	Reactive oxygen intermediates (ROI)	I > N
2.	Transglutaminase	I > N

N0 – Normal feed administered, unchallenged
I0 – IVP administered, unchallenged
NC5 – normal feed administered, IC 5- IVP administered - animals which were challenged with WSSV on 5th day post administration and sampled on 3rd day post challenge
NC10– normal feed administered, IC10- IVP administered - animals which were challenged with WSSV on 10th day post administration and sampled on 3rd day post challenge
C0 – Unchallenged animals
C5 – Challenged with WSSV on 5th day post administration and sampled on 3rd day post challenge
C10 – Challenged with WSSV on 10th day post administration and sampled on 3rd day post challenge
N – Normal feed administered animals
I – IVP administered animals

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Chapter 4

BIO-DEFENCE GENES IN IMMUNOMODULATION OF IVP ADMINISTERED *PENAEUS MONODON*

CHAPTER - 4

Bio-defence genes in immunomodulation of IVP administered *Penaeus monodon*

4.1. Introduction

Knowledge of immune gene expression in response to pathogens is of prime importance to understanding the immune capability of shrimps and also for the establishment of a health monitoring system in shrimp culture. The molecular mechanisms that are underlying the majority of antiviral responses in shrimps are still unknown and are only beginning to be addressed (Liu et al., 2009). Genomic approaches have been used to characterize immune genes in different shrimp species. Expressed sequence tag (EST) programs have been developed from different tissues of shrimps. Suppression subtractive hybridization was used to isolate genes differentially expressed in shrimps. Expression profiles of selected genes have been analysed by real time PCR and micro-array using the relative expression to a constitutive gene (elongation factor-1 alpha).

Tissue specific expression based on ESTs of cephalothorax, eyestalk, hepatopancreas, haematopoietic tissue, pleopods, haemocytes, lymphoid organ and ovary of *P. monodon* has been reported (Lenhert et al., 1999; Supungul et al., 2002; Tassanakajon et al., 2006). Several immune genes, encoding proteins involved in the general biodefense have been discovered and differential gene expression analyzed in penaeid shrimps, *Penaeus monodon* (de la Vega et al., 2006; 2007; Jiravanichpaisal et al., 2007), *Litopenaeus vannamei* (Wang et al., 2007c, 2008c), *Litopenaeus setiferus* (Gross et al., 2001) and *Penaeus japonicus* (He et al., 2004). The gene expression profile of shrimp in response to white spot syndrome virus, yellow head virus, *Vibrio* spp., peptidoglycan, oxytetracycline, oxolinic acid, salinity, and temperature using the high-throughput microarray analysis has been reviewed by Aoki et al. (2010).

The immune related genes identified in *Penaeus monodon* include clottable protein (Yeh et al., 1998; Yeh et al., 1999); transglutaminase (Huang et al., 2004; Yeh et al., 2006); proPO (Sritunyalucksana et al., 1999); β-1,3-glucan binding protein (Sritunyalucksana et al., 2002); *Penaeus monodon* LPS binding lectin - PmLec (Luo et al., 2006); penaeidin-5 (Hu et al., 2006); clip domain serine protease homolog, c-SPH (Lin et al., 2006); heat shock cognate 70, hsc70 (Chuang et al., 2007); heat shock protein 86, hsp86 (Cimino et al., 2002); hsp90 (Jiang et al., 2009); haemocyanin (Colangelo et al., 2004; Zhang et al., 2004b); alpha 2 - macroglobulin (Lin et al., 2007); crustin (Chen et al., 2004); kazal-type serine proteinase inhibitor (Somprasong et al., 2006); astakine (Soderhall et al., 2005); peroxinectin (Sritunyalucksana et al., 2001); haemocyanin (Lenhert et al., 2002); antilipopolysaccharide factors, ALF (Supungul et al., 2002; Tharntada et al., 2008); inhibitor of apoptosis protein, IAP (Leu et al., 2008); Translationally controlled tumor protein, TCTP (Bangrak et al., 2004); ribophorin I (Chotwiwatthanakun et al., 2008); Cathepsin C (Qui et al., 2008); Dicer-1 (Su et al., 2008); *Penaeus monodon*'s Argonaute, Pem-AGO (Dechklar et al., 2008); *Penaeus monodon* Toll, PmToll (Arts et al., 2007b); Crustin-like antimicrobial peptide (Amparyup et al., 2008); lysozyme (Xing et al., 2009); Ribophorin 1 (Chotwiwatthanakun et al., 2008; Molthathong et al., 2008a); Defender against apoptotic death - DAD1 (Molthathong et al., 2008b) and cyclophilin A (Qiu et al., 2009).

Gene expression studies by EST analysis in response to infection with WSSV has been undertaken in *Penaeus japonicus* (Rojtinnakorn et al., 2002), *Penaeus stylirostris* (Astrofsky et al., 2002), *Fenneropenaeus chinensis* (Wang et al., 2006a), *Litopenaeus vannamei* (Reyes et al., 2007; Clavero-Salas et al., 2007; Robalino et al., 2007) and *Penaeus monodon* (Leu et al., 2007; Pongsomboon et al., 2008).

WSSV infected *Penaeus monodon* has revealed the expression of proteins like translationally controlled tumor protein - TCTP / fortilin (Bangrak et al., 2004; Graidist et al., 2006), syntenin (Tonganunt et al., 2005), PmRab7 (Sritunyalucksana et al., 2006), caspase (Wongprasert et al., 2007), *Penaeus monodon* chitin binding protein - PmCBP (Chen et al., 2007b), Pm caspase (Leu et al., 2008b), Signal transducers and activators of transcription - STAT (Chen et al., 2008a), HSP21 (Huang et al., 2008), SWDP (Amparyup et al., 2008b), Cathepsin L and Cathepsin B (Pongsomboon et al., 2008), *Penaeus monodon* lectin - PmLT (Ma et al., 2008), *Penaeus monodon* receptor for activated protein kinase C1 - Pm-RACK1 (Tonganunt et al., 2009), Heat shock cognate 70 - Hsc70 (Xu et al., 2009) and hemocyte homeostasis - associated protein (HHAP) (Prapavorarat et al., 2010) either by up-regulation or by down-regulation.

To understand the overall virus-resistance mechanism in shrimp, suppression subtractive hybridization (SSH) has been employed with WSSV resistant and WSSV sensitive shrimps of each species - *Penaeus japonicus* – haemocytes and hepatopancreas (He et al., 2005; Pan et al., 2005) and *Litopenaeus vannamei* – hepatopancreas (Zhao et al., 2007).

Genes such as *PmAV* - *Penaeus monodon* Anti Viral (Luo et al., 2003), PAP - Phagocytosis Activating Protein (Deachamag et al., 2006), alpha 2 macroglobulin (Chotigeat et al., 2007) and eukaryotic translation initiation factor 5A - eIF5A (Phongdara et al., 2007) were found to be expressed in *Penaeus monodon* which showed resistance to WSSV infection.

Other immune related genes that have been identified in penaeid shrimps with respect to WSSV resistance include PjRab, PjCaspase, hemocyanin, PjQM and PjRab in *Penaeus japonicus* (Wu and Zhang, 2007; Wang et al., 2008b; Lei et al., 2008; Xu et al., 2008; Wu et al., 2008), and anti-lipopolysaccharide factor (ALF) in *Litopenaeus vannamei* (de la Vega et al., 2008). Proteins/genes involved in anti-WSSV responses have been reviewed by Liu et al., (2009).

Shrimp molecular responses to viral pathogens have been reviewed by Flegel and Sritunyalucksana (2010) as innate immune response comprising the humoral interactions (Toll pathway, proPO and Clotting system, Antioxidant enzymes, Lectins, Haemocyanin, etc.) and the cellular responses (Apoptosis pathway, Jak-STAT pathway, RNAi pathway). The mechanism of shrimp innate response by RNA interference has been reviewed by Hirono et al. (2010). The molecular responses for the basis of quasi-immune response (Venegas et al., 2000) and "vaccination" of shrimp have been reviewed as 'Phenomenon with little or no molecular information' by Flegel and Sritunyalucksana (2010) and the relevant issues to be looked into for such studies have been reviewed by Johnson et al. (2008).

Experimental studies to analyze the concept of vaccination in shrimp to WSSV have brought out observations of protection of shrimp using either inactivated virus or recombinant proteins suggesting some aspects of specific immunity. It is reported that haemocytes of *Litopenaeus vannamei* are capable of recognizing and distinguishing different foreign materials, and respond specifically to each treatment (Montano-Perez et al., 2005). Although, different immune related molecules are being identified in shrimps at a fast pace, the identification of the mechanism that provides this sort of short term protection in shrimps has not been explored much. The application of cDNA subtraction and microarray technologies in combination with RT-qPCR expression analysis to reveal information on changes in shrimp gill gene expression to oral vaccination (without a WSSV challenge) with rVP28 have shown the down regulation of HSP70. The study, however, has noted the complexity involved in identifying the mechanism in shrimps (Arts, 2006).

The mechanisms that underpin any potential adaptive invertebrate immune response to viruses may be multiple and varied, and may be unlikely to resemble the adaptive immune responses found in vertebrates (Johnson et al.,

2008). It was hypothesized by Pan et al. (2005), from the profile of up-regulated gene expression in hepatopancreas of WSSV-resistant shrimp, that there might exist a multiple process involved in the defense system in shrimp which is implemented (mediated) by numerous factors with joint action. Hence, in this study semi quantitative RT-PCR analysis of the expression of genes related to the following different immune-related mechanisms identified in shrimp (experimental animals subjected to different treatments) were attempted along with a house keeping gene such as shrimp beta actin gene.

- proPO system (proPO gene)
- Cell adhesion (Peroxinectin gene)
- Clotting / Coagulation (Transglutaminase gene)
- Respiratory- antiviral- storage- melanisation related function (Haemocyanin gene)
- Proteinase inhibition (Alpha 2-macroglobulin gene)
- Apoptosis (Caspase gene)
- Phagocytosis (PAP gene)
- Agglutination (C type lectin gene)
- Cytokine in haematopoeisis (Astakine gene)
- Antimicrobial peptides (Crustin gene, SWD / Crustin III gene, Penaeidin-3 gene)
- Defensive enzyme (Lysozyme gene)
- Antioxidant defence mechanism - Genes of superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPx), Glutathione-s-transferase (GST) genes
- RNAi (Pm argonaute gene)
- Haemocyte adhesion-antimicrobial-PO activation (c-SPH gene)
- Cytoskeleton protein in antiviral phagocytosis (Tropomyosin II gene)
- Signal transduction (Syntenin gene)
- Cell proliferation (eIF5A gene)

- Cysteine proteinase (Cathepsin C)
- Antiviral (*PmAV* gene)
- Virus-host interaction (PmRACK-1 gene)
- Membrane trafficking (Rab7 gene)
- Immunosuppression (Cyclophilin A gene)
- Heat shock related (Hsc70 gene)
- Moulting-chitin digestion (Chitinase gene)

4.2. Materials and Methods

4.2.1. Sample collection for the semiquantitative RT-PCR analysis of bio-defence genes

Haemolymph, gill and hepatopancreas were removed using RNase free (diethyl pyrocarbonate (DEPC) treated) capillary needles and dissectors from the experimental animals which were subjected to the treatments mentioned in Chapter 1. The samples (~250 µL of haemolymph and 50-70mg of gills / hepatopancreas) were stored per mL TRI® Reagent (Sigma) and transferred immediately to -80 ºC, and RNA extraction was carried out within a fortnight.

4.2.2. RNA extraction, cDNA synthesis and semiquantitative RT-PCR of bio-defence genes

RNA was extracted from individual experimental shrimps (6 nos./ treatment) of all treatments mentioned in Chapter 1. Total RNA was isolated using TRI® Reagent (Sigma) following the manufacturer's protocol with slight modifications. Briefly, the samples were macerated and placed at room temperature for 5 mins. to ensure complete dissociation of nucleoprotein complexes. Chloroform (200µL) was added per mL of TRI® Reagent and shaken vigorously for 15 s and allowed to stand for 15 mins. at RT and centrifuged (12,000g, 4ºC, 15 mins.). Of the three layers observed, the colourless top aqueous phase was carefully separated into a fresh MCT. Aliquot of 500µL

isopropanol was added and incubated for 10 mins. at RT and centrifuged (12,000g, 4ºC, 15 mins.). The supernatant was removed and the RNA precipitated at the bottom and sides of the MCT was washed (12,000g, 4ºC, 5 mins. - twice and 12,000g, 4ºC, 10 mins. - once) with 75% ethanol. After removing the supernatant, the pellet was air dried and dissolved in 20µL DEPC treated water and incubated at 55ºC for 10 mins. DNase treatment of the RNA samples were done with 0.2U of the enzyme, RNase free DNase 1 (New England Biolabs), for 1µg of RNA by incubating at 37ºC for 10 mins. The enzyme inactivation was done by incubating at 75ºC for 10 mins. RNA quality and concentration were determined at 260/280nm using a UV – Visible spectrophotometer (Shimadzu).

For cDNA sysnthesis, a sample of 5µg of the extracted RNA was added to a 20µL reaction mixture with RNase Inhibitor (8U), Oligo $(dT)_{12}$ primer (40pmol), dNTP mix (1mM), RTase Buffer (1X) and $MgCl_2$ (2mM). 200U of Moloney Murine Leukemia Virus (M-MuLV) Reverse Transcriptase was added and the reverse transcription was carried out at 42ºC for 1h on a Thermal cycler (Master Cycler personal, Eppendorf). All the reagents were purchased from New England Biolabs.

Bio-defence genes (Table 4) in each treatment were amplified on Master Cycler personal (Eppendorf). The primers selected for these genes included those from literature as well as those designed from sequences deposited in GenBank (Table 4) for which the PCR conditions were standardized on a Mastercycler® Gradient® Eppendorf. Equal volumes of cDNA from each individual of the treatment were pooled and the amplifications of the above mentioned were done. The PCR was done by using 2µL of the cDNA of each treatment with specific primer sets as given in Table 4. Shrimp β-actin was amplified as a reference gene. The 25µL PCR mixture subjected for amplification contained, 200µM dNTP mix, 10pmol each of forward and reverse

primers, 1X PCR buffer (Thermopol), 0.5U Taq DNA Polymerase and 2 µL of cDNA. The hot start PCR programme used for the immune related genes was 94 ºC for 2 mins. followed by 35 cycles of 94 ºC for 2 mins., annealing for 1 min, 72ºC for 1 min and a final extension at 72ºC for 10 mins. The annealing temperature, which was used for the amplification of immune-related genes, is shown along with the primer sequence in the Table. The PCR products were analyzed by horizontal gel electrophoresis of 10µL of the PCR products. The gels were stained with ethidium bromide and documented under UV light with Molecular Imager® Gel Doc™ XR+ Imaging System (Bio-Rad). Band intensity was calculated using Quantity One® software (Bio-Rad) and the significant differences between the treatments were identified from the graphical analysis of the band intensity.

4.3. Results

Semi quantitative RT-PCR analysis of the 30 selected genes in gills, haemolymph and hepatopancreas showed variation in their expression between the selected tissues and between the experimental treatment groups.

4.3.1. Semiquantitative RT-PCR of bio-defence genes in shrimp tissues (gills, haemolymph and hepatopancreas)

Among the tissues analyzed by RT-PCR, gills showed the expression of 28 selected genes followed by haemolymph (25 genes) and hepatopancreas (11 genes) (Table 5). The selected genes were amplified with the same PCR cycling condition with variation in the annealing temperatures only. Primers designed for genes like alpha 2 macroglobulin, crustin, lysozyme, cytosolic MnSOD, catalase, PemAgo, syntenin, eIF5A, Rab7 and chitinase showed amplification in the selected tissues in varying degrees. Some of the genes which have not been reported earlier in the gill, haemolymph and hepatopancreas of *Penaeus monodon* were also found to be amplified. The house keeping gene beta actin showed amplification in all the selected tissues.

4.3.2. Semiquantitative RT-PCR of bio-defense genes in shrimp tissues of different treatments (normal feed and IVP) on 1DPA, 5DPA and 10DPA

4.3.2.1. Gills

4.3.2.1.1. 1st day post administration (1DPA)

Before challenge - The gills of experimental animals administered with different feed types and analyzed on 1st day after the administration, showed amplification of 14 genes (up-regulated-1- cathepsin C; without variation-13) in normal feed administered compared to those of IVP administered animals, and 25 genes (up-regulated-12 – alpha 2 macroglobulin, astakine, chitinase, glutathione-s-transferase, eIF5A, Pen-3, Pem-Ago, transglutaminase, proPO, SWD, Hsc70, tropomyosinII; without variation-13) in IVP administered animals compared to those of normal feed administered animals, as described in Table 6.

After challenge - The semiquantitative RT-PCR analysis of gills of the experimental animals (1st day post administration - 4th day after WSSV challenge) exhibited amplification of 21 genes in normal feed administered group and in IVP administered group it was 14. Among them the normal feed administered group showed up-regulation of 12 genes (alpha 2 macroglobulin, C type lectin, catalase, c-SPH, chitinase, eIF5A, Pen-3, transglutaminase, proPO, Hsc70, tropomyosinII, PemAgo) compared to those of IVP administered group; and 5 genes in IVP administered group (astakine, cathepsin C, PAP, SOD, syntenin) compared to normal feed administered; and no variation in the intensity of expression in 9 genes each as shown in Table 6.

4.3.2.1.2. 5th day post administration (5DPA)

Before challenge - The gills of experimental animals administered with different feed types and analyzed on 5th day after the administration, showed amplification of 26 genes (up-regulated-9- astakine, catalase, cathepsinC, c-SPH, PmAV, SOD, cyclophilinA, Rab7, SWD; without variation-17) in normal

feed administered compared to those of IVP administered group and amplification of 18 genes (up-regulated-1- lysozyme, without variation-17) in IVP administered animals compared to those of normal feed administered group as tabulated in Table 7.

After challenge - The semiquantitative RT-PCR analysis of gills of the experimental animals (5th day post administration - 4th day after WSSV challenge) showed amplification of 10 genes (up-regulated 1 gene - lysozyme; and with out variation 9) in normal feed administered compared to those of control. In IVP administered group, 14 genes got amplified among which 5 were up-regulated such as - alpha 2 macroglobulin, astakine, cyclophilin A, Rab7, SWD and with out variation - 9, compared to normal feed administered group as described in Table 7.

4.3.2.1.3. 10th day post administration (10DPA)

Before challenge - The gills of experimental animals administered with different feed types and analyzed on 10th day after the administration, showed amplification of 25 genes of which 2 genes where found up-regulated such as astakine and lysozyme and 23 remained without variation in normal feed administered group compared to those in IVP administered group. In IVP administered group there was the amplification of 25 genes of which 2 genes (c-SPH, chitinase) were up-regulated and 23 remained without variation in IVP administered animals compared to normal feed administered group as given in Table 8.

After challenge - The semiquantitative RT-PCR analysis of gills of the experimental animals (10th day post administration - 4th day after WSSV challenge) showed amplification of 14 genes in which all genes showed no variation compared to those in IVP administered group. In IVP administered group, 1 gene (SWD) was up-regulated and no variation in 14 genes compared to normal feed administered group (Table 8).

4.3.2.2. Haemolymph

4.3.2.2.1. 1st day post administration (1DPA)

Before challenge - The haemolymph of experimental animals administered with different feed types and analyzed on 1st day after the administration, showed amplification of 9 genes (up-regulated-5- catalase, Hsc70, cathepsin C, proPO, SOD; without variation-4) in normal feed administered, and amplification of 13 genes (up-regulated-9- alpha 2 macroglobulin, PemAgo, cyclophilinA, SWD, lysozyme, Pen-3, eIF5A, glutathione-s-transferase, transglutaminase; without variation- 4) in IVP administered animals as given in Table 9.

After Challenge - The semiquantitative RT-PCR analysis of haemolymph of the experimental animals (1st day post administration - 4th day after WSSV challenge) showed amplification of 9 genes in normal feed administered group where all the genes were found up-regulated compared to IVP administered group. They were peroxinectin, c-SPH, cyclophilinA, Pen-3, lysozyme, eIF5A, glutathione-s-transferase, SOD, PemAgo. In IVP administered group, 4 genes were found amplified among which all got up-regulated compared to those of normal feed administered group. They were SWD, crustin, PAP, PmRACK1 (Table 9).

4.3.2.2.2. 5th day post administration (5DPA)

Before challenge - The haemolymph of experimental animals administered with different feed types and analyzed on 5th day after the administration, showed amplification of 14 genes (up-regulated-6- haemocyanin, cathepsin C, SWD, crustin, eIF5A, PAP and without variation-8)) in the normal feed administered group compared to those of IVP administered group. Meanwhile, in IVP administered group 15 genes got amplified out of which 7 genes got up-regulated such as C type lectin, peroxinectin, lysozyme, Pen-3, Rab7,

PmRACK1, proPO and without variation-8) compared to those of normal feed administered group (Table 10).

After Challenge - The semiquantitative RT-PCR analysis of haemolymph of the experimental animals (5th day post administration - 4th day after WSSV challenge) showed amplification of 7 genes of which 5 genes got up-regulated such as C type lectin, catalase, Hsc70, peroxinectin, Pen-3 and with out variation 2 genes. In IVP administered group, 3 genes got amplified of which 1 gene was up-regulated (cyclophilin A) and 2 remained the same compared to those of normal feed administered animals (Table 10).

4.3.2.2.3. 10th day post administration (10DPA)

Before challenge - The haemolymph of experimental animals administered with different feed types and analyzed on 10th day after the administration, showed amplification of 12 genes of which 4 genes were up-regulated such as C type lectin, cathepsinC, PAP, transglutaminase and 8 genes without variation in normal feed administered compared to those in IVP administered group. In IVP administered animals, 14 genes were amplified of which 6 were up-regulated such as c-SPH, SWD, Rab7, eIF5A, PmRACK1, proPO and 8 remained without variation compared to normal feed administered group (Table 11).

After challenge - The semiquantitative RT-PCR analysis of haemolymph of the experimental animals (10th day post administration - 4th day after WSSV challenge) showed amplification of 4 genes among which there was up-regulation of 1 gene such as Rab7 and no variation in 3 genes in normal feed administered animals compared to those in IVP administered group. In IVP administered group, 13 genes were amplified of which 10 genes were up-regulated such as C-type lectin, catalase, Hsc70, cathepsinC, cSPH, eIF5A, PAP, transglutaminase, syntenin, SOD and 3 genes remained without variation compared to normal feed administered group (Table 11).

4.3.2.3. Hepatopancreas

4.3.2.3.1. 1st day post administration (1DPA)

Before challenge - The hepatopancreas of experimental animals administered with different feed types and analyzed on 1st day after the administration, showed amplification of 7 genes in normal feed administered group (up-regulated-1- SOD; without variation-6) compared to those of IVP administered group, and 7 genes amplified in IVP administered group (up-regulated-1-PmAV; without variation-6) compared to those of normal feed administered (Table 12).

After challenge - The semiquantitative RT-PCR analysis of hepatopancreas of the experimental animals (1st day post administration - 4th day after WSSV challenge) showed amplification of 2 genes compared to those of IVP administered group (1 genes up-regulated - PmAV) and one with out variation. In IVP administered group, 6 genes got amplified among which 5 genes such as C type lectin, PemAgo, PmRACK1, PAP and SOD were up-regulated compared to normal feed administered group and no variation in 1 gene (Table 12).

4.3.2.3.2. 5th day post administration (5DPA)

Before challenge - The hepatopancreas of experimental animals administered with different feed types and analyzed on 5th day after the administration, showed amplification of 8 genes of which 2 were found up-regulated such as tropomyosinII, PmRACK1 and 6 remained with out variation compared to those in IVP administered group. Meanwhile, in IVP administered group, there was the amplification of 8 genes and up-regulation of 2 genes such as PmAV, and SOD, and 6 remained without variation compared to normal feed administered group (Table 13).

After challenge - The semiquantitative RT-PCR analysis of hepatopancreas of the experimental animals (5th day post administration - 4th day after WSSV challenge showed amplification of 6 genes of which 2 genes got up-regulated

(PmAV, SOD) and 4 remained with out variation in normal feed administered animals compared to those of IVP administered group. Meanwhile in the latter 7 genes got amplified of which 3 genes such as PemAgo, tropomyosinII, PmRACK1 were up-regulated and the rest remained with out variation compared to normal feed administered group (Table 13).

4.3.2.3.3. 10th day post administration (10DPA)

Before challenge - The hepatopancreas of experimental animals administered with different feed types and analyzed on 10th day after the administration, showed amplification of 10 genes of which 1 gene such as PmRACK1 was up-regulated and the remaining 9 showed no variation compared to those in IVP administered group. In IVP administered group, 9 genes were amplified all of which showed no variation compared to normal feed administered group (Table 14).

After challenge - The semiquantitative RT-PCR analysis of hepatopancreas of the experimental animals (10th day post administration - 4th day after WSSV challenge) showed amplification of 3 genes in normal feed and IVP administered groups and did not show variation in these 3 genes between the groups (Table 14).

4.4. Discussion

Though research has brought out increasing number of immune relevant genes in WSSV sensitive and WSSV resistant *Penaeus monodon*, much less understanding has been gained on the status of the bio-defence genes in immunomodulation of WSSV 'vaccinated' shrimps after an oral viral challenge. Hence, the semi quantitative RT-PCR analysis of some of the identified immune relevant genes in gills, haemolymph and hepatopancreas of the IVP administered animals was carried out. Bio-defence genes which were unique to

IVP administered animals (when compared to control - normal feed administered) have been discussed.

4.4.1. Gills

4.4.1.1. 1st Day Post Administration (1DPA)

Before challenge, in the gills of 1st day post administration group of animals, 25 genes were amplified in the IVP administered group of which 12 genes such as alpha 2 macroglobulin, astakine, chitinase, glutathione-s-transferase, eIF5A, Pen-3, PemAgo, transglutaminase, proPO, SWD, Hsc70 and tropomyosinII were up-regulated and 13 genes were without variation, while in the normal feed administered group 14 genes were amplified of which 1 gene such as cathepsinC was up-regulated and 13 genes remained without variation.

