# *Molecular Characterization and Gene Expression Profiling of Antimicrobial Peptides in Penaeid Shrimps*

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BY

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March, 2011

*Dedicated to*

*My Family,*

*My Guide*

*&*

*God Almighty*

# *Declaration*

I hereby do declare that the thesis entitled "**Molecular Characterization and Gene Expression Profiling of Antimicrobial Peptides in Penaeid Shrimps**" is an authentic record of research work done by me under the supervision and guidance of Dr. Rosamma Philip, Assistant Professor, Department of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, Cochin University of Science and Technology, for the degree of Doctor of Philosophy in Marine Biotechnology and that no part thereof has been presented before for the award of any other degree, diploma or associateship in any University.

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# *Certificate*

This is to certify that the thesis entitled "**Molecular Characterization and Gene Expression Profiling of Antimicrobial Peptides in Penaeid Shrimps**" is an authentic record of research work carried out by Ms. Swapna P. Antony under my supervision and guidance in the Department of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, Cochin University of Science and Technology, in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Marine Biotechnology and that no part thereof has been presented before for the award of any other degree, diploma or associateship in any University.

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# Abbreviations

| | |
|---|---|
| **α-2M** | Alpha-2-Macroglobulin |
| **ALF** | Anti-lipopolysaccharide Factor |
| **AMP** | Antimicrobial Peptide |
| **APHA** | American Public Health Association |
| **BGBP** | β-1,3 Glucan Binding Protein |
| **BLAST** | Basic Local Alignment Search Tool |
| **cDNA** | Complementary DNA |
| **C-Terminal** | Carboxy-Terminal |
| **DDBJ** | DNA Data Bank of Japan |
| DEPC | Diethylpyrocarbonate |
| **DNA** | Deoxyribonucleic Acid |
| **dNTP** | Deoxyribonucleotide Triphosphate |
| **DSC** | Disulphide Core |
| **EEZ** | Exclusive Economic Zone |
| **ELF** | Elongation Factor |
| **ELISA** | Enzyme Linked Immunosorbent Assay |
| **EST** | Expressed Sequence Tag |
| **FAO** | Food and Agriculture Organization |
| **FCR** | Feed Conversion Ratio |
| **GAPDH** | Glyceroldehyde-3 Phosphate Dehydrogenase |
| **GC** | Granular Cell |
| **HHNBV** | Hypodermal and Haematopoietic Necrosis Baculovirus |
| **HIV** | Human Immunodeficiency Virus |
| **HNP** | Human Neutrophil Peptides |
| **HSV** | Herpes Simplex Virus |

| | |
|---|---|
| **HSV** | Human Herpes Virus |
| **IHHN** | Infectious Hypodermal and Haematopoietic Necrosis |
| **IPTG** | Isopropyl-β-Thio Galactopyranoside |
| **kDa** | KiloDalton |
| **LAB** | Lactic Acid Bacteria |
| **LAMP** | Loop Mediated Isothermal Amplification PCR |
| **LB** | Lauria-Bertani |
| **LGBP** | Lipopolysaccharide and β-1,3 Glucan Binding Protein |
| **LPS** | Lipopolysaccharide |
| **LTA** | Lipo-Teichoic Acid |
| **MAb** | Monoclonal Antibody |
| **mRNA** | Messenger RNA |
| **NACA** | Network of Aquaculture Centres in Asia-Pacific |
| **NCBI** | National Center for Biotechnology Information |
| **NJ** | Neighbor-Joining |
| **NK cells** | Natural Killer Cells |
| **N-Terminal** | Amino-Terminal |
| **OH** | Hydroxyl Radical |
| **OIE** | Office International Des Epizooties |
| **ORF** | Open Reading Frame |
| **PCD** | Post-Challenge Day |
| **PCR** | Polymerase Chain Reaction |
| **PG** | Peptidoglycan |
| **PGBP** | Peptidoglycan Binding Protein |
| **pI** | Isoelectric Point |
| **PK** | Prokineticin |
| **PL** | Postlarvae |
| **PO** | Phenoloxidase |

| | |
|---|---|
| **ppA** | Prophenoloxidase-Activating Enzyme |
| **ppt** | Parts Per Thousand |
| **proPO** | Prophenol Oxidase |
| **PRP** | Pattern Recognition Protein |
| **RNA** | Ribonucleic Acid |
| **RNAi** | RNA Interference |
| **RNase** | Ribonuclease |
| **ROS** | Reactive Oxygen Species |
| **RPLA** | Reverse Passive Latex Agglutination Assay |
| **RT** | Reverse Transcription |
| **RT**-PCR | Reverse Transcription Polymerase Chain Reaction |
| **SDS** | Sodium Dodecyl Sulphate |
| **SEMBV** | Systemic Ectodermal and Mesodermal Baculovirus |
| **SGC** | Semigranular Cells |
| **SOD** | Superoxide Dismutase |
| **SWD** | Single-Whey Domain |
| **TGase** | Transglutaminase |
| **TNF** | Tumor Necrosis Factor |
| **TSV** | Taura Syndrome Virus |
| **UTR** | Untranslated Region |
| **WAP** | Whey-Acidic Protein |
| **WSD** | White Spot Disease |
| **WSSV** | White Spot Syndrome Virus |
| **X-Gal** | 5-Bromo-4-Chloro-3-Indolyl B-D-Galactopyranoside |