Alpha 2 macroglobulin (α-2 macroglobulin) has been defined as a ubiquitous high molecular weight proteinase inhibitor. Alpha 2 macroglobulin is a plasma glycoprotein that arrests proteinases avoiding the degradation of host protein and removing them from circulation. Gollas-Galvan et al. (2003) and Lin et al. (2007) have observed that α-2 macroglobulin expression in haemocytes was significantly induced by administration of peptidoglycan (immunostimulant) in kuruma shrimp and *P. monodon*. The real time RT-PCR m-RNA transcript analysis of α-2 macroglobulin in *P. monodon* haemocytes by Lin et al. (2007) showed upregulation till 48 h post peptidoglycan injection and back to normal value at 72h. In the present study, the oral administration of IVP has caused an up-regulation of α-2 macroglobulin in *P. monodon* showing the activation of a protease inhibitory mechanism.

Astakine is a cytokine which is reported to be involved in cell (haemocyte) proliferation and / or cell differentiation (Soderhall et al., 2005; Jiravanichpaisal et.al., 2007). Jiravanichpaisal et al. (2006) observed that the infectivity of WSSV to haematopoietic cells in crayfish is dependent on temperature and a supplemented growth factor (cytokine) astakine. However, in

the present study, the IVP administered group has shown the activation of cytokine mediated mechanism.

Chitinase have been found to play a key role in the molting process of invertebrates, the digestion of chitinous food, and defense against chitin-bearing pathogens (Zhang et al., 2010). Tan et al. (2000) obtained the full-length cDNA of chitinase-1 (Pmchi-1) from *Penaeus monodon* and found that Pmchi-1 mRNA was detectable in the hepatopancreas and gut, and Pmchi-1 expression during the molt cycle fluctuated markedly. Pan et al. (2005) found that chitinase, was an important immune-relevant factor responsible for the virus resistance in *M. japonicus*. Leu et al. (2007) have observed that a group of immune-related chitin-binding protein genes involving chitinase gene is also likely to be strongly up regulated after WSSV infection. Likewise, the up-regulation of chitinase gene have been noticed in gills of *Penaeus monodon* on the oral administration of IVP to encounter a viral challenge.

Glutathione-s-transferases (GSTs) and glutathione peroxidases (GPxs) are essential components of cellular detoxification systems that defend cells against reactive oxygen species (ROSs). Mathew et al. (2007) reported that in *Penaeus monodon*, WSSV infection induced a significant reduction in the activities of glutathione-dependent antioxidant enzymes (GPx and GST). In this context the upregulation of glutathione-s-transferases in the gills of IVP administered animals, which has caused the activation of a glutathione dependant antioxidant mechanism, has greater significance.

Phongdara et al. (2007) reported that eIF5A (a 157 aminoacid polypeptide implicated in cell proliferation and survival) expression increased during the stage of WSSV infection when there were no gross signs of disease i.e., before moribund stage and hence responsible for supporting viral replication while keeping the cell viable. A perplexing aspect of the eIF5A protein identified is that high expression occurs only in grossly normal, infected shrimp

(GNIS) but not in uninfected normal shrimp (NS) or in moribund/dead shrimp. The up-regulation of eIF5A in the gills of IVP administered group has shown the activation of cell proliferation and survival mechanism in this group.

Penaeidins are a unique family of antimicrobial peptides in crustaceans, originally isolated and characterized in the shrimp, *P. vannamei*. Isoforms of penaeidins are classified according to their similarity in aminoacid sequence into penaeidin 2 (PEN2), penaeidin 3 (PEN3), penaeidin 4 (PEN4) and penaedin 5 (PEN5) (Wang et al., 2007c; Hu et al., 2006). Pen-3, the most abundant and representative member of the penaeidin family is reported to be highly expressed in haemocytes (Munoz et al., 2002; Wang et al., 2007c). Garcia et al. (2009) have observed that Pen-3 isoforms were over expressed in the haemocytes of WSSV infected pre-challenged *P. vannamei*. However, in IVP administered *P. monodon*, Pen-3 was found to be up-regulated and hence an antimicrobial component has been activated.

Pem-ago is a gene identified in *P. monodon* for the argonaute protein. Argonaute protein is a core component of RNA induced silencing complex (RISC) which is involved in RNA interference (RNAi). Pem-ago was found to be required for effective RNAi machinery in penaeid shrimp (Dechklar et al., 2008). In the present study, RNAi mechanism involving Pem-Ago gene was found to be activated on 1st day post administration of IVP.

Transglutaminase can catalyze the cross-linking reaction between soluble clotting protein molecules from the plasma for prevention of excess blood loss from wounds and obstructing micro-organisms from invading the wound in crustaceans. Transglutaminase (TG) stabilizes the clots by intermolecular covalent cross-linking. Of the two types of shrimp transglutaminases (STGI and STGII), STGII was found to be involved in coagulation and that transglutaminase expression was decreased after 24hr post infection in *F. chinensis* (Liu et al., 2007b). The up-regulation of

transglutaminase in the gills of IVP administered *P. monodon* indicates the activation of a coagulation factor on 1st day post administration of IVP.

The prophenoloxidase (proPO) system is considered as a constituent of the immune system and forms an important part of an immuno-recognition process of the defence mechanism in invertebrates (Lai et al., 2005). ProPO can be activated by cell wall components of microbial origins, such as β-1,3-glucan, lipopolysaccharide (LPS) and peptidoglycan. Activation of proPO can be regulated by environmental, biological and experimental factors, including calcium, sodium dodecyl sulfate (SDS), trypsin, pH and temperature. ProPO is an inactive form and is converted to an active form phenoloxidase (PO) after limited proteolysis by serine proteinases. In addition, Ca^{2+} is required for the conversion of the proPO-activating enzyme (ppAE) to an active proteinase that transforms proPO to active phenoloxidase (PO). The pro phenoloxidase system (proPO) is the best-studied crustacean immune defence system that functions as an oxygen transferring enzyme to catalyse the dehydrogenation of catechols to orthoquinones and the orthohydroxylation of phenols to catechols (Strothkamp and Mason, 1974; Sritunyalucksana and Soderhall, 2000). Pro phenoloxidase is capable of converting phenolic substrates such as dihydroxyphenylalanine to dopaquinone, which then polymerise non-enzymatically to melanin. Intermediate compounds in the melanin pathway have bactericidal properties and are involved in immune reactions (Johansson and Soderhall, 1989). ProPO is also involved in cell adhesion, encapsulation and phagocytosis processes (Gillespie et al., 1997) Though the exact mechanism by which WSSV affects phenol oxidase enzyme system in *P.monodon* is still unknown, studies in other crustaceans suggest that viral infection mediated oxidative stress is a major factor responsible for the reduction of phenol oxidase activity (Le Moullac et.al., 1998; Cheng et.al., 2002). The drastic reduction of phenol oxidase in WSSV infected animals is observed by Mathew et.al. (2007). The up-regulation of

proPO gene in the gills of *P. monodon* administered with IVP has shown the activation of proPO system.

Single WAP domain (SWD)-containing proteins are small proteins with a C-terminal region containing a single whey acidic protein (WAP) domain. Proteins containing a whey acidic protein (WAP) domain are small secretory proteins which exhibit a variety of functions including proteinase inhibitory and antimicrobial activities. The SWD proteins are also called crustinIII (Smith et al., 2008). While analyzing the expression of SWDPm1-3 to WSSV challenge, Amparyup et al., (2008b) observed that the SWDPm1 and SWDPm2 mRNA expression showed a significant up-regulation at 6 h after WSSV injection and the results indicated that SWDPm1 and SWDPm2 were highly responsive to WSSV stimulation. However, the IVP administration has caused an up-regulation of the SWD gene in the gills of *P. monodon* before a WSSV challenge.

Heat shock cognate 70 (Hsc70) (differs from HSP70 in having introns) functions as a molecular chaperon and plays an important role in protein folding. Xu et al. (2009) reported that Hsc70 expression in *P. monodon* was enhanced by WSSV infection at the early stage and peaked at 12 h post-infection and decreased drastically at the late stage. In IVP administered animals the gene (Hsc70) was found to be up-regulated.

Tropomyosin II is a muscle protein and its presence in a non-muscle cell is said to be involved in stabilization of actin network by binding with actin filament and controls a dynamic regulation of actin polymer. The decreased level of tropomyosin could affect the ability of actin polymer in maintaining the cellular structure. In addition, tropomyosinII has been reported to bind and inhibit DNase I, a key enzyme in apoptosis cascade (Bourchookarn et al., 2008). Recently, in *P. japonicus*, a four-protein complex consisting of Rab, β-actin, tropomyosin and a white spot syndrome virus (WSSV) envelop protein VP466,

were found to be capable of regulating the hemocytic phagocytosis against virus infection (Wu et al., 2008). The up-regulation of tropomyosinII observed in IVP administered animals might be the activation of phagocytic mechanism in the organism.

The upregulation of cathepsinC was observed in normal feed administered group on 1DPA. Cathepsin C is a lysosomal cysteine protease. In invertebrates, cysteine proteases (cathepsin) represent a major component of the lysosomal proteolytic system, and are responsible for intracellular protein degradation (Zhao et al., 2007). Qui et al. (2008) observed that Pmcathepsin C was up-regulated on LPS stimulation.

Biodefence mechanisms like protease inhibition involving alpha 2 macroglobulin; cytokine related function and hematopoeisis involving astakine; moulting and digestion involving chitinase; antioxidant mechanism involving glutathione-s-transferase; cell proliferation and survival involving eIF5A; antimicrobial activity involving penaedin-3 (Pen-3) and single WAP domain containing protein (SWD); RNA interference - RNAi involving *Penaeus monodon* argonaute (Pem-Ago); coagulation involving transglutaminase; phenol oxidase system involving proPO; heat shock related mechanism involving heat shock cognate 70 (Hsc70) and cytoskeletal system involving tropomyosinII were found to be active as the genes involved in these mechanisms were up-regulated in IVP administered animals on 1DPA (before challenge).

Chen et al. (2008b) have reported that even when the expression level of a gene is not affected by WSSV infection, the response mediated by that gene may still be important. In the present study bio-defence genes were observed without variation in expression in between the normal feed and IVP administered group. Haemagglutination involving C type lectin, antioxidant mechanism involving catalase, haemocyte adhesion involving c-SPH and peroxinectin, antiviral action involving PmAV, antibacterial activity involving lysozyme and crustin,

phagocytosis involving phagocytosis activating protein (PAP), apoptosis involving caspase, immunosuppression effect by Cyclophilin A, receptor activation by *Penaeus monodon* receptor for activated protein kinase C1 (Pm-RACK1), regulation of exocytosis and endocytosis by Rab7 and signal transduction involving syntenin did not show variation in between the IVP and normal feed administered group.

After the challenge, in the gills of 1st day post administration group of animals, 14 genes were amplified in the IVP administered group of which 5 genes such as astakine, cathepsinC, PAP, SOD and syntenin were up-regulated and 9 genes without variation. However, in the normal feed administered group 21 genes were amplified of which 12 genes such as alpha 2 macroglobulin, C type lectin, catalase, c-SPH, chitinase, eIF5A, Pen-3, transglutaminase, proPO, Hsc70, tropomyosinII and PemAgo were up-regulated and 9 genes did not show variation.

Cathepsin C is a lysosomal cysteine protease. In invertebrates, cysteine proteases (cathepsin) represent a major component of the lysosomal proteolytic system, and are responsible for intracellular protein degradation (Zhao et al., 2007). Qui et al. (2008) observed that Pmcathepsin C was upregulated on LPS stimulation. The up-regulation of cathepsinC in the IVP administered group shows that lysosomal protease based mechanism is in action against the virus invasion.

Phagocytosis activating protein (PAP) gene is a ribosomal protein L26 (RPL26) gene, a macrophage activator, isolated from *Penaeus monodon* infected with the white spot syndrome virus. Deachamag et al. (2006) observed that the expression of PAP in the haemolymph of *P. monodon* was induced via the intramuscular injection of the immunostimulants of inactivated WSSV, IVH and fucoidan and that the highest expression occurred after 72 h, 1 week, and 2 weeks, respectively, and in all cases the PAP disappeared completely after 5

weeks. In the IVP administered group a phagocytic action is found to be activated against the viral challenge.

During the course of phagocytosis, the host's NADPH oxidase gets activated which in turn enhances the glycolytic reactions that will increase the consumption of oxygen, and induce the production of a mass of reactive oxygen species (ROS) such as superoxide anion ($O2^{\cdot -}$), hydrogen peroxide (H2O2) and hydroxyl radical ($OH^{\cdot}$). So the rapid elimination of these excessive ROS is essential for the proper functioning of cells and the survival of the animal. This is performed by antioxidant enzymes such as superoxide dismutases (SOD) that scavenges the superoxide anions. SOD detoxifies superoxide radicals by converting them to hydrogen peroxide and oxygen. Hydrogen peroxide is then transformed to water and oxygen by catalase, providing innocuous compounds to the cell. Depending on the metal cofactor used for catalysis SODs are of various kinds of which manganese SOD (MnSOD) is of two types in decapod crustaceans - cytosolic MnSOD (cMnSOD) and mitochondrial MnSOD (mMnSOD) (Zhang et al., 2007). Zhang et al. (2007) observed that in WSSV challenged experiments with *F. chinensis*, up-regulation of mMnSOD expression in shrimp hemocytes (infected and the control groups) in the first 3 h post-challenge showed that mMnSOD might be involved in a transient systemic immune response to the stimulation inflicted during the hemocytes phagocytosing virus or heterogenous granules that would generate a mass of ROS and mMnSOD showing a different expression profile in hepatopancreas after WSSV challenge. They suggested this to the different functions of hepatopancreas and hemocytes in immune defense system. Go´mez-Anduro et al. (2006) found that cMnSOD gene in haemocytes increased transiently 1 h after infection with WSSV and then decreased in *L. vannamei*. The up-regulation of MnSOD supports that the IVP administered animals are encountering the virus with an antioxidant defence mechanism.

Phongdara et al. (2007) identified syntenin of *P. monodon* to be a dynamic responder to WSSV infection and greatly up-regulated in acute phase of infection. Syntenin has been identified as an adaptor protein that couples various kinds of molecules in a signal transduction pathway. It has been observed that an up-regulation of syntenin gene is coupled with an alpha 2 macroglobulin gene in the acute stage of WSSV infection. It has been observed that in IVP administered shrimp, signal transduction involving syntenin was up-regulated compared to the control group.

Astakine gene related to cytokine related function and hematopoeisis, lysosomal protease activity involving cathepsin C, phagocytic action involving PAP, antioxidant mechanism involving SOD, and signal transduction involving syntenin were found to be activated in response to a WSSV challenge.

The normal feed administered group which was challenged on 1st day post administration showed the up-regulation of protease inhibitory activity involving alpha 2 macroglobulin, haemagglutination mechanism involving C type lectin, antioxidant mechanism involving catalase, multifunctional protein domain c-SPH, moulting and digestion involving chitinase, cell proliferation and survival involving eIF5A, antimicrobial activity involving Pen-3, RNA interference - RNAi involving *Penaeus monodon* argonaute (Pem-Ago), coagulation involving transglutaminase, phenol oxidase system involving the proPO, heat shock related mechanism involving Hsc70, and cytoskeletal changes involving tropomyosinII.

Bio-defence mechanisms that did not show variation between the challenged groups of normal feed and IVP administered group include antiviral gene involving PmAV, antibacterial activity involving lysozyme, cell adhesion involving peroxinectin, RNA interference involving Pem-ago, immunosuppression involving Cyclophilin A, antimicrobial involving crustin and SWD and regulation of exocytosis and endocytosis by Rab7.

4.4.1.2. 5th Day Post Administration (5DPA)

Before challenge, in the gills of 5th day post administration group of animals, 18 genes were amplified in the IVP administered group of which 1 gene such as lysozyme was up-regulated and 17 genes were without variation, while in the normal feed administered group 26 genes were amplified of which 9 genes such as astakine, catalase, cathepsinC, c-SPH, PmAV, SOD, cyclophilinA, Rab7 and SWD were up-regulated and 17 genes remained without variation.

Lysozyme has been described in invertebrates as a component of the innate immune system, functioning as an antibacterial protein by hydrolysing the b-1, 4 glycosidic bond of bacterial cell wall peptidoglycans. Zhao et al. (2007) found that lysozyme was upregulated in virus resistant shrimp and may function as immune effectors in humoral immune response against the virus infection. In IVP administered group, the antibacterial, lysozyme was found to be up-regulated on 5DPA (before challenge).

Meanwhile, in the normal feed administered group, biodefence mechanisms such as cytokine related mechanism involving astakine, antioxidant mechanism involving catalase and SOD, lysosomal protease activity involving cathepsinC, cell adhesion involving c-SPH, antiviral gene like PmAV, immunosuppression activation involving cyclophilinA, membrane trafficking involving Rab7 and multifunctional biodefence involving SWD were found up-regulated.

Bio-defence mechanisms that did not show variation between the normal feed and IVP administered group included protease inhibition involving alpha 2 macroglobulin, haemagglutination involving C type lectin, moulting and digestion involving chitinase, antioxidant mechanism involving glutathione-s-transferase, cell proliferation and survival involving eIF5A, cell adhesion involving peroxinectin, phagocytosis activation involving PAP, antimicrobial

activity involving Pen3 and crustin, RNAi involving Pem-Ago, apoptosis involving caspase, receptor activation involving PmRACK1, signal transduction involving syntenin, coagulation involving transglutaminase, phenol oxidase system involving the proPO, heat shock related mechanism involving Hsc70 and cytoskeletal changes involving tropomyosinII.

After the challenge, in the gills of 5th day post administration group of animals, 14 genes were amplified in the IVP administered group of which 5 genes such as alpha 2 macroglobulin, astakine, cyclophilinA, Rab7 and SWD were up-regulated and 9 genes without variation. However, in the normal feed administered group 10 genes were amplified of which 1 gene such as lysozyme was up-regulated and 9 genes did not show variation.

Cyclophilins (Cyps) are a family of proteins that bind the immunosuppressive agent cyclosporin A (CsA) with high affinity and exhibit various biological activities which have important roles in cell cycle events (Ryffel et al., 1991; Galat, 1999). In *P. monodon*, cyclophilin A was found to be an inducible acute phase protein that could be induced by LPS and it could be involved in shrimp immune response (Qiu et al., 2009).

Pan et al. (2005) studied differential gene expression in WSSV resistant shrimp (*Penaeus japonicus*) by subtractive hybridization and showed that genes for small GTPases are up-regulated in virus-resistant shrimp. These small GTP-binding proteins comprise products of the Ras, Rab/YPT, and Rho gene families and are involved in diverse cellular functions, including growth, differentiation, and vesicular traffic (Zerial and MacBride, 2001). Rabs act as molecular switches to control trafficking of endocytic vesicles within cells, as well as their subsequent fusion to endosomes. Sritunyalucksana et al., (2006) reported that *P. monodon* Rab7 (PmRab7) binds to WSSV virions and to the recombinant WSSV envelope protein or VP28 in a dose dependent manner. Wu and Zhang (2007) reported that the mRNA of a Rab-like gene was found to be up-regulated

in the virus-resistant *P. japonicus* indicating that small GTPases might be involved in the shrimp defense response against viruses. Wu et al., (2008) have identified a Rab protein – beta actin – tropomyosin- WSSV protein complex, which might be an intracellular pathway for antiviral phagocytosis by immune signal transduction in shrimp.

In the group of IVP administered animals which was challenged on 5th day post administration, protease inhibition involving alpha 2 macroglobulin, cytokine related mechanism involving astakine, immunosuppression related mechanism involving cyclophilinA, membrane trafficking involving Rab7 and multifunctional bio-defence involving SWD were found to be up-regulated compared to that of the control.

In the gills of normal feed administered group only antibacterial activity involving lysozyme was found to be up-regulated.

Biodefence mechanisms that did not show variation between the challenged groups of normal feed and IVP administered group were haemagglutination involving C type lectin, cell proliferation and survival involving eIF5A, antimicrobial activity involving Pen3, RNAi involving Pem-Ago, receptor activation involving PmRACK1, signal transduction involving syntenin, phenol oxidase system involving the proPO, heat shock related mechanism involving Hsc70 and cell adhesion involving peroxinectin.

4.4.1.3. 10th Day Post Administration (10DPA)

Before challenge, in the gills of 10th day post administration group of animals, 25 genes were amplified in the IVP administered group of which 2 genes such as c-SPH and chitinase were up-regulated and 23 genes were without variation, while in the normal feed administered group 25 genes were amplified of which 2 genes such as astakine and lysozyme were up-regulated and 23 genes remained without variation.

Clip domain serine protease homologs (c-SPHs) are involved in various innate immune functions in arthropods such as antimicrobial activity, cell adhesion, pattern recognition, opsonization, hemocyte adhesion and regulation of the prophenoloxidase system (Lin et al., 2006).

On 10th day post administration, cell adhesion involving c-SPH and molting and digestion mechanism involving chitinase were found to be up-regulated in IVP administered group. In the normal feed administered group, cell adhesion involving astakine and antibacterial activity involving lysozyme were found to be up-regulated.

Bio-defence mechanisms that did not show variation between the normal feed and IVP administered group include protease inhibition involving alpha 2 macroglobulin, haemagglutination involving C type lectin, antioxidant mechanism involving SOD, catalase and glutathione-s-transferase, lysosomal protease activity involving cathepsinC, antiviral gene PmAV, cell proliferation and survival involving eIF5A, cell adhesion involving peroxinectin, phagocytosis activation involving PAP, antimicrobial activity involving Pen3, RNAi involving Pem-Ago, apoptosis involving caspase, immunosuppression related mechanism involving cyclophilinA, antimicrobial mechanism involving crustin and SWD, receptor activation involving PmRACK1, membrane trafficking involving Rab7, signal transduction involving syntenin, coagulation involving transglutaminase, phenol oxidase system involving the proPO, heat shock related mechanism involving Hsc70 and cytoskeletal activation involving tropomyosinII.

After the challenge, in the gills of 10th day post administration group of animals, 15 genes were amplified in the IVP administered group of which 1 gene such as SWD was up-regulated and 14 genes without variation. However, in the normal feed administered group 14 genes were amplified and these did

not show variation. In normal feed administered group none of the selected bio-defence genes were observed to be up-regulated.

Bio-defence mechanisms that did not show variation between the challenged groups of normal feed and IVP administered group were lysosomal protease activity involving cathepsinC, cell adhesion involving c-SPH and peroxinectin, antibacterial activity involving lysozyme, phagocytosis activation involving PAP, antimicrobial activity involving Pen3, immunosuppression related mechanism involving cyclophilinA, receptor activation involving PmRACK1, membrane trafficking involving Rab7, signal transduction involving syntenin, coagulation involving transglutaminase, phenol oxidase system involving the proPO, heat shock related mechanism involving Hsc70 and cytoskeletal activation involving tropomyosinII.

4.4.1.4. Overview of bio-defence mechanism in gill

In the gill tissue of IVP administered animals, the amplification of the number of bio-defence genes was higher on 1DPA; lesser on 5DPA and equal (but different genes) on 10DPA when compared to the normal feed administered control group. The analysis of the up-regulated genes in the IVP administered animals showed that more genes were up-regulated on 1DPA and less number of genes on 5DPA and equal number of genes on 10DPA when compared to the control group. The genes that were up-regulated in IVP administered animals were different on 1DPA, 5DPA and 10DPA except for chitinase which was up-regulated on 1DPA and 10DPA. The higher number of amplified/ up-regulated genes in IVP on 1DPA showed that bio-defence response has been elicited soon after the administration of IVP.

After an oral challenge, the gill tissue of IVP administered animals showed amplification of less number of genes on 1DPA and more number of

genes on 5DPA and 10DPA when compared to the control animals (normal feed administered animals after challenge). An analysis of the IVP administered animals after the WSSV challenge revealed that the up-regulated genes were lesser on 1DPA and higher on 5DPA and 10DPA when compared to the control animals (normal feed administered animals after challenge). The up-regulated genes in the IVP administered animals after an oral challenge were different on 1DPA, 5DPA and 10DPA except for astakine which was up-regulated on 1DPA and 5DPA, and SWD which was up-regulated on 5DPA and 10DPA. The decrease in the number of genes amplified on 1DPA in the animals challenged after the IVP administration compliments the finding (Chapter 2) of lessening the protein spots in 2DE under similar conditions. The underlying mechanism has to be investigated further. The amplification/ up-regulation of more number of genes on 5DPA and lesser on 10DPA shows that the bio-defence mechanisms are more active against WSSV on 5DPA and comparatively lesser on 10DPA.

The comparative analysis of the up-regulated genes between the unchallenged and challenged group of animals on 1DPA, 5DPA and 10DPA showed that after the challenge, the genes which were up-regulated were different from those expressed in the unchallenged condition except for astakine which was up-regulated in the unchallenged and challenged condition in the 1DPA category.

4.4.2. Haemolymph

4.4.2.1. 1st Day Post Administration (1DPA)

Before challenge, in the haemolymph of 1st day post administration group of animals, 13 genes were amplified in the IVP administered group among which 9 genes such as alpha 2 macroglobulin, Pem-Ago, cyclophilinA, SWD, lysozyme, Pen-3, eIF5A, glutathione-s-transferase and transglutaminase were up-regulated and 4 genes remained without variation. While in the normal feed administered group, 9 genes were amplified of which 5 genes such as

catalase, Hsc70, cathepsinC, proPO and SOD were up-regulated and 4 genes remained without variation.

The semiquantitative RT-PCR analysis of haemolymph of the IVP administered animals on 1DPA showed significant variation in bio-defence genes in the IVP administered animals when compared to the normal feed administered animals. Bio-defence mechanisms which were up-regulated in gills (before challenge), like protease inhibition involving alpha 2 macroglobulin, RNAi involving Pem-Ago, antimicrobial activity involving SWD and Pen-3, cell proliferation and survival involving eIF5A; antioxidant mechanism involving glutathione s transferase and coagulation involving transglutaminase were up-regulated in haemolymph as well. However, immunosuppression facilitating mechanism involving cyclophilin A and antibacterial activity involving lysozyme were observed to be up-regulated in haemolymph alone.

In the haemolymph of normal feed administered group on 1st day post administration, antioxidant activity involving catalase and SOD, heat shock related mechanism involving Hsc70, lysosomal protease activity involving cathepsinC, phenol oxidase activity involving proPO were found to be up-regulated in haemolymph.

Bio-defence mechanisms that did not show variation between the normal feed and IVP administered group include haemagglutination involving C type lectin, antimicrobial action involving crustin, phagocytosis activation involving PAP, and membrane traffiking involving Rab7.

After the challenge, in the haemolymph of 1st day post administration group of animals, only 4 genes such as SWD, crustin, PAP and PmRACK1 were amplified and up-regulated in the IVP administered group, while in the normal feed administered group 9 genes such as peroxinectin, c-SPH, cyclophilinA,

Pen-3, lysozyme, eIF5A, glutathione-s-transferase, SOD and Pem-Ago were amplified and up-regulated.

Crustins are antibacterial proteins of 7–14 kDa with a characteristic four-disulphide core-containing whey acidic protein (WAP) domain, expressed by the circulating haemocytes of crustaceans.

The receptor for activated protein kinase C1 (Pm-RACK1) is identified as a specific target for VP9, a nonstructural protein of WSSV (Toganunt et al., 2009). Pm-RACK1 was found to be up-regulated in hepatopancreas, stomach and hemocytes of WSSV infected *P. monodon*.

Antimicrobial activity involving SWD, phagocytosis involving PAP, antimicrobial activity involving crustin, and receptor interaction involving PmRACK1 were up-regulated in haemolymph of IVP administered challenged animals.

The haemolymph of the normal feed administered group showed the up-regulation of cell adhesion activity involving peroxinectin and c-SPH, immunosuppression activation involving cyclophilin A, antimicrobial activity involving Pen-3, antibacterial mechanism involving lysozyme, cell proliferation and survival involving eIF5A, antioxidant activity involving glutathione-s-transferase and SOD, and RNAi involving Pem-Ago.

The presence of bio-defence mechanisms that did not show variation between the challenged groups of normal feed and IVP administered group were absent.

4.4.2.2. 5th Day Post Administration (5DPA)

Before challenge, in the haemolymph of 5th day post administration group of animals, 15 genes were amplified in the IVP administered group among which 7 genes such as C type lectin, peroxinectin, lysozyme, Pen-3,

Rab7, PmRACK1 and proPO were up-regulated and 8 genes remained without variation, while in the normal feed administered group 9 genes were amplified of which 5 genes such as haemocyanin, cathepsinC, SWD, crustin, eIF5A and PAP were up-regulated and 8 genes remained without variation.

Lectins are proteins that have the ability to bind to specific carbohydrates and they are present in almost all living organisms. Lectins have been known as playing a central role in nonself recognition and clearance of invaders in invertebrate immunity by promotion of phagocytosis, antibacterial activity, activation of the proPO system and nodule formation (Luo et al., 2006). In shrimp, Ca dependant (C-type) lectins are the largest group of immune-related ESTs found in hepatopancreas, and it is the major group of PRPs upregulated in *L. vannamei* (Gross et al., 2001; Astrofsky et al., 2002). PmAV, a C-type lectin, was identified from WSSV resistant *P. monodon*. However, of the C-type lectins (PmLT, PmAV, PmLec) identified in *P. monodon*, PmLec was found to bind to bacterial lipopolysaccharide (LPS) to enhance hemocyte phagocytosis (Luo et al., 2006).

On 5th day post administration, haemagglutination involving C type lectin, cell adhesion involving peroxinectin, antibacterial activity involving lysozyme, antimicrobial effect involving Pen-3, membrane trafficking involving Rab7, receptor activation involving PmRACK1 and phenol oxidase system involving proPO were found to be up-regulated in IVP administered animals.

In the normal feed administered group, respiratory mechanism involving haemocyanin, lysosomal protease activity involving cathepsinC, antimicrobial activity involving crustin and SWD, cell proliferation and survival involving eIF5A and phagocytosis mechanism involving PAP were found to be up-regulated.

Bio-defence mechanisms that did not show variation between the normal feed and IVP administered group include protease inhibition involving alpha 2 macroglobulin, apoptosis involving caspase, antioxidant mechanism involving catalase, SOD and glutathione-s-transferase, cell adhesion involving c-SPH, immunosuppression activation involving cyclophilinA and coagulation involving transglutaminase.

After the challenge, in the haemolymph of 5th day post administration group of animals, only 3 genes were amplified among which 1 gene such as cyclophilinA was up-regulated in the IVP administered group, while in the normal feed administered group 7 genes were amplified of which 5 genes such as C type lectin, catalase, Hsc70, peroxinectin and Pen-3 up-regulated and 2 genes remained without variation.

In the group of IVP administered animals which was challenged on 5th day post administration, immunosuppression activation involving cyclophilinA was found to be up-regulated in the haemolymph.

In the haemolymph of normal feed administered group, haemagglutination involving C type lectin, antioxidant mechanism involving catalase, heat shock related activity involving Hsc70, cell adhesion involving peroxinectin and antimicrobial activity involving Pen-3 were found to be up-regulated.

Bio-defence mechanisms that did not show variation between the challenged groups of normal feed and IVP administered group were protease inhibition involving alpha 2 macroglobulin and antioxidant mechanism involving glutathione-s-transferase.

4.4.2.3. 10th Day Post Administration (10DPA)

Before challenge, in the haemolymph of 10th day post administration group of animals, 14 genes were amplified in the IVP administered group among which 6 genes such as c-SPH, SWD, Rab7, eIF5A, PmRACK1 and proPO were up-regulated and 8 genes remained without variation, while in the normal feed administered group 12 genes were amplified of which 4 genes such as C type lectin, cathepsinC, PAP and transglutaminase were up-regulated and 8 genes remained without variation.

On 10th day post administration, cell adhesion involving c-SPH, antimicrobial mechanism involving SWD, membrane trafficking involving Rab7, cell proliferation and survival involving eIF5A, receptor activation involving PmRACK1 and phenol oxidase system activation involving proPO were found to be up-regulated in IVP administered animals.

In normal feed administered animals, haemagglutination involving C type lectin, lysosomal protease activity involving cathepsinC, phagocytosis activation involving PAP and coagulation involving transglutaminase were up-regulated.

Bio-defence mechanisms that did not show variation between the normal feed and IVP administered group include protease inhibition involving alpha 2 macroglobulin, antioxidant mechanism involving SOD, catalase and glutathione-s-transferase, cell adhesion involving peroxinectin, immunosuppression related mechanism involving cyclophilinA, and antimicrobial activity involving crustin and Pen3.

After the challenge, in the haemolymph of 10th day post administration group of animals, 13 genes were amplified among which 10 genes such as C type lectin, catalase, Hsc70, cathepsinC, c-SPH, eIF5A, PAP, transglutaminase,

syntenin and SOD were up-regulated in the IVP administered group, while in the normal feed administered group 4 genes were amplified of which 1 gene such as Rab7 was up-regulated and 3 genes remained without variation.

Catalase is an important antioxidant protein that protects organisms against various oxidative stresses by eliminating hydrogen peroxide. Zhang et al. (2008) observed that in *F. chinensis*, a decrease in catalase expression occurred only after 37hr post infection of WSSV by injection. Catalase activity was found to be decreasing in WSSV infected *P.monodon* (Rameshthangam and Ramasamy, 2006; Mathew et al., 2007).

Haemagglutination involving C type lectin, antioxidant mechanism involving catalase and SOD, heat sock related mechanism involving Hsc70, lysosomal protease activity involving cathepsinC, cell adhesion involving c-SPH and cell proliferation, phagocytosis activation involving PAP, coagulation involving transglutaminase and signal transduction involving syntenin were up-regulated in haemolymph of IVP administered animals challenged on 10^{th} day post administration.

In the normal feed administered group, only the membrane trafficking bio-defence mechanism involving Rab7 was found to be up-regulated.

Biodefence mechanism that did not show variation between the challenged groups of normal feed and IVP administered groups were protease inhibition involving alpha 2 macroglobulin, antimicrobial mechanism involving SWD and Pen3.

4.4.2.4. Overview of bio-defence mechanism in haemolymph

In the haemolymph of IVP administered animals, the amplification of the number of bio-defence genes was higher on 1DPA, 5DPA and 10DPA when compared to the normal feed administered control group. The analysis of the up-

regulated genes in the IVP administered animals showed that more genes were up-regulated on1DPA, 5DPA and 10DPA when compared to the control group. The genes that were up-regulated in IVP administered animals were different on 1DPA, 5DPA and 10DPA except for lysozyme and Pen-3 which were present on 1DPA and 5DPA. Genes for SWD and eIF5A were present on 1DPA and 10DPA. On 5DPA and 10DPA, up-regulated genes such as Rab7, PmRACK1 and proPO were similar. The higher number of amplified/ up-regulated genes in the haemolymph of IVP administered animals on 1DPA, 5DPA and 10DPA showed that bio-defence response has been elicited in the haemolymph unlike that was observed in the gill tissue.

After an oral challenge, the haemolymph of IVP administered animals showed amplification of less number of genes in the IVP administered animals on 1DPA and 5DPA, and higher on 10DPA when compared to the control (normal feed administered group after the challenge). The decrease in the up-regulated genes on 1DPA and 5DPA, and subsequent increase in the up-regulated genes on 10DPA point that in the haemolymph the bio-defence mechanisms are more active on 10DPA unlike the condition observed in the gill tissue. The up-regulated genes in the IVP administered animals after an oral challenge were different on 1DPA, 5DPA and 10DPA except for PAP which was up-regulated on 5DPA and 10DPA. The up-regulation of different genes in different groups showed that the immune response elicited against WSSV in IVP administered animals varied with respect to time post administration in haemolymph.

The comparative analysis of the up-regulated genes between the unchallenged and challenged group of animals on 1DPA, 5DPA and 10DPA showed that after the challenge, the genes which were up-regulated were different from those expressed in the unchallenged condition except for the up-

regulation of SWD in the 1DPA group and c-SPH in the 10DPA group in the unchallenged and challenged condition category.

4.4.3. Hepatopancreas

4.4.3.1. 1st Day Post Administration (1DPA)

Before challenge, in the hepatopancreas of 1st day post administration group of animals, 7 genes were amplified in the IVP administered group among which 1 gene such as PmAV was up-regulated and the remaining 6 genes remained without variation, while in the normal feed administered group 7 genes were amplified of which 1 gene such as SOD was up-regulated and the remaining 6 genes remained without variation.

The semiquantitative RT-PCR analysis of hepatopancreas of the experimental animals on 1DPA showed up-regulation of PmAV when compared to the normal feed administered animals.

PmAV mRNA was found to be up regulated in virus-resistant shrimps, indicating that *PmAV* gene is inducible in contrast to the genes of shrimp penaeidin (shrimp antibacterial peptide) with constitutive expression (Destoumieux et al., 1997, 2000). This result suggests that the transcriptional regulation of *PmAV* is important for viral defense. When shrimps were challenged by WSSV under the fatal dose by Luo et al. (2007), the *PmAV* gene expression in the hepatopancreas was down-regulated within the first day post-infection, while the viral load was low. This may be due to inhibition of shrimp immunity by WSSV at the early stage of infection. From the second day, a significant increase in both viral load and *PmAV* expression appeared. It indicates that the shrimp antiviral mechanism may be triggered by virus through a complicated process: a delayed response, or a response that correlated to viral load. The *PmAV* gene involved in antiviral defense is then induced to express.

In the normal feed administered group, antioxidant mechanism involving SOD was found to be upregulated in the hepatopancreas on 1st day post administration.

Biodefence mechanisms that did not show variation between the normal feed and IVP administered group included RNAi involving PemAgo, antioxidant defence involving catalase and glutathione s transferase, cytoskeletal activation involving tropomyosinII, phagocytic mechanism involving PAP, and heat shock related mechanism involving Hsc70.

After the challenge, in the hepatopancreas of 1st day post administration group of animals, 6 genes were amplified in the IVP administered group of which 5 genes such as C type lectin, Pem-Ago, PmRACK1, PAP and SOD were upregulated and 1 gene did not show variation. However, in the normal feed administered group 2 genes were amplified of which 1 gene such as PmAV was upregulated and the remaining 1 gene (glutathione-s-transferase) showed no variation.

In the group of IVP administered animals which were challenged on 1st day post administration, bio-defence mechanisms such as haemagglutination involving C type lectin, RNAi involving PemAgo, receptor activation involving PmRACK1, phagocytosis activation involving PAP and antioxidant defence involving SOD were up-regulated in the hepatopancreas.

Bio-defence mechanisms that did not show variation between the challenged groups of normal feed and IVP administered group include antioxidant mechanism involving glutathione-s-transferase.

4.4.3.2. 5th Day Post Administration (5DPA)

Before challenge, in the hepatopancreas of 5th day post administration group of animals, 8 genes were amplified in the IVP administered group among

which 2 genes such as PmAV and SOD were up-regulated and the remaining 4 genes remained without variation, while in the normal feed administered group 8 genes were amplified of which 2 genes such as tropomyosinII and PmRACK1 were up-regulated and the remaining 6 genes remained without variation.

On 5th day post administration, antiviral gene involving PmAV and antioxidant mechanism involving SOD were found to be up-regulated in IVP administered group.

In the normal feed administered group, cytoskeletal mechanism involving tropomyosinII and receptor activation involving PmRACK1 was found to be up-regulated.

Bio-defence mechanisms that did not show variation between the normal feed and IVP administered group include haemagglutination involving C type lectin, RNAi involving Pem-Ago, antioxidant mechanism involving catalase and glutathione-s-transferase, phagocytosis mechanism involving PAP and heat shock related mechanism involving Hsc70.

After the challenge, in the hepatopancreas of 5th day post administration group of animals, 7 genes were amplified in the IVP administered group of which 3 genes such as PemAgo, tropomyosinII and PmRACK1 were up-regulated and 4 genes did not show variation. However, in the normal feed administered group 6 genes were amplified of which 2 genes such as PmAV and SOD were up-regulated and the remaining 4 genes showed no variation.

In the group of IVP administered animals which was challenged on 5th day post administration, RNAi involving PemAgo, cytoskeletal mechanism involving tropomyosinII and receptor activation involving PmRACK1 were found to be up-regulated.

In the hepatopancreas of normal feed administered group, antiviral gene PmAV and antioxidant activity involving SOD were found to be up-regulated.

Bio-defence mechanisms that did not show variation between the challenged groups of normal feed and IVP administered group were haemagglutination involving C type lectin, antioxidant mechanism involving glutathione-s-transferase, phagocytosis mechanism involving PAP and heat shock related mechanism involving Hsc70.

4.4.3.3. 10th Day Post Administration (10DPA)

Before challenge, in the hepatopancreas of 10th day post administration group of animals, 9 genes were amplified in the IVP administered group and all of them remained without variation, while in the normal feed administered group 10 genes were amplified of which 1 gene such as PmRACK1 was up-regulated and the remaining 9 genes remained without variation.

On 10th day post administration, only the normal feed administered group showed up-regulation with the bio-defence mechanism like receptor activation involving PmRACK1.

Bio-defence mechanisms that did not show variation between the normal feed and IVP administered group include haemagglutination involving C type lectin, RNAi involving Pem-Ago, antioxidant mechanism involving SOD, catalase and glutathione s transferase, antiviral gene PmAV, cytoskeletal activation involving tropomyosinII, phagocytosis activation involving PAP, and heat shock related mechanism involving Hsc70.

After the challenge, in the hepatopancreas of 10th day post administration group of animals, 3 genes were amplified in the IVP administered group and in the normal feed administered group and these showed no variation in between the groups.

In the group of IVP and normal feed administered animals, none of the selected bio-defence genes was found to be up-regulated after the challenge.

Bio-defence mechanisms that did not show variation between the challenged groups of normal feed and IVP administered group were antioxidant mechanism involving catalase and glutathione-s-transferase and phagocytosis activation involving PAP**.**

4.4.3.4. Overview of bio-defence mechanism in hepatopancreas

In the hepatopancreas of IVP administered animals, the amplification of the number of bio-defence genes was equal (different genes) on 1DPA and 5DPA and lower on 10DPA when compared to the normal feed administered control group. The analysis of the up-regulated genes in the IVP administered animals showed that equal number but different genes were up-regulated on 1DPA and 5DPA and no up-regulation in 10DPA when compared to the control group. The genes that were up-regulated in IVP administered animals were PmAV on 1DPA and 5DPA, and MnSOD on 5DPA. The amplification/ up-regulation of equal number of bio-defence gene in the hepatopancreas on 1DPA and 5DPA shows that though specific genes have been activated on these days, not much immune activation has taken place in the tissue in response to the IVP administration unlike the conditions observed in the gills and haemolymph. The lesser number of genes amplified/ up-regulated on 10DPA shows that the immune response may be getting weaker after a specific period after the administration.

After an oral challenge, the hepatopancreas of IVP administered animals showed amplification of higher number of genes in the IVP administered animals on 1DPA and 5DPA, and equal number of genes on 10DPA when compared to the control (normal feed administered group after the challenge). The analysis of the up-regulated genes in the IVP administered animals showed that more genes were up-regulated on 1DPA and 5DPA when compared to the

control group, and on 10DPA there was no difference from the control group. The pattern of up-regulation of genes on 1DPA and 5DPA observed in the hepatopancreas of animals challenged after IVP administration is similar to that in the gills. The up-regulated genes in the IVP administered animals after an oral challenge were different on 1DPA and 5DPA except for Pem-Ago and PmRACK1. The amplification/ up-regulation of higher number of bio-defence gene in the hepatopancreas on 1DPA and 5DPA showed that the tissue had elicited an up-regulatory mechanism for some of the bio-defence genes to combat the WSSV infection in these periods.

The comparative analysis of the up-regulated genes between the unchallenged and challenged group of animals on 1DPA, 5DPA and 10DPA showed that after the challenge, the genes which were up-regulated were different than that was expressed in the unchallenged condition. The observation suggests that the bio-defence genes that are activated in the hepatopancreas of IVP administered animals after a WSSV challenge are unlike those in gills and haemolymph.

The variation in the number and type of genes amplified/ up-regulated in the IVP administered animals over a period of time, and in different tissues points to the complexity involved in the bio-defence mechanism in *P. monodon*.

Although a number of bio-defence genes were up-regulated in the normal feed administered and IVP administered groups, there was specific difference in the genes which were up-regulated thus showing the different mechanisms adapted by the two groups in response to the WSSV challenge.

It was observed from the analysis of bio-defence genes which were amplified/ up-regulated/ without variation that the various cellular and humoral immune responses elicited on 1DPA, 5DPA and 10DPA in gills, haemolymph

and hepatopancreas of IVP administered animals were not similar or comparable showing that some part of different bio-defence mechanisms were simultaneously activated in different tissues to modulate the WSSV succession in *P. monodon,* which is unlike the classical concept of shrimp immune defence.

Table 4. Primer sequences of the bio-defence genes selected for the study

Gene	Primer sequences (5'-3') and Annealing Temperature (ºC)	PCR amplicon (~bp)	Reference & GenBank Sequences used for primer designing by GeneTool & Primer3
Prophenol oxidase (proPO)	F-TGGCACTGGCACTTGATCTA R-GCGAAAGAACACAGGGTCTCT (56)	590	
Astakine	F-GTCGCGCATTTAACAAGGAG R-CCCTGTGGATTGAGCTCACT (56)	455	
Peroxinectin	F-CGAAGCTTCTTGCAACTACCA R-GCAGGCTGATTAAACTGGCTT (56)	547	Jiravanichpaisal et al., 2007
Haemocyanin	F-GTCGACGAACTTCACTGGGA R-GTTCAGTGTCATCAACGGCA (56)	598	
Transglutaminase	F-TGGGYCTTCGGGCAGTT R-CGAAGGGCACGTCGTAC (56)	627	
Penaeidin-3	F-AGGATATCATCCAGTTCCTG R-ACCTACATCCTTTCCACAAG (55)	240	
Alpha 2 macroglobulin	F-ATGGCCAATCCCGAGAGGTACCTACTG R-TGTTGCTGCAGAAGTTTGTTATCCTCAT(65)	345	Nucleotide sequence AY826818.1
Superoxide dismutase (cytosolic MnSOD)	F-CGAGGCTTGCGCGTCAC R-TTTGGTTGCCCCGAGGAGTC (61)	924	Nucleotide sequence AY726542.1
Catalase	F-ACTCCCATTGCTGTTCGT R-ATCCCAATTTCCTTCTTCTG (47)	130	EST Sequence GO082433.1
Glutathione peroxidase	F-AGTCGATGTCAACGGGTCAAC R-GCTGAACCTCTTAAACGCCTG (57)	985	Ren et al., 2009
Glutathione-s-transferase	F-TTCGCCGGAGACAAGCTAACC R-GCGATCGTAAACTGAGCGTAC (57)	247	Ren et al., 2009
HSC70	F-TGTCGGTATTGATCTGGGAA R-ACGCTCTGTGTCTGTGAAGG (55)	125	Chuang et al., 2007

Caspase	F-GGAGGAACCTGCGAAGAAC R-AGCGTCGAGTGGATGTAAGG	(57)	825	Wongprasert et al., 2007
Rab7	F-TTCCCTCCCAAAGTACATCATG R-AGGCCAATCCCCATGTGAAAT	(55)	887	DQ231062.1
Cyclophilin A	F-CGTAAAGGTGGTGAAACAAGA R-GTGAGAAGACGAAATGGTGAAT	(55)	125	Lihua et al., 2009
Chitinase	F-GAGAAGCTGAACGTGAACGAC R-CTTGATCTTGAGACTGTCAGG	(55)	350	AF157503.1
Single WAP domain/crustin III	F-CGATATCTTCTCCATCTGCGTC R-GAGCCAACCGCGATGACGTCAG	(57)	190	Amparyup et al., 2008
Lysozyme	F-CCGCCATCAACCGCAACCGC R-GCGGACAGTCTCGGAGCACT	(57)	165	AF539466.2
Argonuate (Pem-ago)	F-CAAGAATTTGGTCTGACGAT R-GGTGAGGTTTTGTTCACATT	(47)	495	DQ531711.1
PAP	F-ATTGCATCATCCACCATG R-GGGACTTTGTCATCTTCA	(47)	360	Deachamag et al., 2006
C-type lectin (PmLec)	F-CGTGGATCCCAACCTCTTCAGGGTAGG R-CGTGAATTCAGCATTGTACTGGCACAC	(57)	546	Luo et al., 2006
cSPH	F-CATGGACAGGCTGCGGTTGC R-GTGTCTTCGTTTGTCGCCGG	(61)	1093	Lin et al., 2006
Tropomyosin II	F-GCTGCTCGAGGAGGACCTGG R-TCCTGTCGGCTTCCTCAGCC	(61)	240	Tan et al., 2000
Syntenin	F-GATTTGGCCGTCTCACAAGTGCAG R-GCCTTAAGTTACAGGTCGGGAATGG	(55)	750	AF335106.1
eIF5A	F-GCTCTCTCGCTCCTCCTTTCAT R-GCCCATGGACAAAGCAAAGGT	(55)	737	DQ851145.1

PmAV	F- GATGGTGGCTCCTCAGAGAACT R- GTAGCAAGACACATCATGGATATGG (62)	~100	Luo et al., 2007
PmRACK-1	F-CTGCGCGGGACCCTGGTG R-CACGGGAAGTAACGCTGACCTGC (60)	937	EF569136.1
Crustin	F-CGCACAGCCGAGAGAAACACTATCAAGAT R-GGCCTATCCCTCAGAACCCAGCACG (55)	430	EF654659.1
PmCathepsin C	F-5' GATTCTGACCAGCAACCACCA 3' R-5' TACAGGCTCCATAGTAACCTCCAA 3' (56)	240	Qui et al., 2008
Beta Actin	F-CTTGTGGTTGACAATGGCTCCG R-TGGTGAAGGAGTAGCCACGCTC (55)	520	Zhang et al., 2007

Table 5. Bio-defence genes expressed in different tissues

Gene	bp	Gill	Haemolymph	Hepatopancreas	Primers-new
Beta actin	520	E	E	E	
Pro PO	590	E	E	NE	
Peroxinectin	547	E	E	NE	
Transglutaminase	627	E	E	NE	
Haemocyanin	598	NE	E	NE	
Alpha 2 macroglobulin	345	E*	E	E	AY826818.1
Caspace	825	E	E	NE	
Cathepsin C	240	E	E	NE	
PAP	360	E	E	E	
C type lectin	546	E	E	E	
Astakine	455	E	-	NE	
Crustin	430	E	E	NE	EF654659.1
SWD/ crustin III	190	E	E	NE	
Penaedin 3	240	E	E	NE	
lysozyme	165	E	E	NE	AF539466.2
SOD (cytosolicMn)	924	E	E	E	AY726542.1
catalase	130	E	E	E	EST Sequence GO082433.1
Glutathione peroxidase	985	-	-	-	
Glutathione s transferase	247	E	E	E	
Pm Argonaute (PemAgo)	495	E	E	E	DQ531711.1
c-SPH	1093	E*	E	NE	
Tropomyosin II	240	E	NE	E	
syntenin	750	E	E	NE	AF335106.1
eIF5A	737	E	E	NE	DQ851145.1
PmAV	100	E	E	E	
PmRACK1	937	E*	E	E	
Rab7	887	E	E	NE	DQ231062.1
Cyclophilin A	125	E*	E	NE	
Hsc70	125	E	E	E	
Chitinase	350	E	-	-	AF157503.1

E – Expressed; NE – Not Expressed; * - bp vary

Table 6. Bio-defence genes in gills on 1st day post administration

1DPA	Before challenge		After challenge	
	Normal	**IVP**	**Normal**	**IVP**
Upregulated	Cathepsin C	Alpha 2 macroglobulin	Alpha 2 macroglobulin	Astakine
		Astakine	C type lectin	Cathepsin C
		Chitinase	Catalase	PAP
		Glutathione s transferase	c-SPH	SOD
		eIF5A	Chitinase	Syntenin
		Pen-3	eIF5A	
		PemAgo	Pen-3	
		Transglutaminase	PemAgo	
		proPO	Transglutaminase	
		SWD	proPO	
		Hsc70	Hsc70	
		TropomyosinII	TropomyosinII	
Without variation	C type lectin	C type lectin	PmAV	PmAV
	Catalase	Catalase	Lysozyme	Lysozyme
	cSPH	cSPH	Peroxinectin	Peroxinectin
	PmAV	PmAV	PemAgo	PemAgo
	Lysozyme	Lysozyme	Cyclophilin A	Cyclophilin A
	Peroxinectin	Peroxinectin	Crustin	Crustin
	PAP	PAP	PmRACK1	PmRACK1
	Caspase	Caspase	Rab7	Rab7
	Cyclophilin A	Cyclophilin A	SWD	SWD
	Crustin	Crustin		
	PmRACK1	PmRACK1		
	Rab7	Rab7		
	Syntenin	Syntenin		