# *CHAPTER-1*

## *General Introduction*

## 1.1 Introduction

Aquaculture represents one of the fastest growing food-producing sectors in the world. As the world population grows, demand for aquaculture products will also continue to increase. Aquaculture is increasingly filling the void left as yield from capture fisheries and world population continues to increase (FAO, 2004). World aquaculture production has increased an average of 8.8% per year since 1950 (FAO, 2006). Shrimp farming has become a major aquaculture activity and source of investment over the past two to three decades. Since the late 1980s, farmed shrimp has become a major contributor to overall shrimp supplies in the world, making up for the declining wild catch and meeting the steadily increasing demand. Several elements have driven the rapid expansion of shrimp culture. They include potentially high profits, buoyant demand for high value seafood products, increasing demand for farmed shrimp due to limitations and fluctuations from capture fisheries, and the industry's capacity to generate foreign exchange and employment in poor coastal areas. Benefits of shrimp farming to poor coastal communities thereby reduce poverty. However, this industry also has confronted many of the developmental problems in this relatively short period including sector competition, over production, trade restrictions, over capitalization and concerns over environmental impacts. The significance of aquaculture in the context of global food production sector, the management of aquatic resources and the socio-economic development of coastal rural areas is now fully appreciated world-wide. Significant advances have also been made globally to make shrimp aquaculture development responsible and sustainable.

Shrimp is one of the most popular types of seafood in the world and is now the most important internationally traded fishery commodity in terms of value. The global shrimp market has increased considerably over the last couple of decades, in particularly the aquaculture. Shrimp farms are being created throughout the world to help meet the demand for shrimp. According to the FAO (2006), the shrimp aquaculture growth was 10% per year over the past three decades. When the FAO first compiled production statistics on shrimp in 1950, production came solely from wild catches (FAO, 1995). Export-oriented shrimp aquaculture is a fairly recent industry that took off in the mid 1970s. With improved technologies and the introduction of formulated feeds, the industry boomed in the following decade (Rosenberry, 1999). Today almost one-third of world shrimp production is from farmed shrimp. Approximately 5 million metric tons of shrimp are produced annually. The total world production of shrimp, both captured and farmed, is about 6 million tons, of which about 60% enters the world market (FAO, 2008). In many tropical developing countries, it is the most valuable fishery export; the employment aspect is also significant. In 1984, only 29 countries had reported any shrimp production and to date more than 63 countries are listed in FAO aquaculture statistics as having produced shrimp at one time or another.

## *1.2 Major groups of shrimps cultured world wide*

The popular names, shrimps and shrimps, have been used variously to denote decapod crustaceans of the families Penaeidae and Palaemonidae. But in the recent aquaculture literature, a distinction has been drawn between the two groups. The name shrimp is used for freshwater forms of Palaemonids and shrimp for the marine penaeids (Pillay, 1998). There are more than 50 species/varieties of shrimps available in marine waters, with a very wide distribution in both tropical and temperate ecosystems. Most are very small and not suitable for farming or human consumption. However,

the giant tiger shrimp (*Penaeus monodon*), which is internationally known as tiger shrimp, has been and continues to be the leading cultured species. *P. monodon* is also the largest (maximum length 363 millimeters) and fastest growing of the farmed shrimp species and accounts for more than half of the total shrimp aquaculture output. In India, other than *P. monodon*, species such as *Fenneropenaeus indicus* (Indian white shrimp), *P. penicillatus* (like white shrimp), *P. semisulcatus* (green tiger shrimp) and *P. merguiensis* (banana shrimp) are also farmed. However, the two shrimp species – *P. monodon* and *F. indicus* form the mainstay of shrimp aquaculture in the country. In other parts of the world, *L. vannamei* is the dominant species in South American countries and *F. chinensis* is the most popular farmed species in China. Most of the south and south-east Asian countries farm only *P. monodon*. Kuruma shrimp (*Marsupenaeus japonicus*), native to the Indian Ocean and the South-eastern Pacific Ocean is commonly farmed in Japan and Australia (FAO, 2002) (Fig. 1.1).