Table 7. Bio-defence genes in gills on 5th day post administration

5DPA	Before challenge		After challenge	
	Normal	**IVP**	**Normal**	**IVP**
Upregulated	Astakine	Lysozyme	Lysozyme	Alpha 2 macroglobulin
	Catalase			Astakine
	Cathepsin C			Cyclophilin A
	cSPH			Rab7
	PmAV			SWD
	SOD			
	Cyclophilin A			
	Rab7			
	SWD			
Without variation	Alpha 2 macroglobulin	Alpha 2 macroglobulin	C type lectin	C type lectin
	C type lectin	C type lectin	eIF5A	eIF5A
	Chitin	Chitin	Pen-3	Pen-3
	Glutathione s transferase	Glutathione s transferase	Pem-Ago	Pem-Ago
	eIF5A	eIF5A	PmRACK1	PmRACK1
	Peroxinectin	Peroxinectin	Syntenin	Syntenin
	PAP	PAP	proPO	proPO
	Pen3	Pen3	Hsc70	Hsc70
	PemAgo	PemAgo	Peroxinectin	Peroxinectin
	Caspase	Caspase		
	Crustin	Crustin		
	PmRACK1	PmRACK1		
	Syntenin	Syntenin		
	Transglutaminase	Transglutaminase		
	proPO	proPO		
	Hsc70	Hsc70		
	TropomyosinII	TropomyosinII		

Table 8. Bio-defence genes in gills on 10th day post administration

10DPA	Before challenge		After challenge	
	Normal	**IVP**	**Normal**	**IVP**
Upregulated	Astakine	cSPH		SWD
	Lysozyme	Chitinase		
Without variation	Alpha 2 macroglobulin	Alpha 2 macroglobulin	Cathepsin C	Cathepsin C
	C type lectin	C type lectin	c-SPH	c-SPH
	Catalase	Catalase	Lysozyme	Lysozyme
	Cathepsin C	Cathepsin C	Peroxinectin	Peroxinectin
	PmAV	PmAV	PAP	PAP
	Glutathione s transferase	Glutathione s transferase	Pen-3	Pen-3
	eIF5A	eIF5A	Cyclophilin A	Cyclophilin A
	Peroxinectin	Peroxinectin	PmRACK1	PmRACK1
	PAP	PAP	Rab7	Rab7
	SOD	SOD	Syntenin	Syntenin
	Pen3	Pen3	Transglutamina se	Transglutamina se
	PemAgo	PemAgo	proPO	proPO
	Caspase	Caspase	Hsc70	Hsc70
	Cyclophilin A	Cyclophilin A	Tropomyosin II	Tropomyosin II
	Crustin	Crustin		
	PmRACK1	PmRACK1		
	Rab7	Rab7		
	Syntenin	Syntenin		
	Transglutaminase	Transglutaminase		
	proPO	proPO		
	SWD	SWD		
	Hsc70	Hsc70		
	TropomyosinII	TropomyosinII		

Table 9. Bio-defence genes in haemolymph on 1st day post administration

1DPA	Before challenge		After challenge	
	Normal	**IVP**	**Normal**	**IVP**
Upregulated	Catalase	Alpha 2 macroglobulin	Peroxinectin	SWD
	Hsc70	PemAgo	c-SPH	Crustin
	Cathepsin C	Cyclophilin A	Cyclophilin A	PAP
	proPO	SWD	Pen-3	PmRACK1
	SOD	Lysozyme	Lysozyme	
		Pen-3	eIF5A	
		eIF5A	Glutathione s transferase	
		Glutathione s transferase	SOD	
		Transglutaminase	PemAgo	
Without variation	C type lectin	C type lectin		
	Crustin	Crustin		
	PAP	PAP		
	Rab7	Rab7		

Table 10. Bio-defence genes in haemolymph on 5th day post administration

5DPA	Before challenge		After challenge	
	Normal	**IVP**	**Normal**	**IVP**
Upregulated	Haemocyanin	C type lectin	C type lectin	Cyclophilin A
	Cathepsin C	Peroxinectin	Catalase	
	SWD	Lysozyme	Hsc70	
	Crustin	Pen-3	Peroxinectin	
	eIF5A	Rab7	Pen-3	
	PAP	PmRACK1		
		proPO		
Without variation	Alpha 2 macroglobulin	Alpha 2 macroglobulin	Alpha 2 macroglobulin	Alpha 2 macroglobulin
	Caspase	Caspase	Glutathione s transferase	Glutathione s transferase
	Catalase	Catalase		
	c-SPH	c-SPH		
	Cyclophilin A	Cyclophilin A		
	Glutathione s transferase	Glutathione s transferase		
	Transglutaminase	Transglutaminase		
	SOD	SOD		

Table 11. Bio-defence genes in haemolymph on 10th day post administration

10DPA	Before challenge		After challenge	
	Normal	**IVP**	**Normal**	**IVP**
Upregulated	C type lectin	c-SPH	Rab7	C type lectin
	Cathepsin C	SWD		Catalase
	PAP	Rab7		Hsc70
	Transglutaminase	eIF5A		Cathepsin C
		PmRACK1		cSPH
		proPO		eIF5A
				PAP
				Transglutaminase
				Syntenin
				SOD
Without variation	Alpha 2 macroglobulin	Alpha 2 macroglobulin	Alpha 2 macroglobulin	Alpha 2 macroglobulin
	Catalase	Catalase	SWD	SWD
	Peroxinectin	Peroxinectin	Pen-3	Pen-3
	Cyclophilin A	Cyclophilin A		
	Pen-3	Pen-3		
	Crustin	Crustin		
	Glutathione s transferase	Glutathione s transferase		
	SOD	SOD		

Table 12. Bio-defence genes in hepatopancreas on 1st day post administration

1DPA	Before challenge		After challenge	
	Normal	**IVP**	**Normal**	**IVP**
Upregulated	SOD	PmAV	PmAV	C type lectin
				PemAgo
				PmRACK1
				PAP
				SOD
Without variation	PemAgo	PemAgo	Glutathione s transferase	Glutathione s transferase
	Catalase	Catalase		
	Glutathione s transferase	Glutathione s transferase		
	TropomyosinII	TropomyosinII		
	PAP	PAP		
	Hsc70	Hsc70		

Table 13. Bio-defence genes in hepatopancreas on 5th day post administration

5DPA	Before challenge		After challenge	
	Normal	**IVP**	**Normal**	**IVP**
Upregulated	TropomyosinII	PmAV	PmAV	PemAgo
	PmRACK1	SOD	SOD	TropomyosinII
				PmRACK1
Without variation	C type lectin	C type lectin	C type lectin	C type lectin
	PemAgo	PemAgo	Glutathione s transferase	Glutathione s transferase
	Catalase	Catalase	PAP	PAP
	Glutathione s transferase	Glutathione s transferase	Hsc70	Hsc70
	PAP	PAP		
	Hsc70	Hsc70		

Table 14. Bio-defence genes in hepatopancreas on 10th day post administration

10DPA	Before challenge		After challenge	
	Normal	**IVP**	**Normal**	**IVP**
Upregulated	PmRACK1			
Without variation	C type lectin	C type lectin	Catalase	Catalase
	PemAgo	PemAgo	Glutathione s transferase	Glutathione s transferase
	Catalase	Catalase	PAP	PAP
	Glutathione s transferase	Glutathione s transferase		
	PmAV	PmAV		
	TropomyosinII	TropomyosinII		
	PAP	PAP		
	Hsc70	Hsc70		
	SOD	SOD		

Pages 153-169 contain the figures of the PCR products of different bio-defence genes in gills, haemolymph and hepatopancreas. Each gene amplicon is documented in the left side of the page and the band intensity is graphically represented on the right side of the page. Each lane of the documented gel contain the following:

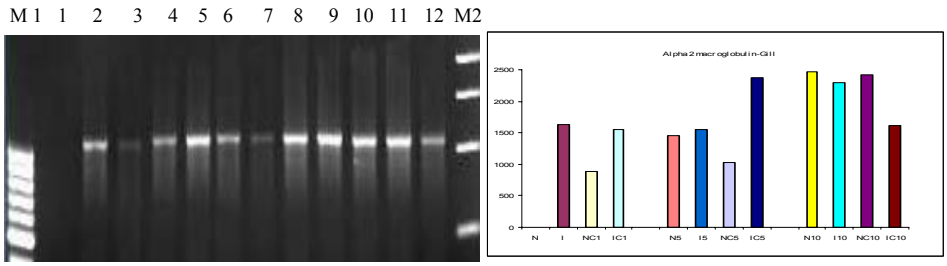
M1 – 100bp ladder

1. Normal (fed with normal feed, 1st day after 7 days of administration)
2. IVP (fed with IVP coated feed, 1st day after 7 days of administration)
3. Normal (fed with normal feed, challenged 1st day after 7 days of administration and samples collected on 4th day post challenge)
4. IVP (fed with IVP coated feed, challenged 1st day after 7 days of administration and samples collected on 4th day post challenge)
5. Normal (fed with normal feed, 5th day after 7 days of administration)
6. IVP (fed with IVP coated feed, 5th day after 7 days of administration)
7. Normal (fed with normal feed, challenged 5th day after 7 days of administration and samples collected on 4th day post challenge)
8. IVP (fed with IVP coated feed, challenged 5th day after 7 days of administration and samples collected on 4th day post challenge)
9. Normal (fed with normal feed, 10th day after 7 days of administration)
10. IVP (fed with IVP coated feed, 5th day after 7 days of administration)
11. Normal (fed with normal feed, challenged 10th day after 7 days of administration and samples collected on 4th day post challenge)
12. IVP (fed with IVP coated feed, challenged 10th day after 7 days of administration and samples collected on 4th day post challenge)

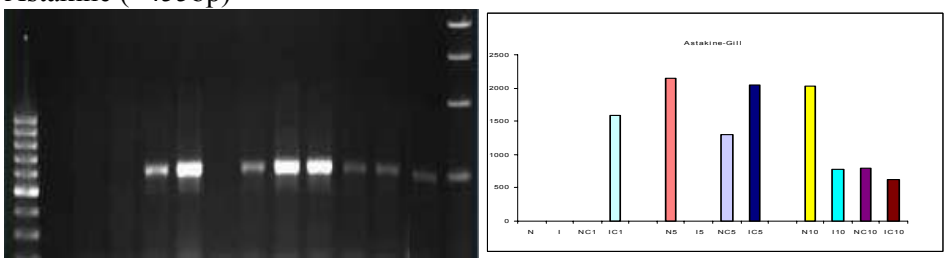
M2 – 1000bp ladder

Gill

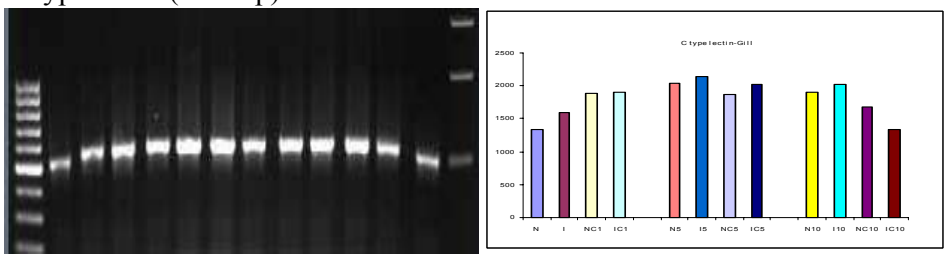
Alpha 2 macroglobulin (~1015bp)



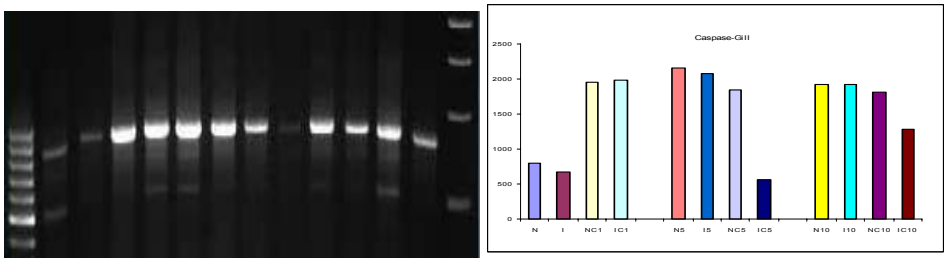
Astakine (~455bp)



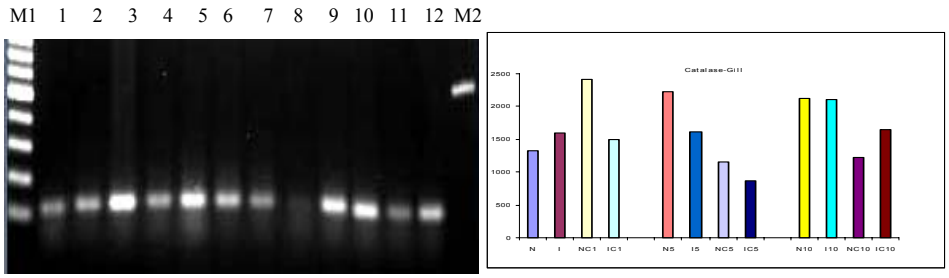
C-type lectin (~546bp)



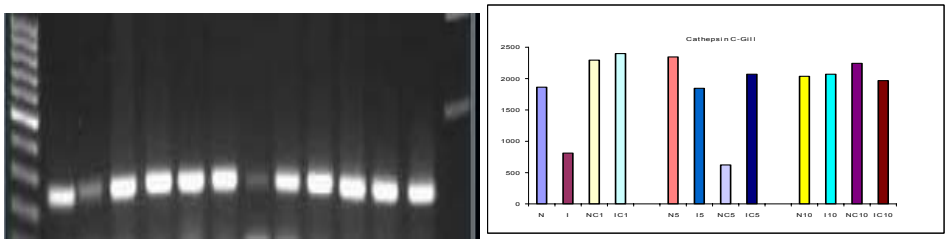
Caspase (~825bp)



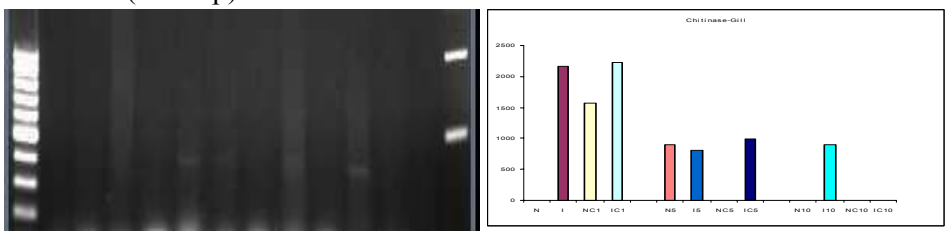
Catalase (~130bp)



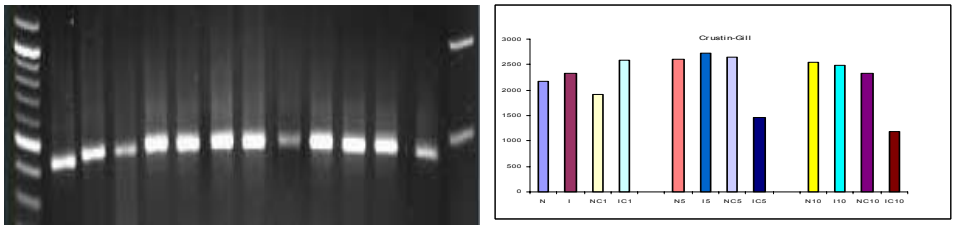
Cathepsin C (~240bp)



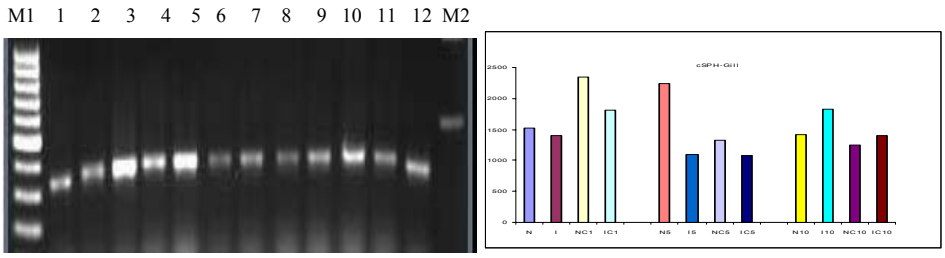
Chitinase (~350bp)



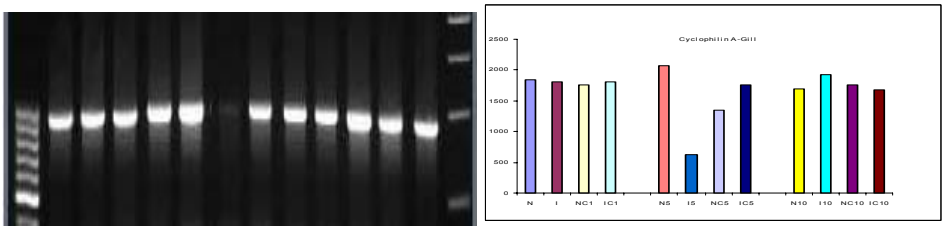
Crustin (~430bp)



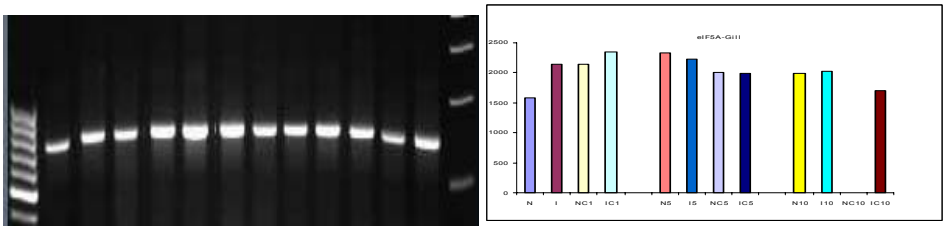
c-SPH (~320bp)



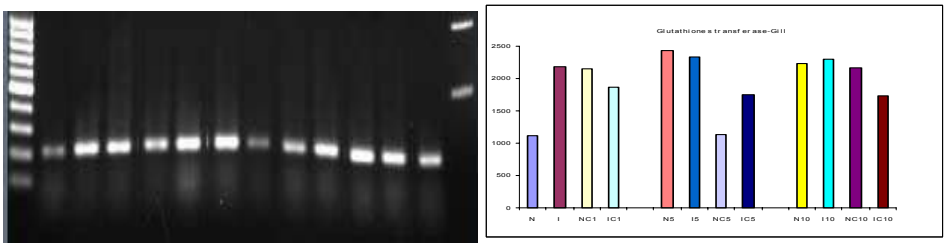
Cyclophilin A (~940bp)



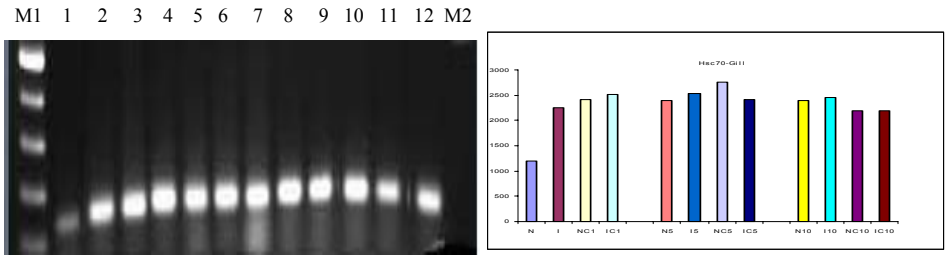
eIF5A (~737bp)



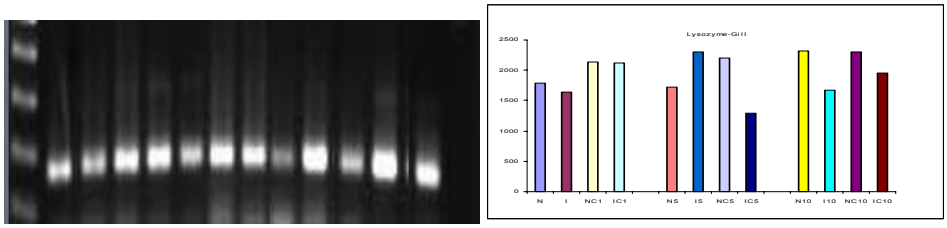
Glutathione-s-transferase (~247bp)



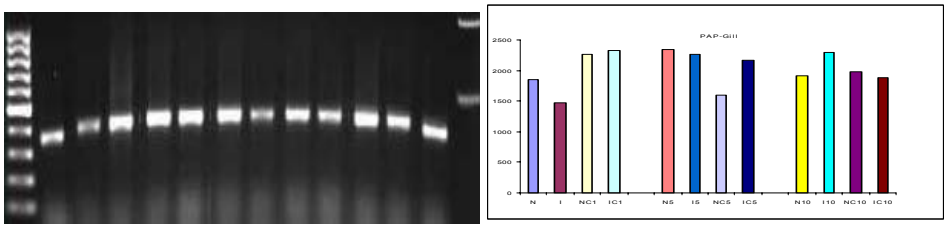
Hsc70 (~125bp)



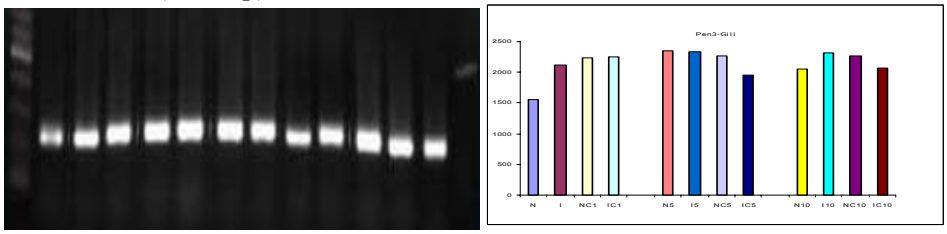
Lysozyme (~165bp)



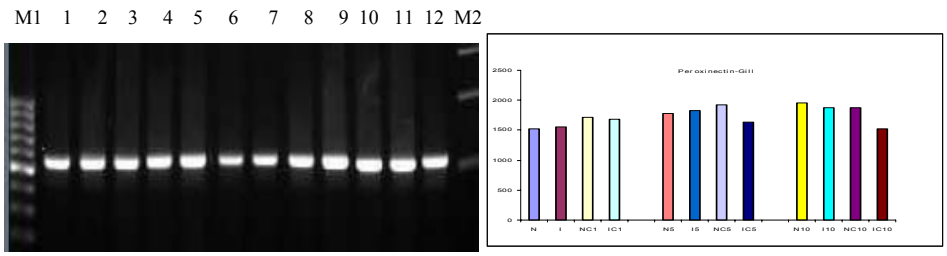
PAP (~360bp)



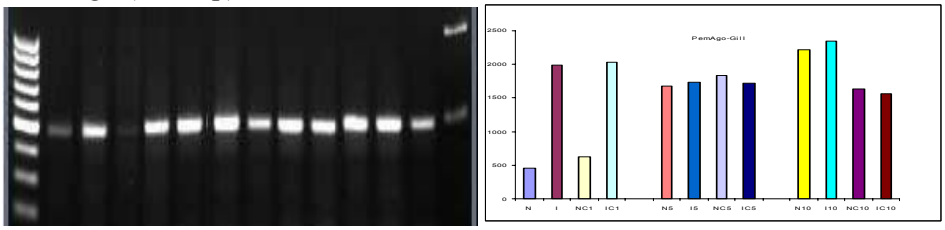
Penaeidin-3 (~240bp)



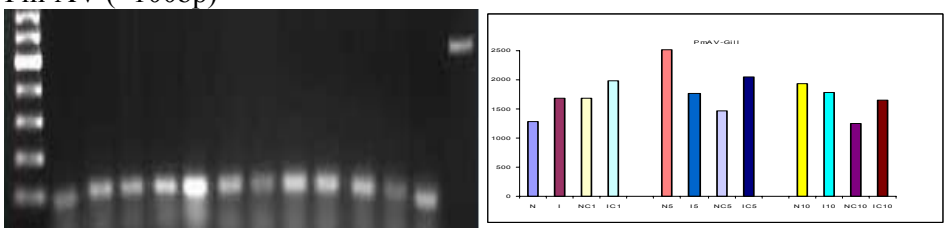
Peroxinectin (~547bp)



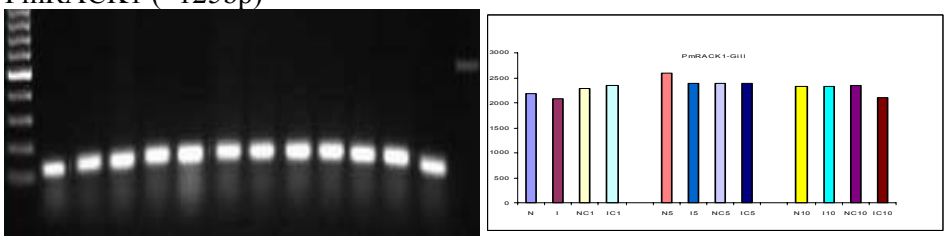
Pem-Ago (~495bp)



Pm-AV (~100bp)

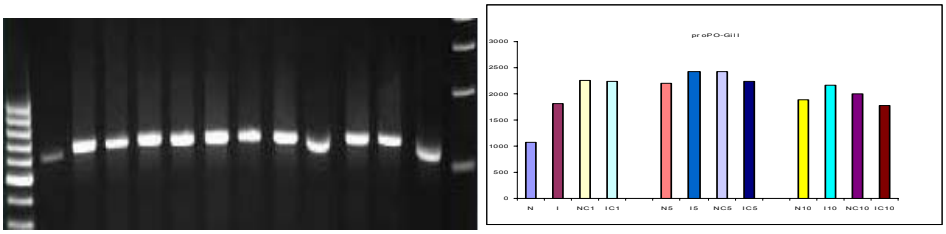


PmRACK1 (~125bp)

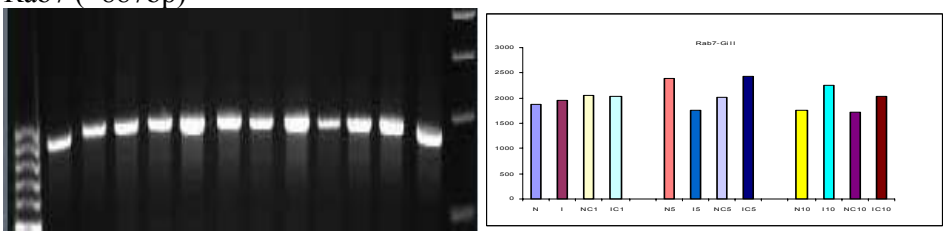


ProPO (~590bp)

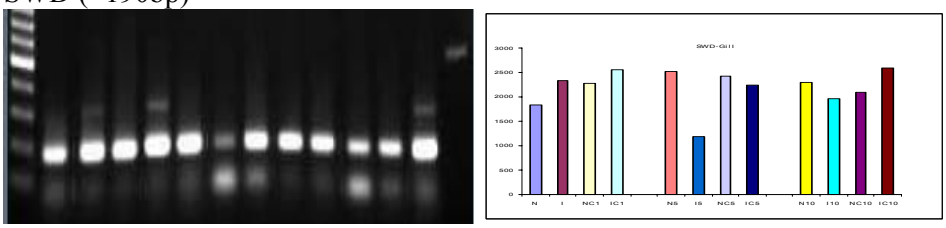
M1 1 2 3 4 5 6 7 8 9 10 11 12 M2



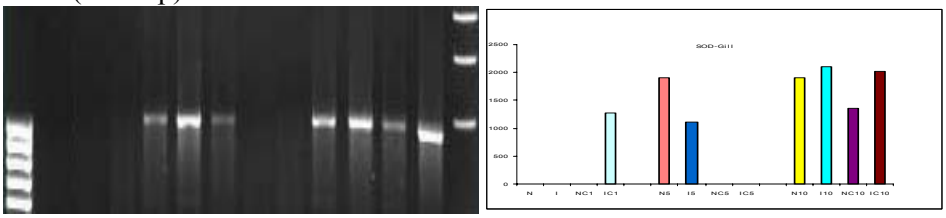
Rab7 (~887bp)



SWD (~190bp)

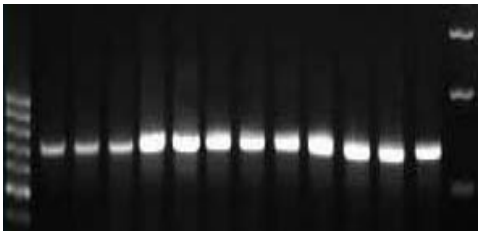


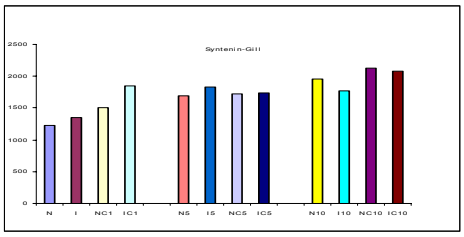
SOD (~924bp)



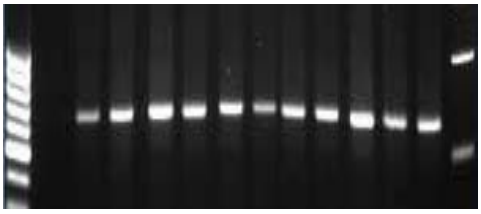
Syntenin (~750bp)

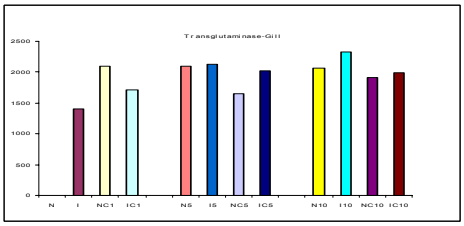
M1 1 2 3 4 5 6 7 8 9 10 11 12 M2



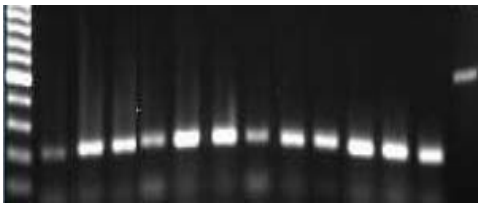


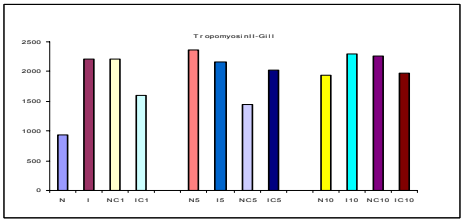
Transglutaminase (~627bp)



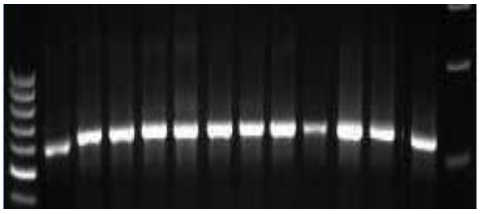


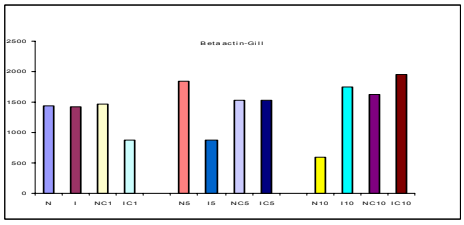
TropomyosinII (~240bp)





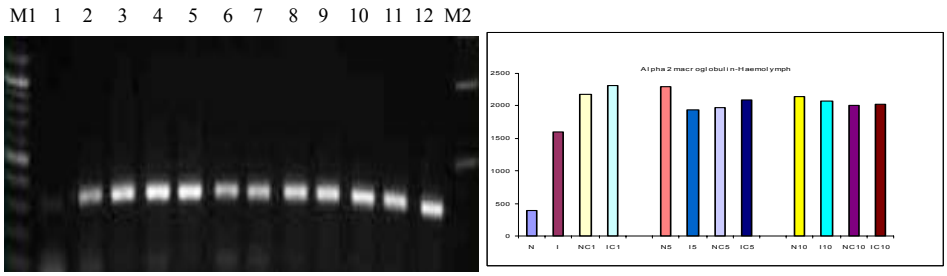
beta-actin (~520bp)



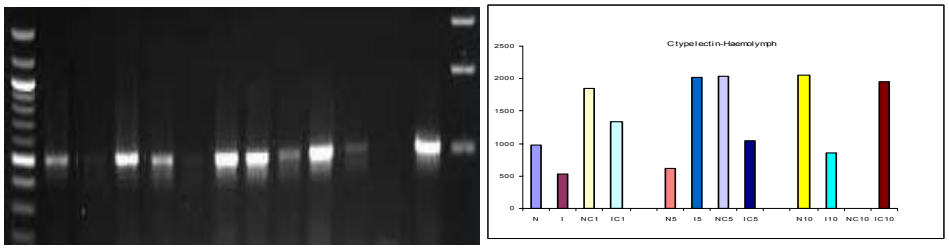


Haemolymph

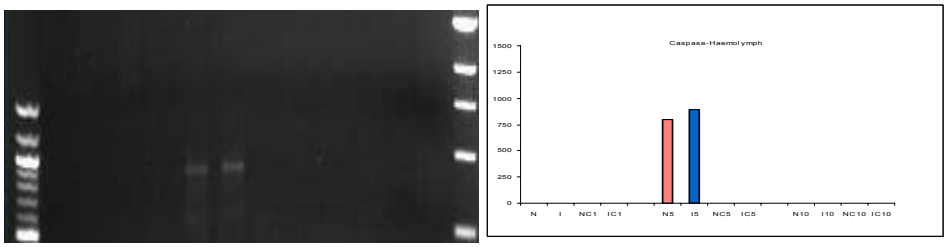
Alpha 2 macroglobulin (~345bp)



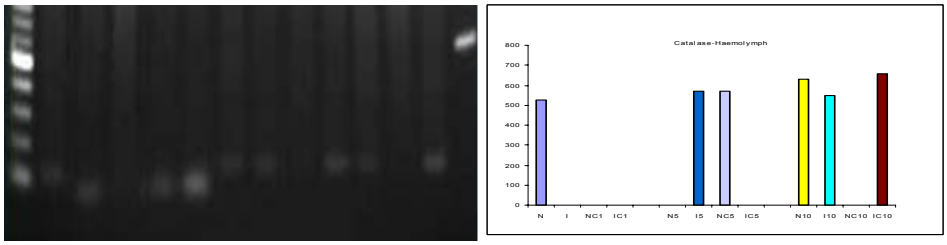
C-type lectin (~546bp)



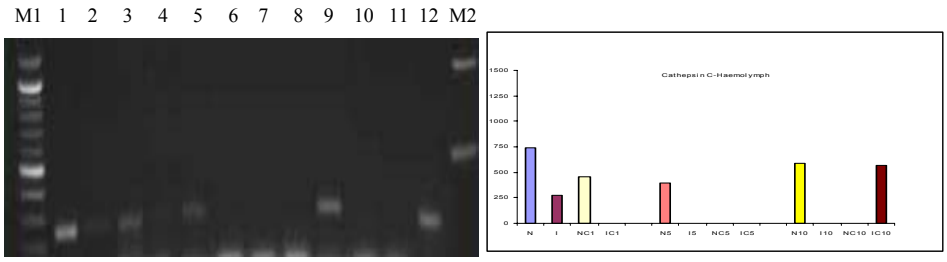
Caspase (~825bp)



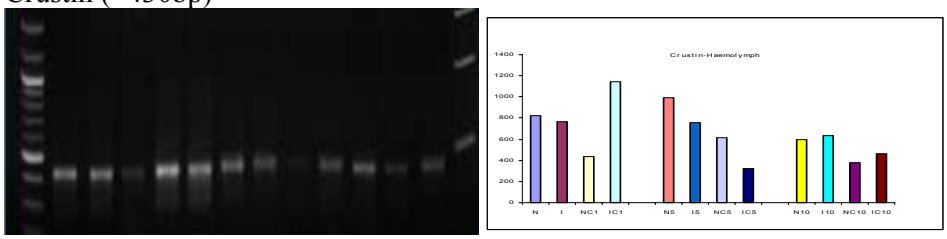
Catalase (~130bp)



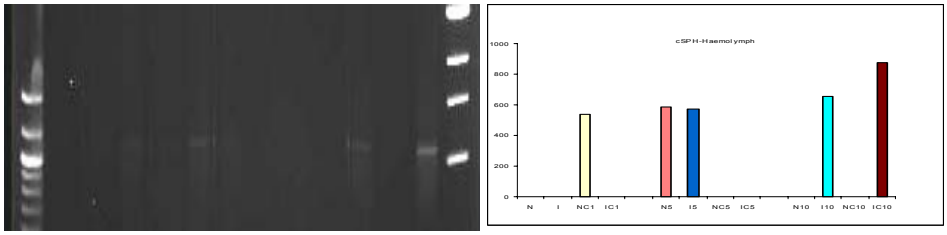
Cathepsin C (~240bp)



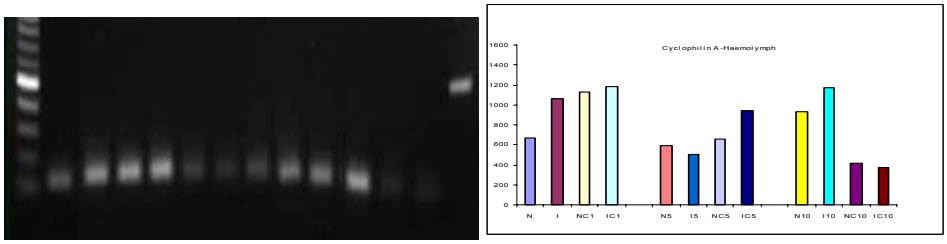
Crustin (~430bp)



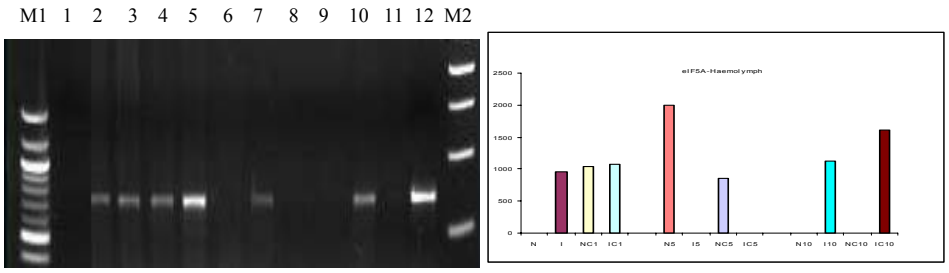
c-SPH (~1093bp)



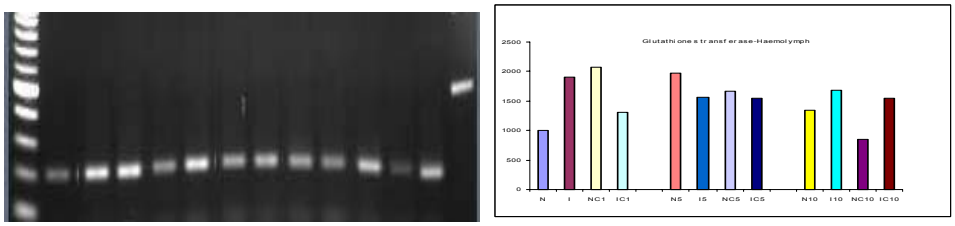
CyclophilinA (~125bp)



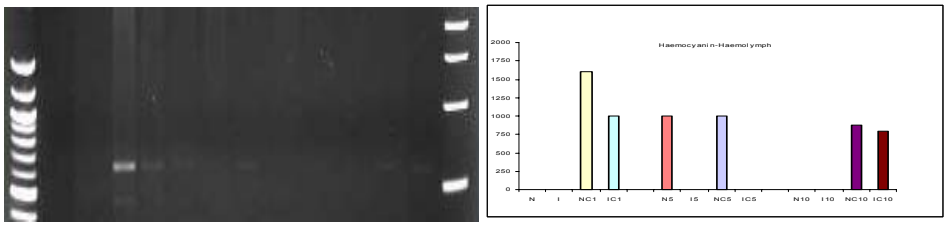
eIF5A (~727bp)



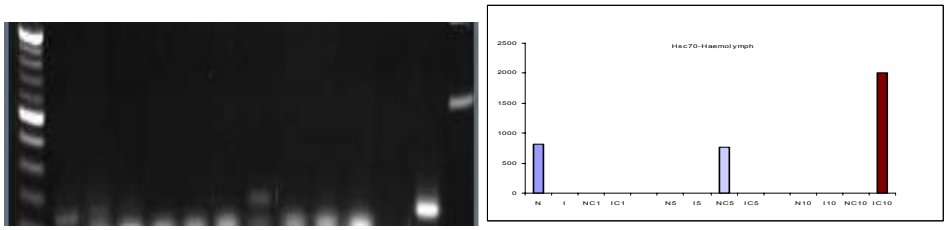
Glutathione-s-transferase (~247bp)



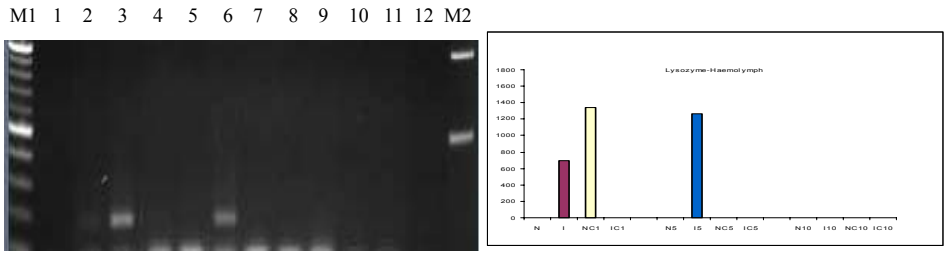
Haemocyanin (~598bp)



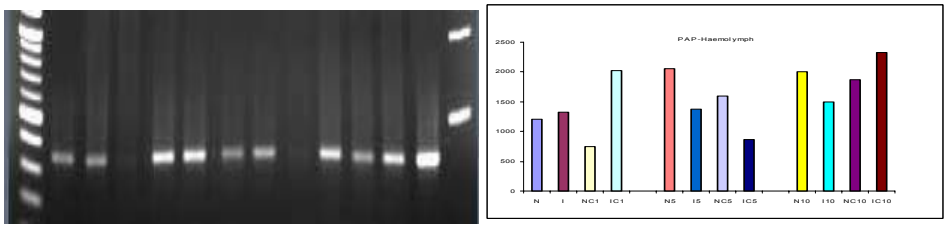
Hsc70 (~125bp)



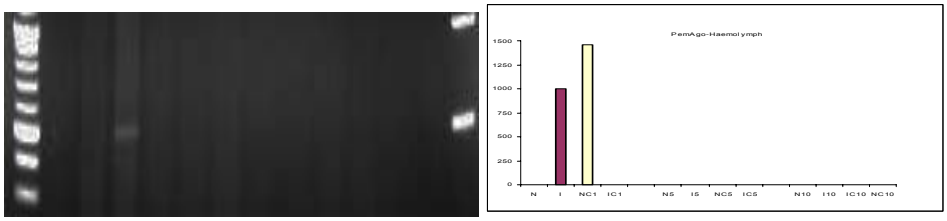
Lysozyme (~165bp)



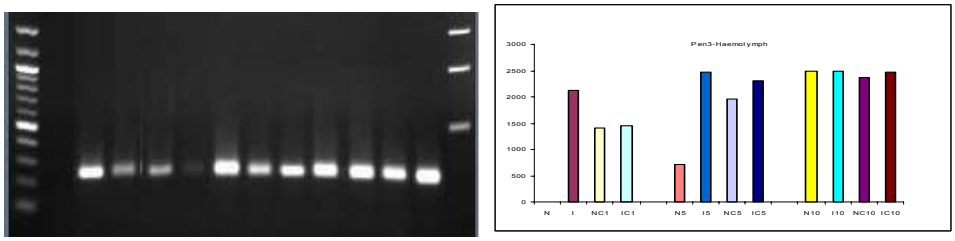
PAP (~360bp)



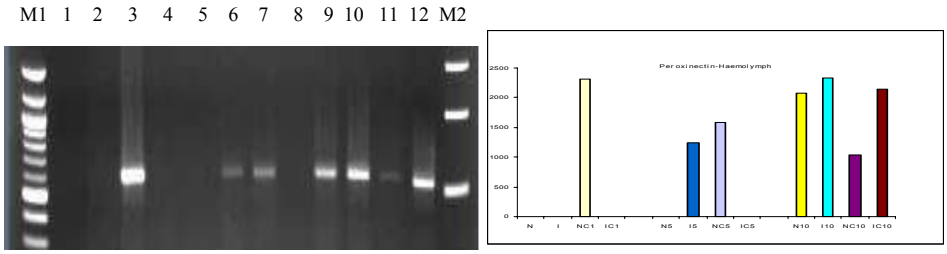
Pem-Ago (~495bp)



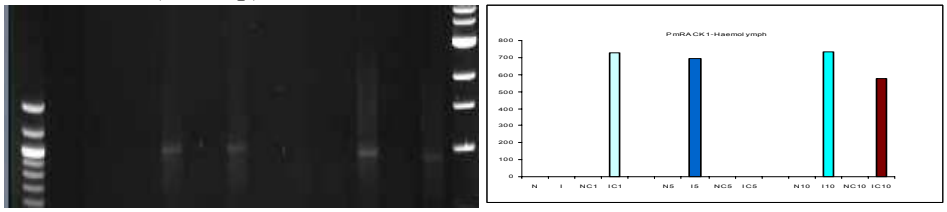
Pen-3 (~240bp)



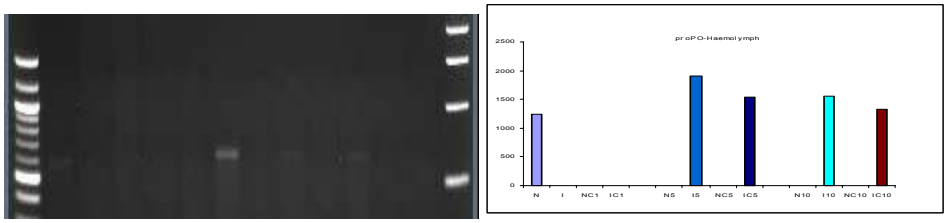
Peroxinectin (~547bp)



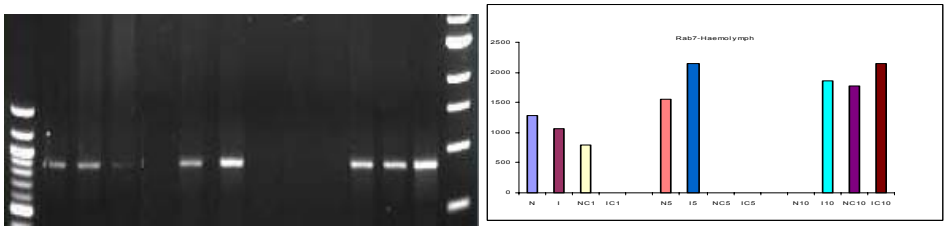
PmRACK1 (~937bp)



ProPO (~590bp)

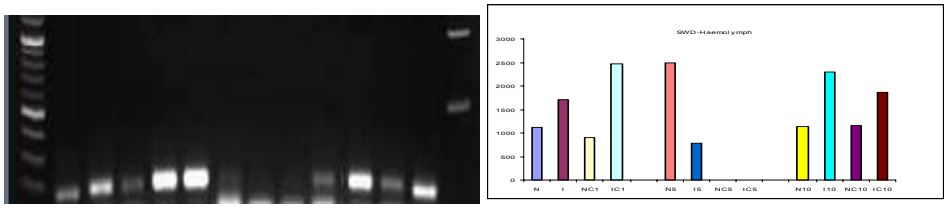


Rab7 (~887bp)

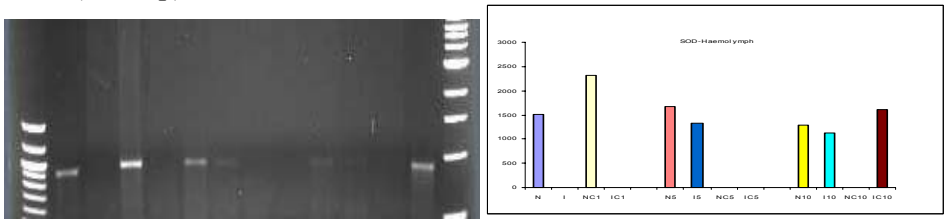


SWD (~190bp)

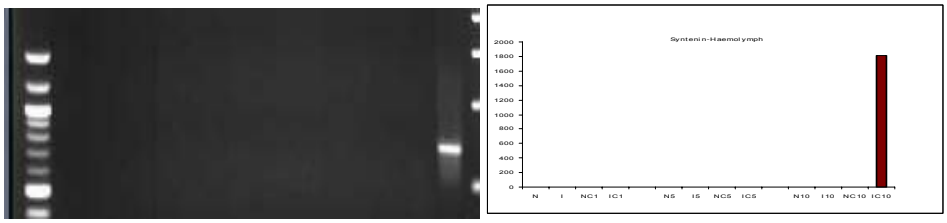
M1 1 2 3 4 5 6 7 8 9 10 11 12 M2



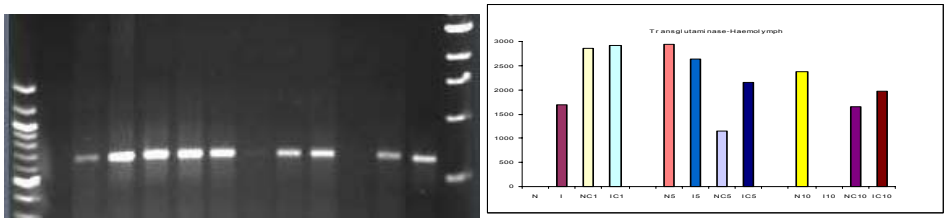
SOD (~924bp)



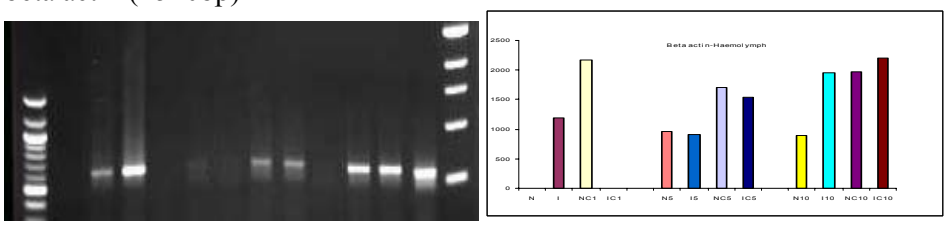
Syntenin (~750bp)



Transglutaminase (~627bp)

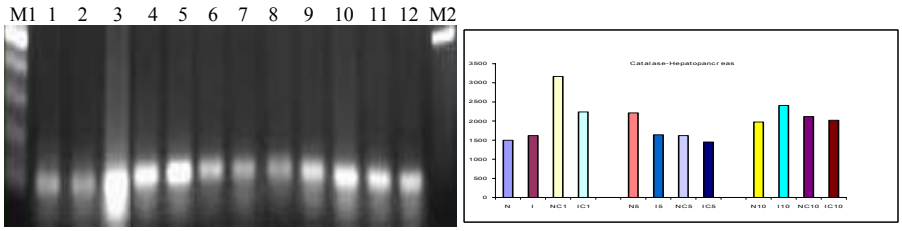


beta actin (~520bp)