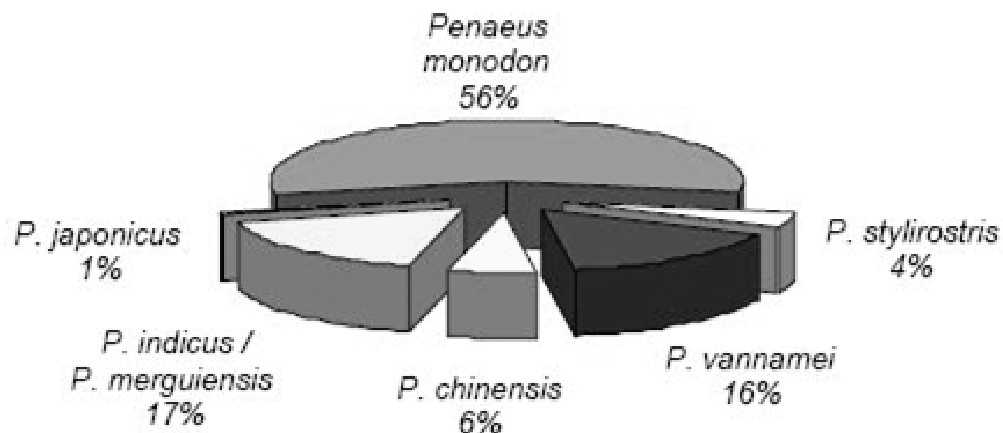



**Fig. 1.1. Relative importance of penaeid shrimp species to global aquaculture production. (Adapted from Rosenberry, 1999)**

### 1.2.1 *Penaeus monodon (Black tiger shrimp)*

Named for its huge size and banded tail, the black tiger shrimp is one of the most common penaeid shrimp species currently being cultured in the world and the primary species farmed in Asia (excluding China and Japan).

This species accounts for more than half of the total world shrimp aquaculture output, and it is the largest and fastest growing of all shrimp species. *P. monodon* is the largest, reaching 330 mm or more in body length, and exhibits the highest growth rate of all cultured penaeids (Lee and Wickins, 1992). Even at high stocking densities, *P. monodon* can reach marketable sizes of 20 cm and 35 g in three to six months, tolerant to a wide range of salinities and therefore holds the promise for the commercial aquaculturists of a more rapid cash flow than other cultured fish and shellfish (Muir and Roberts, 1982). All these together make the black tiger shrimp an interesting species to culture (Rosenberry, 1997, 1999).

## *Taxonomy:*

Penaeid shrimp belong to the largest phylum in the animal kingdom, the Arthropoda. The subphylum Crustacea is made up of 42,000, predominantly aquatic, species, that belong to 10 classes. Within the class Malacostraca, shrimp, together with crayfish, lobsters and crabs, belong to the order Decapoda.

Phylum : Arthropoda

Subphylum : Crustacea
Class : Malacostraca
Order : Decapoda
Superfamily : Penaeoidea
Family : Penaeidae Rafinesque, 1815
Genus : Penaeus Fabricius, 1798
Species : monodon

***Other scientific names appearing in the literature of this species:***

*Penaeus carinatus* Dana, 1852; *Penaeus caeruleus* Stebbings, 1905; *Penaeus bubulus* Kubo, 1949; *Penaeus monodon* var. *manillensis* Villaluz and Arriola, 1938.

***Common names:***

Giant tiger shrimp, Jumbo tiger shrimp, Blue tiger shrimp, Leader shrimp, Panda shrimp (Australia), Jar-Pazun (Burma), Bangkear (Cambodia), Ghost shrimp (Hong Kong), Udang windu (Indonesia), Ushi-ebi (Japan), Kambandogo (Kenya), Jinga (India, Bombay region), Kara chemmeen (Kerala).

## *1.2.2 Fenneropenaeus indicus– Indian White Shrimp*

*F. indicus* is raised on extensive farms throughout Asia. This species have attracted attention recently because it tolerates low water quality compared to *P. monodon*, can be grown at high densities, and is readily available as post-larvae (PL) in the wild. Furthermore, *F. indicus* regularly reaches sexual maturity in the grow-out ponds. Native to the Indo-Pacific region, this shrimp species are among the most commercially important wild caught shrimp commodities in East Africa, South Asia, Southeast Asia and Australia (Rosenberry, 1999).