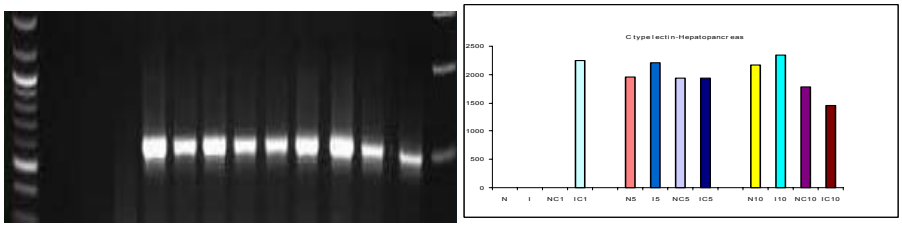


Hepatopancreas

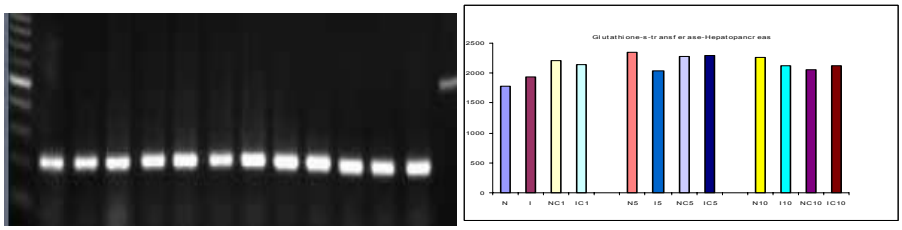
Catalase (~130bp)



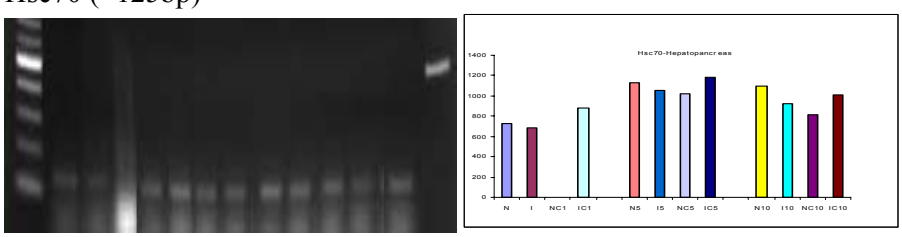
C-type lectin (~546bp)



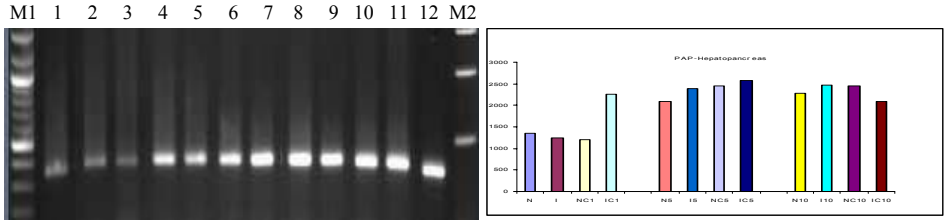
Glutathione-s-transferase (~247bp)



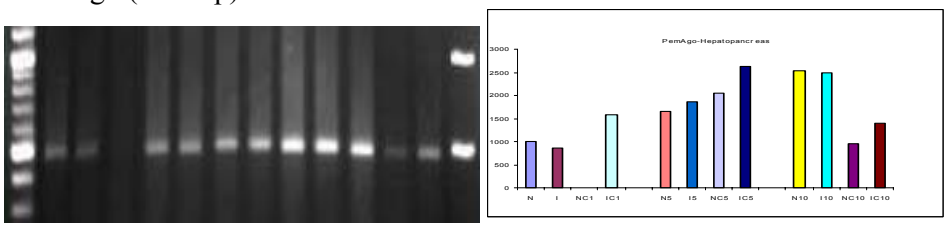
Hsc70 (~125bp)



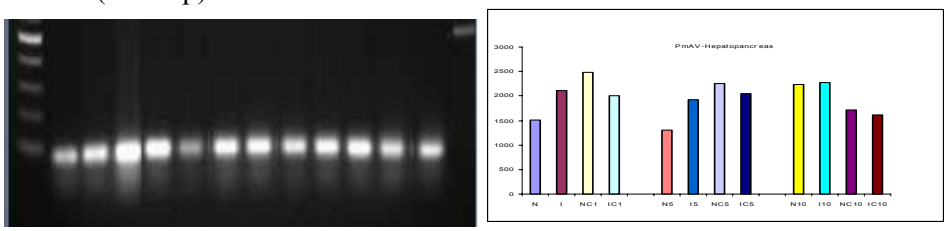
PAP (~360bp)



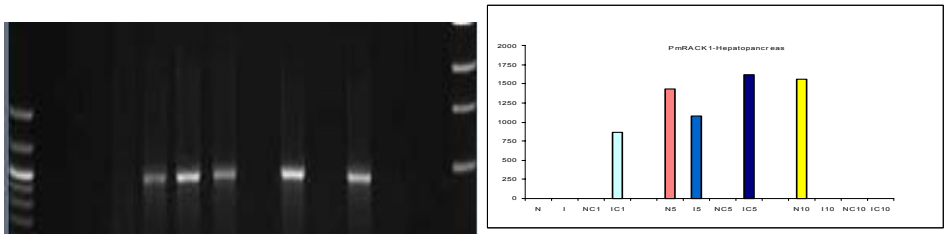
Pem-Ago (~495bp)



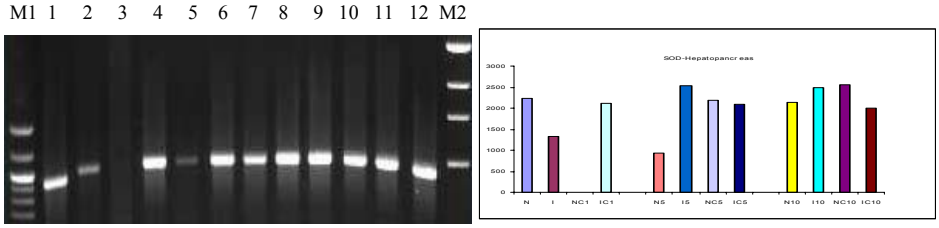
PmAV (~100bp)



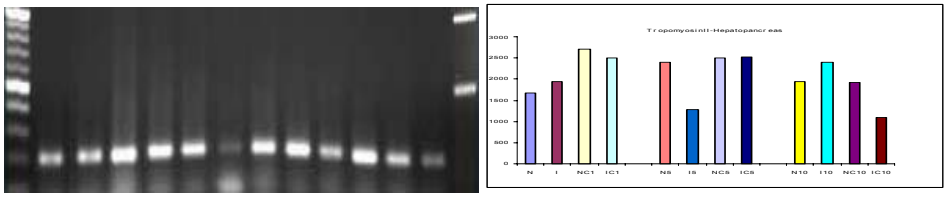
PmRACK1 (~937bp)



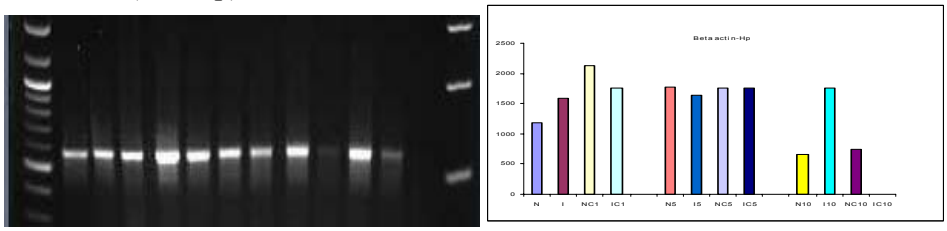
SOD (~924bp)



TropomyosinII (~240bp)



beta actin (~520bp)



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Chapter 5

VIRAL GENE EXPRESSION OF *PENAEUS MONODON* TO IVP ADMINISTRATION

CHAPTER - 5

Viral gene expression of *Penaeus monodon* to IVP administration

5.1. Introduction

The striking contrast between the interaction of viruses that infect vertebrates and invertebrates is that it is usual for invertebrates to carry persistent viral infections. It is suggested that the factors involved in this tolerance may also underlie the protective response observed following previous exposure to virus (Flegel, 2007). While this may be the case in some circumstances, it seems likely that sub-unit vaccines in shrimp offer protection via mechanisms that differ from persistent infection. One departure from the tolerance model is that shrimps vaccinated with sub-unit vaccines do not have any detectable virus in them when screened by PCR (Witteveldt et al., 2004a, 2004b). Thus, the observed mechanisms involved in protection following previous exposure to live virus are likely to be different from those following subunit or DNA vaccination (Johnson et al., 2008). However, *Fenneropenaeus indicus*, when challenged with WSSV after the administration of inactivated virus preparation (IVP) for 7 consecutive days, had shown the absence of WSSV by nested PCR on 5^{th} day after challenge and the presence of WSSV on 10^{th} day after challenge (Singh et al., 2005). In studies on DNA vaccine of WSSV in *Litopenaeus vannamei*, protein expression analysis indicated the presence of VP28 in the immunized animals (Li et al., 2010). However, most of the vaccination experiments have relied on the relative percentage survival rather than to the presence or absence of WSSV in the experimental animals.

WSSV is a lytic virus, and in the late stage of infection, the infected cells disintegrate, causing the destruction of affected tissues. Most of the ORFs in the 181 ORFs of the WSSV genome encode proteins that show no homology to known proteins. A few ORFs encode proteins with identifiable features, and

these are mainly involved in nucleotide metabolism and DNA replication. Nine homologous regions with highly repetitive sequences occur in the genome. Based on temporal expression profiles, WSSV genes can be classified as early or late or latency associated genes, and they are regulated as coordinated cascades under the control of different promoters (Liu et al., 2009). The WSSV genes involved in pathogenesis have been reviewed in Chapter 1.

Histopathological evidence has shown that WSSV replicates in tissues of ectodermal and mesodermal origin only (Chou et al. 1995; Lightner 1996; Karunasagar et al. 1997; Mohan et al. 1997; Rajendran et al. 1999, Yoganandhan et al. 2003c; Vijayan et al. 2003). Evidence of histopathological manifestations in the target tissues is one of the criteria used in the diagnosis of WSSV infection (Lightner 1996; Wang et al., 1997). Investigations on the histopathology of the WSSV infection by Rajendran et al. (2005), have revealed obvious histological changes characteristic of WSSV in the cuticular epidermis of eyestalk and pleopod (Rajendran et al., 1998, 1999), which is in agreement with the findings that epithelial cells of the eyestalk and pleopod are the preferred tissues of WSSV, and the initial sites of viral infection (Chang et al., 1996; Lo et al., 1997).

Immunological methods are incorporated to histopathological studies for WSSV detection based on monoclonal or polyclonal antibodies produced against viral antigens or recombinant viral antigens. Monoclonal and polyclonal antibodies produced against VP28 or rVP28 were used to develop several methods including immunofluorescence and immunohistochemistry (Poulos et al., 2001; Anil et al., 2002; Escobedo-Bonilla et al., 2005, 2007; Rahman, 2007).

Expressions of an immediate early gene (*ie1*), 5 early genes (*pk1*, *tk-tmk*, *rr1*, *dnapol*, *endonuclease*) and 3 late genes (*vp28*, *latency 1*, *icp11*) were analyzed in experimental animals which were subjected for different treatments in the present study. Histopathological and immunohistochemistry methods

were also adopted to study the presence or absence of virions in the gill tissues of the experimental animals.

5.2. Materials and Methods

5.2.1. Viral gene expression in *P. monodon* to IVP administration

5.2.1.1. Sample collection for the semiquantitative RT-PCR analysis of WSSV genes

Haemolymph, gill and hepatopancreas were removed using RNase free (diethyl pyrocarbonate (DEPC) treated) capillary needles and dissectors from the experimental animals which were subjected to the treatments as mentioned in Chapter 1. The samples (~250 µL of haemolymph and 50-70mg of gills / hepatopancreas) were transferred to per mL TRI® Reagent (Sigma) and immediately stored at -80ºC and RNA extraction was carried out within a fortnight.

5.2.1.2. RNA extraction, cDNA synthesis and semiquantitative RT-PCR of WSSV genes

RNA was extracted from individual experimental shrimps (6 nos./ treatment) of all the treatments as mentioned under Chapter 1. Total RNA was isolated using TRI® Reagent (Sigma) following the manufacturer's protocol with slight modifications. Briefly, the samples were macerated in TRI® Reagent and placed at room temperature for 5 minutes to ensure complete dissociation of nucleoprotein complexes. Chloroform (200µL) was added per mL of TRI® Reagent and shaken vigorously for 15 seconds and allowed to stand for 15 minutes at RT and centrifuged at 12,000g for 15 minutes at 4ºC. Of the three layers observed, the colourless top aqueous phase was carefully separated into a fresh MCT. An aliquot of 500µL of isopropanol was added and incubated for 10min at RT and centrifuged at 12,000g for 15 minutes at 4ºC. The supernatant

was removed and the RNA precipitated at the bottom and sides of the MCT was washed twice at 12,000g for 15 minutes at 4ºC, and once at 12,000g for 15 minutes at 4ºC) with 75% ethanol. After removing the supernatant, the pellet was air-dried and dissolved in 20µL DEPC treated water and incubated at 55ºC for 10 minutes. DNase treatment of the RNA samples was done with 0.2U of the enzyme, RNase free DNase 1 (New England Biolabs), for 1µg of RNA by incubating at 37ºC for 10 minutes. The enzyme inactivation was done by incubating at 75ºC for 10 minutes. RNA quality and concentration were determined at 260/280nm using a UV – Visible spectrophotometer (Shimadzu).

For cDNA sysnthesis, a sample of 5µg of the extracted RNA was added to a 20µL reaction mixture with RNase Inhibitor (8U), Oligo $(dT)_{12}$ primer (40pmol), dNTP mix (1mM), RTase Buffer (1X) and $MgCl_2$ (2mM). 200U of Moloney Murine Leukemia Virus (M-MuLV). Reverse Transcriptase was added and the reverse transcription was carried out at 42ºC for 1hr. on a Thermal cycler (Master Cycler personal, Eppendorf). All the reagents were purchased from New England Biolabs.

WSSV genes (Table 15) in each treatment were amplified on Master Cycler personal (Eppendorf). The primers selected for these genes were those reported by Liu et al. (2005) and *icp11* (the WSSV gene for the nonstructural protein ICP11 WSSV; Wang et al., 2007). Equal volumes of cDNA from each individual of the treatment were pooled and the amplifications of the above mentioned genes were done. The PCR was done by using 2µL of the cDNA of each treatment with specific primer sets as given in Table 15. Shrimp β-actin was amplified as a reference gene. The 25µL PCR mixture subjected for amplification contained, 200µM dNTP mix, 10pmol each of forward and reverse primers, 1X PCR buffer (Thermopol), 0.5U Taq DNA Polymerase and 2 µL of cDNA. The hot start PCR programme used for the WSSV genes was 94 ºC for 2mins. followed by 35 cycles of 94 ºC for 30 s, annealing for 30 s, 68ºC for 30 s

and a final extension at 72ºC for 10 mins. The annealing temperatures were 50ºC for endonuclease, 53ºC for latency 1 and ribonucleotide reductase (rr1), 54ºC for DNA polymerase (dnapol), immediate early gene 1 (ie1), vp28 and thymidine kinase – thymidilate kinase (tk-tmk) and 55ºC for protein kinase 1 (pk1) and β-actin. The hot start PCR programme for ICP11 was 94 ºC for 2mins. followed by 35 cycles of 94 ºC for 2 mins., annealing at 62ºC for 1 min, 72ºC for 1 min and a final extension at 72ºC for 10 mins. The PCR products were analyzed by horizontal gel electrophoresis of 10µL of the PCR products. The gels were stained with ethidium bromide and documented under UV light with Molecular Imager® Gel Doc™ XR+ Imaging System (Bio-Rad).

5.2.2. Nested PCR analysis of experimental animals

The nested PCR for WSSV detection was done using Lo et al., (1996) primers as adopted by OIE for WSSV (details in Chapter 2).

5.2.3. Histopathology and Immunohistochemistry of experimental animals

5.2.3.1. Histopathology

The experimental animals were fixed in Davidson's AFA (alcohol, formalin, glacial acetic cid) fixative and the gills were transferred to 70% ethanol for 24hrs. They were dehydrated in a series of alcohol – 70%, 80%, 90%, 95%, 100% and finally dipped in acetone for 2mins. and cleared in two changes of xylene for 10mins. The tissues were infiltrated with paraffin wax through xylene-wax mixture in the ratio 1:3 and in three changes of pure wax and finally embedded in paraffin wax. The tissue blocks were preserved at room temperature till sectioning.

The tissues were cut into 5µm thin sections and the sections were transferred to clean microscopic slides previously coated with Chrome Alum Gelatin. The sections were then dewaxed in two changes of xylene and

rehydrated through 95% to 70% alcohol and finally in distilled water. The staining of sections was done using haematoxylin and differentiated in acid alcohol. Bluing was done in Scott's tap water and the sections were counter stained with eosin. After another series of dehydration of the sections with alcohol, clearing was done with xylene and finally mounted in DPX (Distyrene, Plastisizer, Xylene mixture). The sections were observed under a light microscope.

5.2.3.2. Indirect Immunohistochemistry of experimental animals

Gill tissue of experimental animals were fixed in Davidson's AFA, embedded in paraffin wax, cut into 5µm sections and transferred to Chrome Alum Gelatin coated microscopic slides and fixed overnight in room temperature. The sections were dewaxed using xylene and re-hydrated through series of alcohol and distilled water. The slides were incubated for 1hr. with 3% BSA in PBS in a humidified chamber to block free sites. These were washed in PBS – Tween-20 mixture (0.01%) three times (3 mins. each) and incubated with WSSV specific monoclonal (C38) antibody (Anil et al., 2002) for 1 hr. The sections were then washed in PBS – Tween-20 mixture thrice (3 mins. each). Anti mouse IgG FITC [fluorescein-5-isothiocyanate–labeled goat anti–mouse IgG antibody – (Sigma)], diluted to 1:40 in BSA-PBS was added and incubated for 1 hr. in dark in a humidified chamber. Subsequently, the sections were washed with PBS – Tween-20, stained with the nuclear stain DAPI (4', 6-diamidino-2-phenylindole) (10 µL, 0.02 µg/mL) and incubated for 3 mins. The slides were finally rinsed with distilled water, air dried, and mounted with mounting media (Vectasheild, U. S. A.). The sections were observed at 360-370 nm (for DAPI) and 470-490 nm (for FITC) under a UV fluorescence microscope (Olympus BX51, Germany) and the images were processed and merged using the Image-Pro® Express software (Media Cybernectics Inc., MD, U. S. A).

5.3. Result

5.3.1. Semiquantitative RT-PCR of WSSV genes

Semiquantitative RT-PCR analysis of the 9 selected viral genes in gills, haemolymph and hepatopancreas showed variation in their expression between the selected tissues and between the experimental treatment groups.

5.3.1.1. Semiquantitative RT-PCR of WSSV genes in shrimp tissues (gills, haemolymph, hepatopancreas)

Among the tissues analyzed by RT-PCR, gills of the experimental animals (both challenged and unchallenged group) showed cumulative expression of 8 selected genes (*ie1*, *tk-tmk*, *rr1*, *dnapol*, *endonuclease*, *vp28*, *icp11, latency1*) followed by haemolymph (6 genes – *ie1*, *pk1*, *tk-tmk*, *dnapol*, *vp28*, *icp11*) and hepatopancreas (5 genes – *ie1*, *rr1*, *dnapol*, *latency1*, *icp11*) (Table 16). The unchallenged group of (both IVP administred and normal feed administered) animals showed amplification of 3 genes (*icp11, tk-tmk, vp28*) in gills. Meanwhile, the haemolymph of unchallenged normal feed administered animals showed amplification of 3 genes (*ie1, tk-tmk, vp28*), and IVP administered animals 4 genes (*icp11, ie1, tk-tmk, vp28*). In the hepatopancreas of normal feed administered animals, viral genes were not amplified while in the IVP administered animals, 2 genes such as *dnapol* and *latency1* were found to be expressed in the unchallenged animals.

In the WSSV challenged animals, 8 genes (*ie1*, *tk-tmk*, *rr1*, *dnapol*, *endonuclease*, *vp28*, *icp11, latency1*) were found amplified in gill tissue and 6 (*ie1*, *tk-tmk*, *dnapol*, *vp28*, *icp11, pk1*) in haemolymph, in both normal fed, and IVP administered group. In the hepatopancreas of normal feed administered animals viral genes were not found to be expressed, however, in the IVP administered animals, 4 genes such as *rr1, ie1, icp11* and *latency1* were found to be expressed after the challenge. The house keeping gene, beta actin, showed amplification in all the selected tissues.

5.3.1.2. Semiquantitative RT-PCR of viral genes in shrimp tissues (gills, haemolymph and hepatopancreas) subsequent to different treatments (normal feed and IVP administered) on 1DPA, 5DPA and 10DPA

5.3.1.2.1. Gill

5.3.1.2.1.1. 1st day post administration (1DPA)

Before challenge - The gills of experimental animals administered with different feed types and analyzed on 1st day after the administration, showed amplification of 3 genes (*icp11*, 2-*tk-tmk and vp28*) in normal feed administered and amplification of 2 genes (*tk-tmk, vp28*) in IVP administered animals (Table 17).

After challenge - The semiquantitative RT-PCR analysis of gills of the experimental animals challenged on 1st day post administration (1DPA) and sampled on 4th day after WSSV challenge showed amplification of only three genes (*icp11, tk-tmk, vp28*) in normal control animals even after challenge. In the IVP administered group, 8 genes such as *rr1, ie1, dnapol, tk-tmk, latency1, endonuclease, icp11* and *vp28* were amplified (Table 17).

5.3.1.2.1.2. 5th day post administration (5DPA)

Before challenge - The gills of experimental animals administered with different feed types and analyzed on 5th day after the administration, showed amplification of 3 genes (*tk-tmk, icp11, vp28*) in normal feed administered and IVP administered animals (Table 17).

After challenge - The semiquantitative RT-PCR analysis of gills of the experimental animals on 5th day post administration (5DPA) and sampled on 4th day after WSSV challenge showed expression of 8 genes (*rr1, ie1, dnapol, tk-tmk, latency1, endonuclease, icp11, vp28*) in normal feed and IVP administered group (Table 17).

5.3.1.2.1.3. 10th day post administration (10DPA)

Before challenge - The gills of experimental animals administered with different feed types and analyzed on 10th day after the administration, showed amplification of 3 genes such as *icp11*, *tk-tmk and vp28*) in normal feed administered and 2 genes such as *tk-tmk* and *vp28* in IVP administered animals (Table 17).

After challenge - The semiquantitative RT-PCR analysis of gills of the experimental animals on 10th day post administration (10DPA) and sampled on 4th day after WSSV challenge showed expression of 8 genes (*rr1, ie1, dnapol, tk-tmk, latency1, endonuclease, icp11, vp28*) in both normal feed and IVP administered group (Table 17).

5.3.1.2.2. Haemolymph

5.3.1.2.2.1. 1st day post administration (1DPA)

Before challenge - The haemolymph of experimental animals administered with different feed types and analyzed on 1st day after the administration, showed amplification of 3 genes (*icp11*, *tk-tmk and vp28*) in normal feed administered and 3 genes (*ie1*, *tk-tmk, vp28*) in IVP administered animals (Table 18).

After challenge - The semiquantitative RT-PCR analysis of haemolymph of the experimental animals on 1st day post administration (1DPA) and sampled on 4th day after WSSV challenge showed expression of 4 genes (*dnapol, ie1, vp28, tk – tmk)* in normal animals and 4 genes (*icp11, tk-tmk, ie1 and vp28)* in IVP administered animals (Table 18).

5.3.1.2.2.2. 5th day post administration (5DPA)

Before challenge - The haemolymph of experimental animals administered with different feed types and analyzed on 5th day after the administration, showed amplification of 3 genes (*ie1*, *tk-tmk and vp28*) in normal feed administered and in IVP administered animals (Table 18).

After challenge - The semiquantitative RT-PCR analysis of haemolymph of the experimental animals on 5th day post administration (5DPA) and sampled on 4th day after WSSV challenge showed expression of 5 genes such as *icp11, tk-tmk, dnapol, vp28, ie 1* in normal animals and 5 genes such as *icp11, tk-tmk, pk1, vp28 and ie 1* in IVP administered group (Table 18).

5.3.1.2.2.3. 10th day post administration (10DPA)

Before challenge - The haemolymph of experimental animals administered with different feed types and analyzed on 10th day after the administration, showed amplification of 2 genes such as *tk-tmk*, and *vp28* in normal feed administered and 4 genes such as *icp11, ie1*, *vp28* and *tk-tmk* in IVP administered animals (Table 18).

After challenge - The semiquantitative RT-PCR analysis of haemolymph of the experimental animals on 10th day post administration (10DPA) and sampled on 4th day after WSSV challenge showed expression of 4 genes such as *tk – tmk, pk – 1, icp 11, ie -1 and vp 28*, in normal animals, and 6 genes in IVP administered group such as *tk – tmk, pk1, dnapol, vp28, icp11 and ie1* (Table 18).

5.3.1.2.3. Hepatopancreas

5.3.1.2.3.1. 1st day post administration (1DPA)

Before challenge - The hepatopancreas of experimental animals administered with different feed types and analyzed on 1st day after the administration, did not show amplification of viral genes in normal feed administered and IVP administered animals.

After challenge - The semiquantitative RT-PCR analysis of hepatopancreas of the experimental animals on 1st day post administration (1DPA) and sampled on 4th day after WSSV challenge showed expression of 4 genes

(*rr1,ie1,icp11,latency1)* in IVP administered group and normal feed administered did not show the expression of viral genes (Table 19).

5.3.1.2.3.2. 5th day post administration (5DPA)

Before challenge - The hepatopancreas of experimental animals administered with different feed types and analyzed on 5th day after the administration, showed amplification of 2 genes such as *dnapol and latency1* in IVP administered animals and no viral gene in normal feed administered group (Table 19).

After challenge - The semiquantitative RT-PCR analysis of hepatopancreas of the experimental animals on 5th day post administration (5DPA) and sampled on 4th day after WSSV challenge did not show amplification of any of the selected WSSV genes.

5.3.1.2.3.3. 10th day post administration (10DPA)

Before challenge - The hepatopancreas of experimental animals administered with different feed types and analyzed on 10th day after the administration, showed amplification of one viral gene such as *latency1* in the IVP administered animals (Table 19).

After challenge - The semiquantitative RT-PCR analysis of hepatopancreas of the experimental animals on 10th day post administration (10DPA) and sampled on 4th day after WSSV challenge did not show expression of WSSV genes in normal feed administered group. In the IVP administered group *latency1* gene expressed after the challenge.

5.3.2. Nested PCR analysis of experimental animals before WSSV challenge

The expression of viral genes in the unchallenged animals (which were negative for WSSV in post larval stage) prompted for the nested PCR analysis of the samples for WSSV. It was observed that 76% of the shrimps were second

step positive for WSSV. Since they did not show any disease signs (evident from histopathology with no hypertrophied nuclei, and immunohistochemistry with no WSSV protein), the work was continued with those animals.

5.3.3. Histopathology and Immunohistochemistry

The gill tissue sections were observed under light microscope and the hypertrophied nuclei were absent in normal feed and IVP administered animals (Figs. 9a, 9b). In the challenged condition, hypertrophied nuclei could be demonstrated in the gill tissue of the control group of animals (normal feed administered) as well as in the test (IVP administered) group of animals (Figs. 10a, 10b).

Immunohistochemistry of the gill tissue using monoclonal antibody raised against VP 28 antigen in the unchallenged experimental animals gave no signals demonstrating the absence of virions or viral antigens in these animals (Figs. 11a, 11b). However, in animals after the WSSV challenge, green positive signals were detected with FITC conjugated monoclonal antibodies against VP28 in the gills of control and test animals (normal feed and IVP administered) (Figs. 12a, 12b).

5.4. Discussion

5.4.1. WSSV genes

Sanchez-Paz (2010) reported that WSSV transcribes its genes in a temporal manner. During an infection, viral genes are transcribed in an ordered cascade of events, which can be broadly divided into three kinetic phases of gene expression: immediate-early (IE), early (E) and late (L). IE genes are expressed relying primarily on host proteins and factors for their expression, which occur in the absence of viral DNA replication (eg: *ie1*, *pk1*). E gene expression, dependent on the preceding expression of IE genes, mainly encodes enzymes required for viral DNA synthesis, plus a number of proteins that can

regulate the expression of L genes (eg: *endonuclease*, *rr1*, *tk-tmk*, *dnapol*). After viral DNA synthesis is initiated, L genes are expressed and encode enzymes and structural proteins necessary for virion assembly (eg: *vp28*, *icp11*, *latency1*).

IE genes encode proteins involved in activating the expression of viral early and late genes, altering the functions of host genes and eliminating host immune defense, their expression is especially important in determining host range. Functional analysis of the WSSV IE *ie1* gene promoter showed that a 23-nt fragment is critical for a strong promoter activity. This cis-acting element contains a sequence (ATTCCTAGAAA) that is recognized, and strongly activated, by the shrimp STAT binding motif, consequently enhancing the expression of viral IE genes (Liu et al., 2005). Thus, WSSV annexes the defensive activity of STAT to its benefit. It is now accepted that WSSV reduces STAT transcription in the host (Chen et al., 2008a). Hence, this may imply that transcription levels above a certain minimum would be beneficial to the virus in order to drive the expression of viral genes such as *ie1*, while it may also be beneficial to prevent STAT transcription to increase above the basal level to avoid eliciting a global antiviral immune response.

Protein phosphorylation in virus-infected cells may depend on cellular PK or virally encoded PK (Li et al., 1995). An earlier expression of Ser/Thr PK may indicate a higher susceptibility of hemocytes to be infected than cells of the cuticular epidermis.

Ribonucleotide reductases (RR) are key enzymes in living cells since they catalyze the reduction of ribonucleotides to the four deoxyribonucleoside triphosphates (dNTP) required for DNA synthesis. WSSV genome revealed the presence of the genes encoding the large (RR1) and small (RR2) subunits of the RR. The synthesis of WSSV RR in WSSV-infected shrimp may explain the efficient replication of this virus not only in young/actively proliferating cells but also in old/resting cells (Sanchez-Paz, 2010).

The WSSV genome contains a gene encoding TK-TMK chimeric protein (WSSV *tk-tmk*, wsv395). RT-PCR analysis revealed the presence of transcripts of the *tk-tmk* transcript as early as 4 h p.i., and it continued to be found up to 60 h p.i. (Tsai et al., 2000b).

The WSSV genome comprises a DNA polymerase gene (*dnapol*) identified by the presence of three highly conserved motifs (Exo I, II, and III). It is possible that, as in most of the insect baculovirus early genes having one or both of these basal elements, the WSSV *dnapol* transcription may be mediated by host RNA polymerase II (Chen et al., 2002a). The analysis of the temporal expression of the structural WSSV protein genes *vp28* during infection was studied in experimentally infected shrimp (*P. monodon*) and found that transcripts of *vp28* were detected from 1 day p.i., onwards.

By using a DNA microarray analysis, latency related genes (ORF 151, 366 and 427) were detected in SPF shrimps, indicating that these shrimps had been carriers of the virus and were actively expressing viral genes (Khadijah et al., 2003). More recently, interactions between the ORF427 protein and a novel shrimp serine/threonine protein phosphatase (PP) were found (Lu and Kwang, 2004), suggesting that shrimp PP may be involved in the latent-lytic life cycle of WSSV through interacting with ORF427. The latency stage of WSSV occurs before infection and proliferation. Latency genes show maximal activity rates of transcription following viral DNA synthesis, and are involved in the persistence of the virus within a host cell (Flint, et al., 2000). Their function is to keep a low number of viruses and inactivating host genes, until the optimal conditions of pH, salinity and temperature or population density are present (Sanchez-Martinez et al., 2007).

WSSV gene *icp11* (also identified as vp9) is the most highly expressed viral gene at both transcriptional and translational levels (it was 3.5-fold more

highly expressed than the major envelope protein gene *vp28*). Its encoded protein, ICP11, is a nonstructural protein localised in both cytoplasmic and nuclear compartments (Wang et al., 2007a), and contains a fold and a negative charge comparable with those recognised in dsDNA, suggesting that it may function by mimicking the DNA shape and chemical character (Wang et al., 2008a). Furthermore, it was found that ICP11 binds directly to the DNA binding site of nucleosome-forming histones (H3 and H2A.x), interfering, thus, with critical functions of DNA damage repair, and nucleosome assembly, which has been reported as a mechanism to manipulate cellular chromatin to ensure viral genome survival and propagation.

In the experimental animals, gill tissue was found to show the expression of eight of the nine selected WSSV genes followed by haemolymph (6 genes) and hepatopancreas (5 genes). From the observation, gill tissue can be considered as the sample tissue for all the selected viral genes except *pk1*. Of the three selected tissues (heamolymph, gill and hepatopancreas), only haemolymph was found to express *pk1*.

In the gill tissue of IVP and control group (normal feed administered group) before a challenge, WSSV genes such as *tk-tmk, icp11* and *vp28* were found to be expressed. However, after the challenge, all genes (*ie1*, *tk-tmk*, *rr1*, *dnapol*, *endonuclease*, *vp28*, *icp11, latency1*) were found to be expressed in the gill tissue of IVP and control group (normal feed administered group) on 5DPA and 10DPA. However, only *icp11, tk-tmk* and *vp28* were found to be expressed in normal feed administered animals, and IVP administered animals expressed all the genes ((*ie1*, *tk-tmk*, *rr1, dnapol*, *endonuclease*, *vp28*, *icp11, latency1*) after the challenge on 1DPA.

In the haemolymph of experimental animals, WSSV genes such as *ie1*, *tk-tmk* and *vp28* in the control group (normal feed administered group), and *ie1*,

tk-tmk, *vp28* and *icp11* in the IVP administered group were found to be expressed before the challenge. However, after the challenge, *ie1*, *tk-tmk* and *vp28* on 1DPA, 5DPA and 10DPA; *icp11* on 5DPA and 10DPA, and *pk1* on 10DPA were expressed in the IVP and control group (normal feed administered group). After the WSSV challenge, apart from the common genes expressed, *dnapol* was expressed in the control group and *icp11* in IVP administered group on 1DPA; *dnapol* was expressed in the control group and *pk1* in IVP administered group on 5DPA, and *dnapol* was expressed in IVP administered group on 10DPA.

In the hepatopancreas of normal feed administered animals, none of the viral genes was expressed before and after the WSSV challenge on 1DPA, 5DPA and 10DPA. However, in the IVP administered animals, before the challenge none of the viral genes was expressed on 1DPA, while viral genes, such as *dnapol,* were expressed on 5DPA and 10DPA and *latency1* on 10DPA. After the WSSV challenge, viral genes were not expressed on 5DPA group, but genes such as *rr1, ie1, icp11* and *latency1* were expressed on 1DPA group, and *latency1* was expressed in 10DPA group.

The presence of early and late WSSV genes in the gills and haemolymph of unchallenged animals points to the presence of WSSV genes in the experimental animals. The presence of these genes (*tk-tmk, vp28*) in the experimental animals might be masking the WSSV challenge from proceeding into an infection lead mortality as observed during the experimental period. All the expressed genes were unique to the treatment groups thus showing the variability of tissues in response to WSSV challenge. The variations observed in the expression of viral genes in different groups points to the possibility of adoption of different pathways for WSSV infection in the host. The absence of a temporal pattern of viral gene expression might be facilitated within *P. monodon* either to induce or dissuade the WSSV infection on 4th day after the challenge.

The absence of any of the selected viral genes in the hepatopancreas of the normal feed administered group after WSSV challenge on 1DPA is intriguing. This variation in response to WSSV in normal feed administered group on 1DPA and 5DPA might be due to the variation among the individual samples. In this case, the IVP administered, might be acting as overdose of 'inactivated antigen' as the animals already have a very low copy number of WSSV genes (not as virions as confirmed by histology and immunohistochemistry).

5.4.2. Nested PCR

Though the larvae obtained for rearing were negative for WSSV, 76% of the animals which were subjected for the experiment were found to be second step positive to WSSV. However, neither mortality due to WSSV infection was observed in unchallenged animals nor hypertrophied nuclei and viral proteins detected by histology and immunohistochemistry respectively. Yoganandhan et al. (2003a) have observed that PCR susceptibility to WSSV disease increases with age of *P. monodon* which has also been reported by Venegas et al. (1999) in *P. japonicus*. Lo et al. (1997) have reported that two-step WSSV PCR-positive brooders produced two-step WSSV PCR-negative eggs and nauplii, but that the subsequent zoeal stage became two-step WSSV PCR-positive. Yoganandhan et al. (2003a) have observed that in *Penaeus monodon*, surviving larvae, post-larvae and juveniles showed WSSV-positive signals by nested PCR (and without signs of WSSV) while moribund animals were positive by single-step PCR. Jang et al. (2009) have identified by TaqMan real-time PCR that 75.5% of the brooders in South Korea to be positive to WSSV ie, carrying more than two copies of WSSV ng−1 of DNA. During the period of study it was almost impossible to get nested PCR negative WSSV for the experimentation and hence was forced to utilize the juveniles reared under biosecurity after confirming that virions were not existing in the animals.

5.4.3. Histopathology and Immunohistochemistry

Histological and immunohistochemical analysis of gill tissues of the experimental animals (before WSSV challenge) revealed the absence of hypertrophied nuclei as well as viral protein (VP28) in the group of animals administered with normal feed and IVP even though they had expressed *tk-tmk*, *vp28 and icp11*. Gill tissues were chosen as it was reported that WSSV infects tissues of ectodermal and mesodermal origin (Wongteerasupaya et al., 1995). The absence of histological and immunofluorescence evidence for WSSV might be due to the lack of translation product of the viral gene. Here, there might have happened a partial integration of WSSV genome in to the host genome. Meanwhile in the experimental animals subjected to WSSV challenge, the histological and immunohistochemical analysis of gill tissues revealed the presence of hypertrophied nuclei and immunofluorescence for VP28 in the group of animals fed on normal feed and IVP. It is hypothesized that, in IVP administered animals, IVP might have caused an over-stimulation in the context of having viral genes integrated in to the host genome and expressed, and thus might have caused an 'immune fatigue' state in the animals leading to favourable condition for virus multiplication.

The expression of early gene (*tk-tmk*) and late gene (*vp28*) was predominant in gills and haemolymph of the experimental animals before a WSSV challenge. The persistence of WSSV genes without leading into mortality was observable. Persistence has been defined as the state in which a virus maintains its capacity for either continued or episodic reproduction in an individual host, subsequent to an initial period of productive infection and occurrence of an antiviral host response. This definition also includes the condition known as latency in which virus reproduction can be partially or completely suppressed for prolonged periods, but the capacity for reactivation is maintained (Sanhez-Paz, 2010). However, in the present study, latency related gene (latency1) was found to be predominantly expressed in hepatopancreas of

IVP administered animals. The WSSV challenge on the experimental animals (with different WSSV genes) did produce the expression of majority of selected genes in gills, less number of genes in haemolymph and lesser number of genes in hepatopancreas. The variation observed in different tissues, in different treatments and in different animal samples with respect to a WSSV challenge could not be directed to a temporal pattern of WSSV transcription as some of the late genes (eg: *vp28* and *icp11*) were already present without leading the animals to mortality. However, with respect to IVP administered animals, IVP might be having an over-stimulating effect on the animals which expressed viral genes and reported as nested positive.

The results obtained from the study on semi-quantitative viral gene expression (before WSSV challenge) shows inclination to the hypothesis put forward by Flegel (2009) that shrimp uses the reverse transcriptase (RT) in their genome to recognize "foreign" mRNA of both RNA and DNA viruses and uses integrases (IN) to randomly insert short cDNA sequences into their genomes and by chance, some of these sequences result in production of immunospecific RNA (imRNA) capable of stimulating RNAi that suppresses viral propagation and the individuals with protective inserts would pass these on to the next generation.

The present study gives clues about unique ways WSSV adopts to interact with *P. monodon* without leading into mortality and by which it might be taking a unique and complex pathway for an ‘infectious tolerence’ (Cobbold and Waldmann, 1998) or aiming a ‘trans-generational immunological priming’ (Little and Kraaijeveld, 2004; Rowley and Powell, 2007) at genetic level in its host population.

Table 15. Primer sequences of WSSV genes

Gene	Primer sequence (5'-3')	PCR amplicon size (bp)	Reference
immediate early gene (*ie1*)	F-GACTCTACAAATCTCTTTGCCA R-CTACCTTTGCACCAATTGCTAG	502	
protein kinase (*pk1*)	F- TGGAGGGTGGGGGACCAACGGACAAAAC R-CAAATTGACAGTAGAGAATTTTGCAC	512	
thymidine kinase – thymidylate kinase (*tk-tmk*)	F-GAGCAGCCATACGGGTAAAC R-GCGAGCGTCTACCTTAATCC	412	
ribonucleotide reductase (*rr1*)	F-ATCTGCTAGTCCCTGCACAC R-AAAGAGGTGGTGAAGGCACG	408	Liu et al., 2005
DNA polymerase (*dnapol*)	F-TGGGAAGAAAGATGCGAGAG R-CCCTCCGAACAACATCTCAG	586	
endonuclease	F-TGACGAGGAGGATTGTAAAG R-TTATGGTTCTGTATTTGAGG	408	
vp28	F-CTGCTGTGATTGCTGTATTT R-CAGTGCCAGAGTAGGTGAC	555	
latency 1	F-CTTGTGGGAAAAGGGTCCTC R-TCGTCAAGGCTTACGTGTCC	647	
icp11	F-CCATATGGCCACCTTCCAGACTGAC R-CCTCGAGTTCTGTTGTTGGCACAATC	249	Wang et al., 2007
beta actin	F-CTTGTGGTTGACAATGGCTCCG R-TGGTGAAGGAGTAGCCACGCTC	520	Zhang et al., 2007

E - Expressed; NE – No Expression

Table 16. WSSV genes expressed in gills, haemolymph and hepatopancreas

Gene	bp	Gill	Haemolymph	Hepatopancreas
ie1	502	E	E	E
pk1	512	NE	E	NE
tk-tmk	412	E	E	NE
rr1	408	E	NE	E
dnapol	586	E	E	E
endonuclease	408	E	NE	NE
vp28	555	E	E	NE
latency1	647	E	NE	E
icp11	249	E	E	E

Table 17. WSSV genes expressed in gills

Gills	**Before challenge**		**After challenge**	
	Normal	IVP	Normal	IVP
1DPA	*tk-tmk*	*tk-tmk*	*tk-tmk*	*tk-tmk*
	vp28	*vp28*	*vp28*	*vp28*
	icp11		*icp11*	*icp11*
				rr1
				ie1
				dnapol
				latency1
				endonulease
5DPA	*tk-tmk*	*tk-tmk*	*tk-tmk*	*tk-tmk*
	vp28	*vp28*	*vp28*	*vp28*
	icp11	*icp11*	*icp11*	*icp11*
			rr1	*rr1*
			ie1	*ie1*
			dnapol	*dnapol*
			latency1	*latency1*
			endonulease	*endonulease*
10DPA	*tk-tmk*	*tk-tmk*	*tk-tmk*	*tk-tmk*
	vp28	*vp28*	*vp28*	*vp28*
	icp11		*icp11*	*icp11*
			rr1	*rr1*
			ie1	*ie1*
			dnapol	*dnapol*
			latency1	*latency1*
			endonulease	*endonulease*

Table 18. WSSV genes expressed in haemolymph

Haemolymph	Before challenge		After challenge	
	Normal	IVP	Normal	IVP
1DPA	*tk-tmk*	*tk-tmk*	*tk-tmk*	*tk-tmk*
	vp28	*vp28*	*vp28*	*vp28*
	ie1	*icp11*	*ie1*	*ie1*
			dnapol	*icp11*
5DPA	*tk-tmk*	*tk-tmk*	*tk-tmk*	*tk-tmk*
	vp28	*vp28*	*vp28*	*vp28*
	ie1	*ie1*	*icp11*	*icp11*
			ie1	*ie1*
			dnapol	*pk1*
10DPA	*tk-tmk*	*tk-tmk*	*tk-tmk*	*tk-tmk*
	vp28	*vp28*	*vp28*	*vp28*
		icp11	*icp11*	*icp11*
		ie1	*pk1*	*pk1*
			ie1	*ie1*
				dnapol

Table 19. WSSV genes expressed in hepatopancreas

Hepatopancreas	Before challenge		After challenge	
	Normal	IVP	Normal	IVP
1DPA				*rr1*
				ie1
				icp11
				latency1
5DPA		*dnapol*		
		latency1		
10DPA		*latency1*		*latency1*

Fig.9
Histology of Gill tissue (Before WSSV challenge)

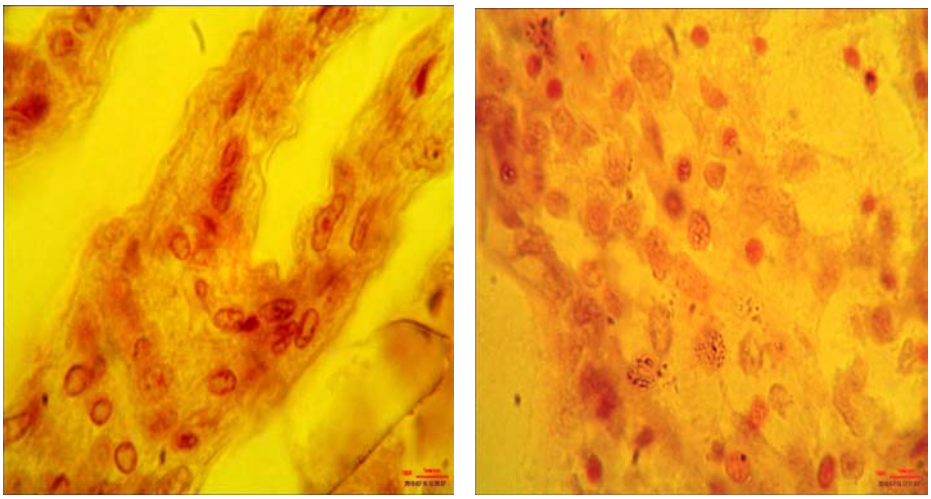


Fig.9a. Normal feed administered **Fig.9b.** IVP administered

Fig.10
Histology of Gill tissue (After WSSV challenge)

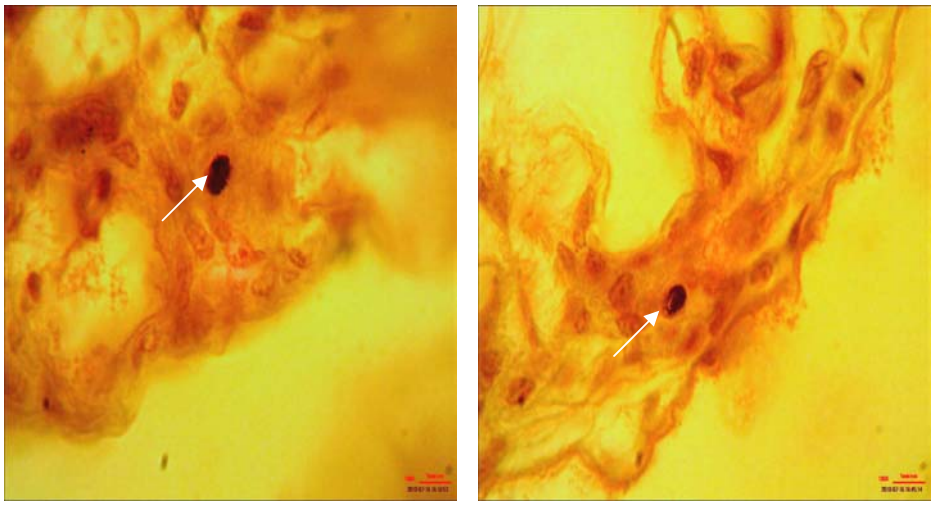


Fig.10a. Normal feed administered **Fig.10b.** IVP administered

- White arrows point to hypertrophied nuclei

Fig. 11

Immunohistochemistry of Gill tissue (Before WSSV challenge)

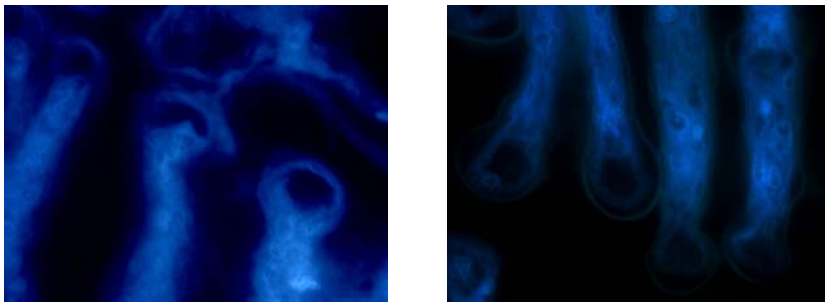


Fig.11a. Normal feed administered **Fig.11b.** IVP administered

Fig. 12

Immunohistochemistry of Gill tissue (After WSSV challenge)

Fig.12a. Normal feed administered

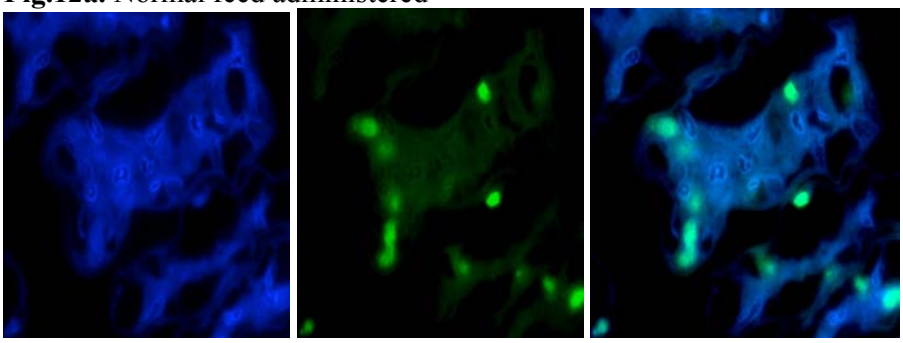
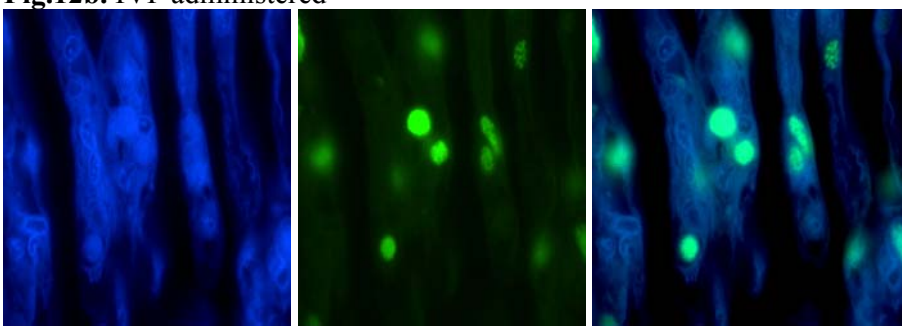


Fig.12b. IVP administered



DAPI **FITC** **DAPI + FITC**

Pages 196-200 contain the figures of the PCR products of different viral genes in gills, haemolymph and hepatopancreas. Each gene amplicon is documented in the left side of the page and the band intensity is graphically represented on the right side of the page. Each lane of the documented gel contain the following:

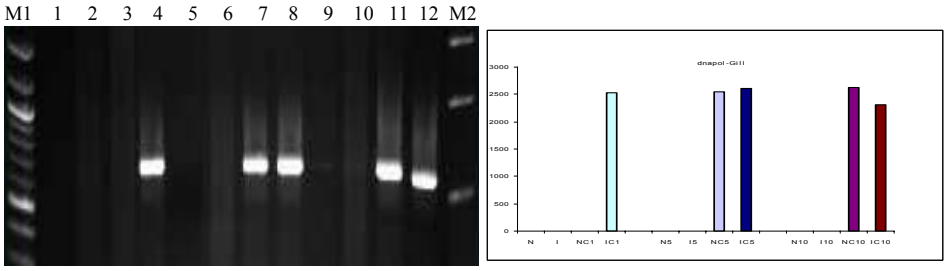
M1 – 100bp ladder

1. Normal (fed with normal feed, 1st day after 7 days of administration)
2. IVP (fed with IVP coated feed, 1st day after 7 days of administration)
3. Normal (fed with normal feed, challenged 1st day after 7 days of administration and samples collected on 4th day post challenge)
4. IVP (fed with IVP coated feed, challenged 1st day after 7 days of administration and samples collected on 4th day post challenge)
5. Normal (fed with normal feed, 5th day after 7 days of administration)
6. IVP (fed with IVP coated feed, 5th day after 7 days of administration)
7. Normal (fed with normal feed, challenged 5th day after 7 days of administration and samples collected on 4th day post challenge)
8. IVP (fed with IVP coated feed, challenged 5th day after 7 days of administration and samples collected on 4th day post challenge)
9. Normal (fed with normal feed, 10th day after 7 days of administration)
10. IVP (fed with IVP coated feed, 5th day after 7 days of administration)
11. Normal (fed with normal feed, challenged 10th day after 7 days of administration and samples collected on 4th day post challenge)
12. IVP (fed with IVP coated feed, challenged 10th day after 7 days of administration and samples collected on 4th day post challenge)

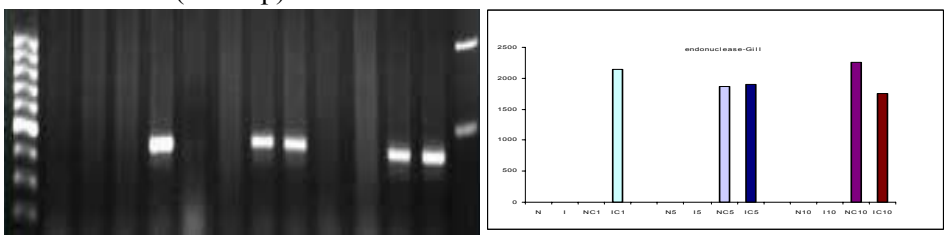
M2 – 1000bp ladder

Gill

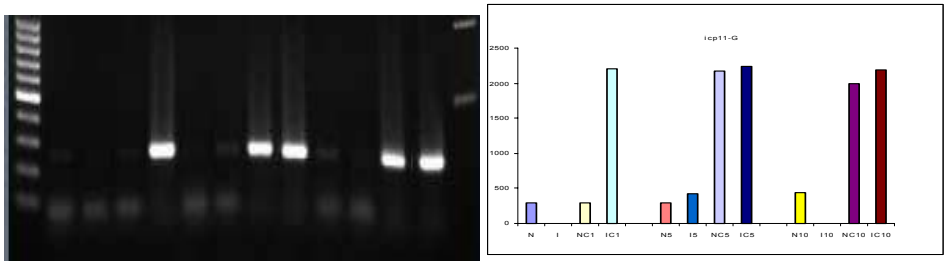
dnapol (~586bp)



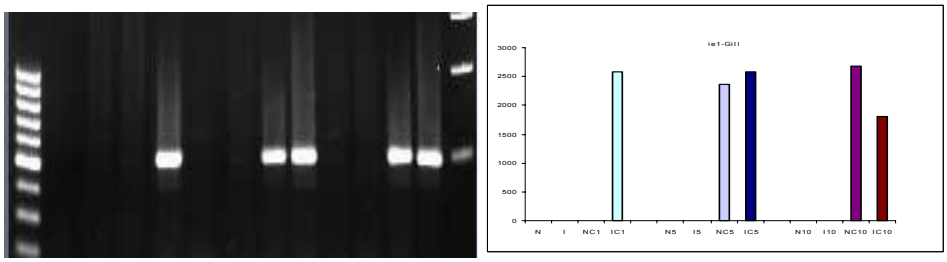
endonuclease (~408bp)



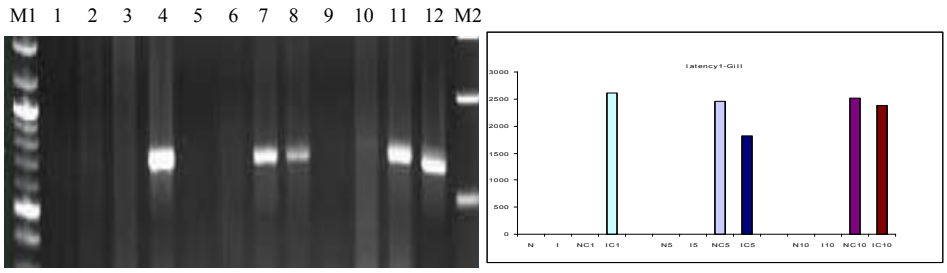
icp11 (~249bp)



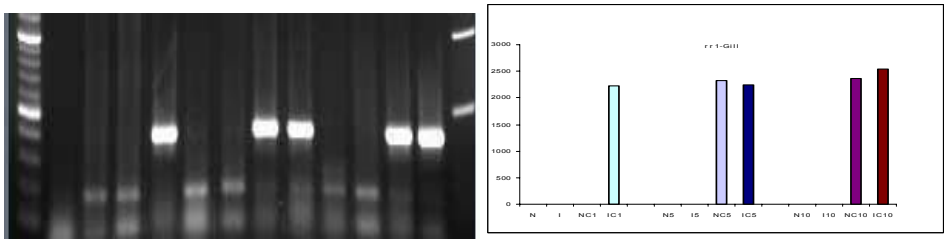
ie1 (~502bp)



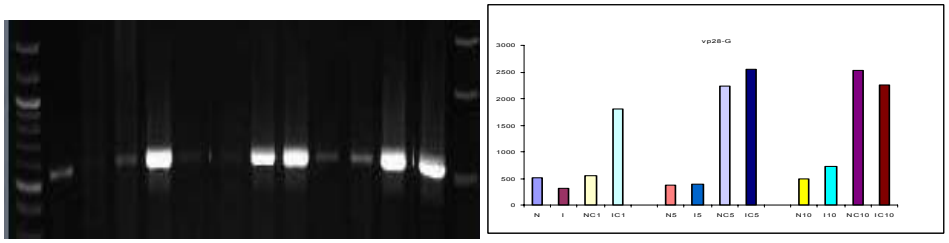
latency1 (~647bp)



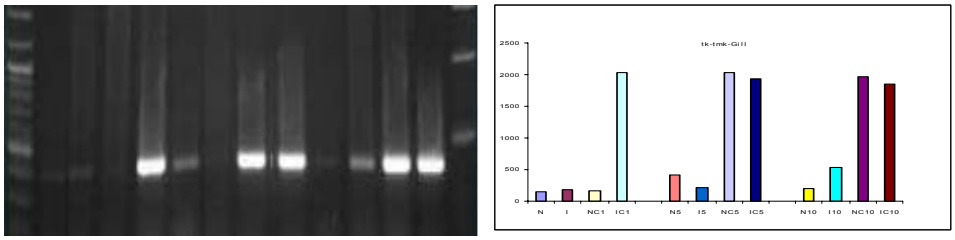
rr1 (~408bp)



vp28 (~555bp)

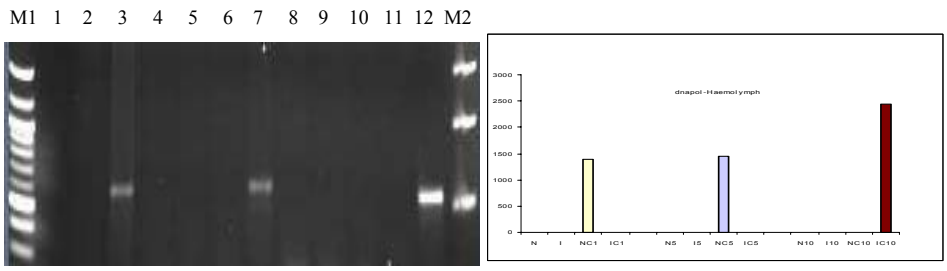


tk-tmk (~412bp)

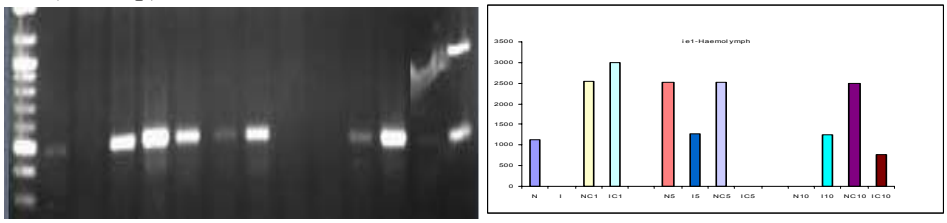


Haemolymph

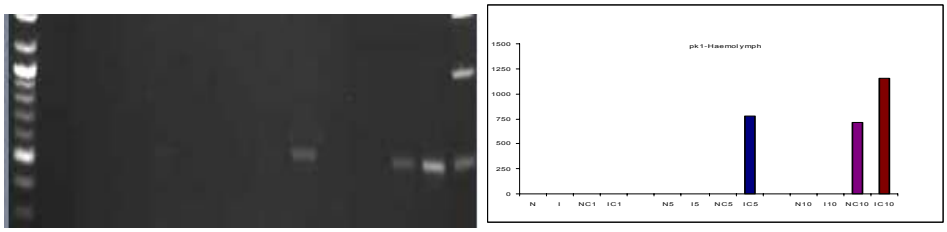
dnapol (~586bp)



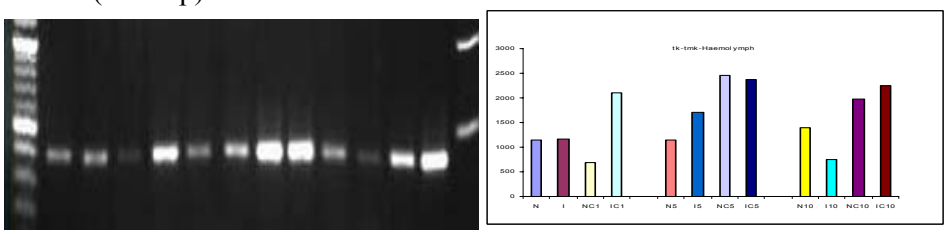
ie1 (~502bp)



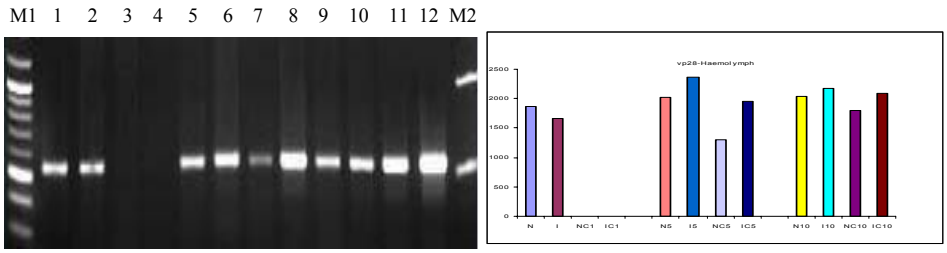
pk1 (~512bp)



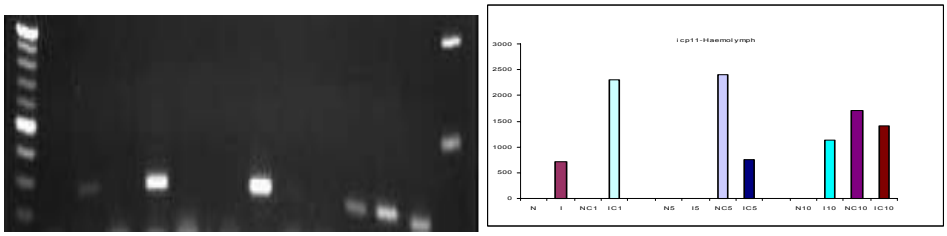
tk-tmk (~412bp)



vp28 (~555bp)

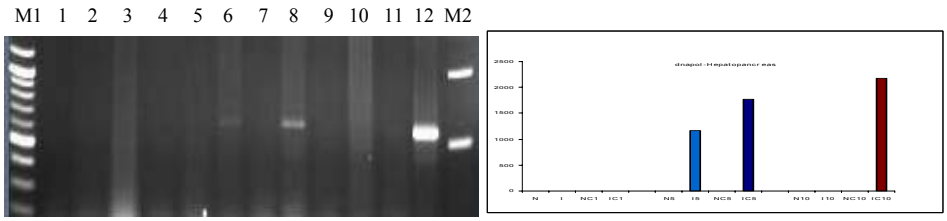


icp11 (~249bp)

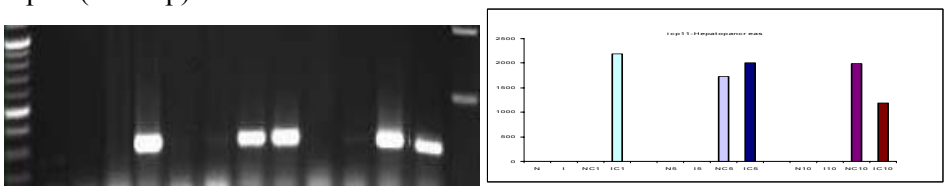


Hepatopancreas

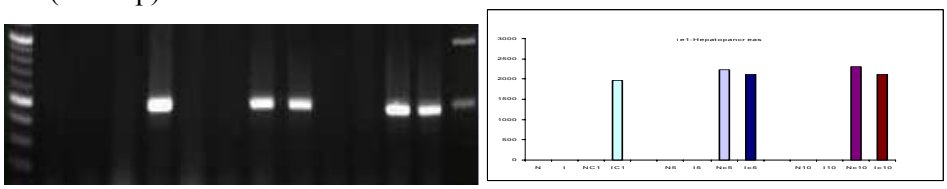
dnapol (~586bp)



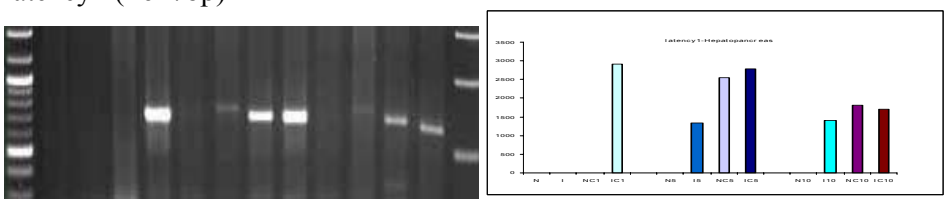
icp11 (~249bp)



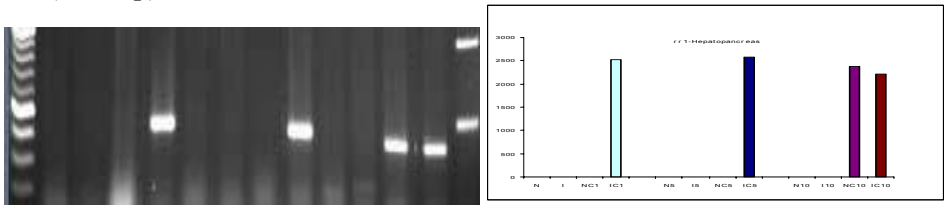
ie1 (~502bp)



latency1 (~647bp)



rr1 (~408bp)



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Chapter 6

CONCLUSION

CHAPTER - 6

Conclusion

Aquaculture, is perceived as having the greatest potential to meet the growing demand for aquatic food as it accounts for almost 50 percent of the world's food fish with its socio-economic and ecological impacts. Crustaceans form one of the main value added components in aquaculture and among them, shrimp aquaculture is the predominant one. The lucrative shrimp aquaculture suffered set backs in many countries mainly due to white spot syndrome virus (WSSV) (Genus – *Whispovirus*, Family – *Nimaviridae*), a rapidly replicating and extremely virulent shrimp pathogen, which has emerged globally as one of the most prevalent and widespread.

Prophylaxis include maintaining water quality of the environment, application of immunostimulants or vaccines to enhance the immune mechanism in shrimp, and/or selection of specific pathogen free animals.

Types of aquaculture shellfish vaccines for combating White Spot Disease (WSD) that have been experimented include administration of heat inactivated WSSV (Namikoshi et al., 2004); formalin inactivated WSSV (Namikoshi et al., 2004; Singh et al., 2005; Melena et al., 2006); recombinant viral proteins of WSSV - VP19, VP28, VP26, VP292, VP466 (Witteveldt et al., 2004a, 2004b; Namikoshi et al., 2004; Vaseeharan et al., 2006; Ha et al., 2008) and DNA vaccine for WSSV (Rout et al., 2007; Rajeshkumar et al., 2008; Li et al., 2010).

The protection seen in the above studies appears to be dependent on the shrimp species and on the time of application of the competing agents (Melena et al., 2006). The durations of protection (maximum seven weeks) and efficacies varied with viral proteins and between studies. Until now, most of the promising results obtained from experimental bioassays have been achieved largely on

empirical grounds as all the vaccine related studies in shrimp have used largely, survival as a parameter, mostly after a challenge (van de Braak, 2002). These studies have focused on the practical issues of vaccination, rather than targeting at the understanding of the mechanisms that are involved in this immune response (Johnson et al., 2008).

In the present study, the following objectives were undertaken to gather information on the immune response of tiger shrimp, *Penaeus monodon*, against the administration of inactivated virus preparation (IVP):

- Partial protein profiling of *Penaeus monodon* in response to IVP administration.
- Non-specific immune response of *Penaeus monodon* to IVP administration.
- Bio-defence genes in immunomodulation of IVP administered *Penaeus monodon*.
- Viral gene expression of *Penaeus monodon* to IVP administration.

The findings of the work are summarized below:

- The protein profile of IVP administered *P.monodon* before and after oral challenge was investigated employing 1 Dimensional Electrophoresis (1DE) and 2 Dimensional Electrophoresis (2DE).

- There were no remarkable variations in the 1D protein profile of gill and hepatopancreas of the IVP administered shrimp with the control which was maintained on normal diet.

- Protein profile of haemocyte lysate of IVP administered *P. monodon* by 2DE was exhibiting variations from that of the control animals. This comparison was made before and after the challenge with WSSV, and in the latter the protein spots were lesser compared to those of the control suggesting the response of haemocyte to the IVP administration

indicating the down regulation of certain proteins under situations of IVP administration and challenge. This situation requires more investigations.

- In gill tissue, the 2D protein profile was following a pattern of disappearance of protein spots after challenge on the first day and fifth day of challenge in IVP administered animals compared to that of the control. Meanwhile on 10^{th} day, the spots were comparatively higher than those of the control group. It was apparent that by 10^{th} day of administration of IVP, its efficacy had been getting reduced reaching to a situation equivalent to that of the control group.

- The partial protein profile using 2DE has revealed that *P. monodon* haemocytes and gill tissue, exposed to a particular dose of IVP through oral route, respond to a WSSV challenge through a protein related mechanism.

- The reactive oxygen intermediates (ROI) and lipid peroxides were significantly higher in the haemolymph of the experimental animals (*P.monodon*) after the administration of IVP (before challenge).

- The total haemocyte count (THC) and phenol oxidase activity were significantly higher in the group which was challenged on 5^{th} day post administration of IVP. Significant difference was not observed in animals which were challenged on 10^{th} day post administration.

- Among the non-specific immune indices analysed, only THC was found to show significant difference between the unchallenged and challenged animals with higher value for unchallenged group and the lower value for the group challenged on 5th and 10th day post administration. Possibly, a reduction in THC subsequent to challenge both in normal and

IVP administered group suggests that WSSV may be acting on the heamocytes inspite of IVP administration reducing their count.

- ROI and transglutaminase activity were found to be significantly higher in IVP administered group. In earlier studies wherever feed incorporated with β 1,3 glucan, certain Indian herbs, Vitamin C with its derivatives, copper and Vitamin E were applied, ROI was found to be higher. This has not been reported along with the application of WSSV vaccine in any form. Meanwhile, the higher transglutaminase activity in IVP administered group suggests that it has positively influenced the coagulation mechanism.

- All above observations indicate that the non-specific immune response of *P.monodon* on administering IVP and after the challenge differs from previous such studies where a high level of proPO, SOD, lysozyme and alkaline phosphatase activity in the haemolymph of vaccinated group (without WSSV challenge) was observed.

- Analysis of the bio-defence genes, related to the different immune related mechanisms such as proPO, peroxinectin, transglutaminase, haemocyanin, alpha 2-macroglobulin, caspase, PAP, C type lectin, astakine, crustin, SWD, penaeidin-3, lysozyme, superoxide dismutase, catalase, glutathione peroxidase, glutathione-s-transferase, Pm-argonaute, c-SPH, tropomyosin II, syntenin, eIF5A, cathepsin C, *PmAV*, PmRACK-1, Rab7, cyclophilin A, Hsc70 and chitinase were done.

- The higher number of amplified/ up-regulated genes was seen in gill tissue of IVP administered animals on 1DPA (before challenge) which indicated that the bio-defence response was elicited soon after the administration of IVP.

- The amplification/ up-regulation of more number of genes observed in gill tissue of IVP administered animals on 5DPA (after challenge) and lesser number on 10DPA indicated that the bio-defence mechanisms were more active against WSSV on 5DPA compared to 10DPA.

- The comparative analysis of the up-regulated genes in the gill tissue between the unchallenged and challenged group of animals on 1DPA, 5DPA and 10DPA showed that after the challenge, the genes which were up-regulated were different from those which were expressed in the unchallenged condition, except for astakine which was upregulated both in the unchallenged and challenged condition in the 1DPA.

- The higher number of amplified/ up-regulated genes in the haemolymph of IVP administered animals when compared to control group on 1DPA, 5DPA and 10DPA (before challenge) showed that the bio-defence response was elicited primarily in the haemolymph than that was observed in the gill tissue.

- The upregulation of different bio-defence genes in haemolymph in different groups showed that the immune response elicited against WSSV in IVP administered animals (after challenge) varied with time post administration of IVP such as 1st day, 5th day and 10th day post administration respectively where the highest activity was observed on 10th day. The decrease in the up-regulated genes on 1st DPA and 5th DPA, and subsequent increase in the up regulated genes on 10th DPA pointed that in the haemolymph, the bio-defence mechanisms were more active on 10th DPA unlike the condition observed in the gill tissue.

- The comparative analysis of the up-regulated genes in the haemolymph between the unchallenged and challenged group of animals on 1DPA, 5DPA and 10DPA showed that after the challenge, the genes which were

up-regulated were different from those expressed in the unchallenged condition, except for the up-regulation of single whey domain (SWD) in the 1DPA group and (clip domain sereine protease homologue (c-SPH) in the 10DPA group.

- The amplification/ up-regulation of bio-defence genes in the hepatopancreas (before challenge) were PmAV on 1DPA and 5DPA and MnSOD on 5DPA which indicated poor expression of the bio-defence gene in hepatopancreas.

- However, after the challenge, amplification/ up-regulation of higher number of bio-defence gene in the hepatopancreas could be observed, five genes on 1DPA and three genes on 5DPA, which indicated that the tissue had elicited an up-regulatory mechanism of some of the bio-defence genes to combat the WSSV infection in these periods.

- The comparative analysis of the up-regulated genes in the hepatopancreas between the unchallenged and challenged group of animals on 1DPA, 5DPA and 10DPA showed that after the challenge, the genes which were up-regulated were different from those which were expressed in the unchallenged condition.

- From the analysis of amplified genes/ up-regulated genes/ genes without variation it could be observed that various cellular and humoral immune responses elicited on 1st day post administration, 5th day post administration and 10th day post administration in gills, haemolymph and hepatopancreas of IVP administered animals were varying from each other and were not comparable indicating that different bio-defence mechanisms were simultaneously getting activated in different tissues to modulate the WSSV succession in *P. monodon*.

- Semiquantitative RT-PCR of WSSV genes (*ie1*, *pk1*, *tk-tmk*, *rr1*, *dnapol*, *endonuclease*, *vp28*, *latency1* and *icp11*) in IVP administered *P. monodon*, before and after WSSV challenge were analyzed.

- The animals which were stocked in the RAS and reared under biosecured condition were PCR negative for WSSV. However, prior to the experiment 76% of them were found to be nested PCR positive for WSSV. Nevertheless, histopathological examiniations and immunohistochemistry did not show the presence of virions on the tissues which prompted us to go ahead with the study.

- Among the tissues analyzed by RT-PCR, gills of the experimental animals (both challenged and unchallenged group) showed cumulative expression of 8 selected genes (*ie1*, *tk-tmk*, *rr1*, *dnapol*, *endonuclease*, *vp28*, *icp11, latency1*) followed by haemolymph (6 genes – *ie1*, *pk1*, *tk-tmk*, *dnapol*, *vp28*, *icp11*) and hepatopancreas (5 genes – *ie1*, *rr1*, *dnapol*, *latency1*, *icp11*).

- The unchallenged group of (both IVP administered and normal feed administered) animals showed amplification of 3 genes (*icp11, tk-tmk, vp28*) in gills. Meanwhile, the haemolymph of unchallenged normal feed administered animals showed amplification of 3 genes (*ie1, tk-tmk, vp28*), and IVP administered animals 4 genes (*icp11, ie1, tk-tmk, vp28*). In the hepatopancreas of normal feed administered animals, viral genes were not amplified while in the IVP administered animals, 2 genes such as *dnapol* and *latency1* were found to be expressed in the unchallenged animals.

- This suggest viral gene integration with the host genome and their expression. However by histopathology and immunohistochemistry,

absence of virions was confirmed in experimental animals before challenge.

- In the WSSV challenged animals, 8 genes (*ie1*, *tk-tmk*, *rr1*, *dnapol*, *endonuclease*, *vp28*, *icp11, latency1*) were found amplified in gill tissue and 6 (*ie1*, *tk-tmk*, *dnapol*, *vp28*, *icp11, pk1*) in haemolymph, in both normal feed and IVP administered group. In the hepatopancreas of normal feed administered animals viral genes were not found to be expressed. However, in the IVP administered animals, 4 genes such as *rr1, ie1, icp11* and *latency1* were found to be expressed after the challenge.

- After the WSSV challenge, hypertrophied nuclei and VP28 were detected in gill tissues of the experimental animals by histopathology and immunohistochemistry respectively (both in control and IVP administered).

- The variations observed in different tissues, in different treatments and in different animal samples with respect to a WSSV challenge could not be directed to a temporal pattern of WSSV transcription as some of the late genes (eg: *vp28* and *icp11*) were already present without leading the animals to mortality during the time of experimentation.

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- It was observed that *P. monodon* when administered with IVP, adopted a unique immune mechanism with complexity in inter play of various pathways which warrants further studies.

- This complexity is seen in the protein profiling by 2DE, expression of non-specific immune parameters, immune gene expression, existence of viral gene integration with out virion, and presence of hypertrophied nuclei and VP28 protein in IVP administered and challenged group with

out mortality during the experimental period both in the control and experimental group.

- Precisely this work opens up new avenues of research to explore the status of WSSV gene integration in to the host genome with out having the virion per se and the immune status of such animals in protecting themselves from a horizontal active virus invasion. In this context the 'Vaccination' has different dimensions.

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