## *Taxonomy:*

Phylum : Arthropoda
Subphylum : Crustacea
Class : Malacostraca
Order : Decapoda
Superfamily : Penaeoidea
Family : Penaeidae Rafinesque, 1815
Genus : Fenneropenaeus
Species : indicus

***Other scientific names appearing in the literature of this species:***

*Penaeus indicus* H. Milne Edwards, 1837; *Penaeus indicus longirostris* De Man, 1892

***Common names:***

Indian white shrimp, Tugela shrimp, White shrimp, Banana shrimp, Indian banana shrimp, Red leg banana shrimp and Naran chemmeen (Kerala).

## *1.3. Major constraints in aquaculture*

Disease has always played a critical role in determining the success or failure of any aquacultural effort, but its importance is increased in semi-intensive operations (Flegel, 1997). Fig. 1.2 shows a model of shrimp aquatic disease developed by Dr. Sniezko (Lightner and Redman, 1998). This model includes to three adjacent spheres—the host, the environment and the pathogens themselves. When one sphere grows in importance and size, this sphere begins to intersect with the others, such that the greater intersection creates a tendency for disease. If all three spheres are to intersect, such that the environmental conditions are right, there are suitable pathogens and the population is large enough the disease will begin to spread. Wild populations tend to avoid these problems, even though all factors may be present, while aquaculture encourages them by amplifying the presence of each variable (McClennen, 2004).

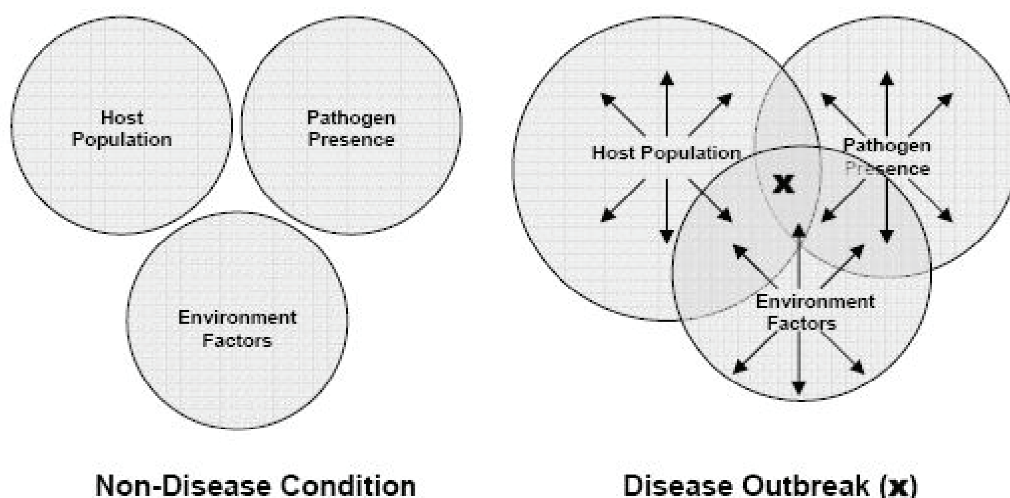



**Fig. 1.2. The classic three circles of host-pathogen-environment interactions (Adapted from Schnieszko, 1974)**

## 1.3.1. Diseases

Diseases are one of the major constraints for the sustainable increase of shrimp production. Shrimp diseases can be divided into non-infectious and infectious in origin (Lightner and Redman, 1998). Infectious diseases are caused by viruses, bacteria, fungi and parasites. Environmental symptoms result from mismanagement of pond water quality and shrimp health. Overstocking and environmental stress are the major causes of disease outbreaks. Moreover, the practice of transfer of seed, larvae and broodstock at national and international level has exacerbated the spread of many pathogens. Biological factors such as microbial flora present in the pond also play a role on the susceptibility of shrimp to pathogens (Horowitz and Horowitz, 2001). All the main species of shrimps, such as *P. monodon, F. chinensis, L. vannamei* and *M. japonicus* are affected by viral diseases with various etiologies (Lightner et al., 1983): baculoviruses, e.g. *Baculovirus penaei,* and parvoviruses or picorna-like viruses such as infectious hypodermal and hematopoietic necrosis (IHHN) (Owens et al., 1992). Also, fungi and bacteria cause severe mortalities in hatcheries (Baticados et al., 1990; Song et al., 1993) and the regular use of antibiotics has progressively led to a critical situation of resistance.

Fungi represent potential pathogens for reared crustaceans, as the filamentous fungus *Lagedinium* sp. that affects the larval stages of shrimp and lobster (Crisp and Bland, 1989) or fungi from the *Fusarium* genus. Fungal diseases, such as Rickettsia, Larval mycosis and Fusariosis again are treatable and of low impact. In shrimps, few protozoans have been recorded in mortalities, but more as epibionts reflecting poor management in hatcheries and ponds. Parasital or protozoan diseases, such as Epicommensals, Gregarines and Microsporidians latch on to farmed shrimp and are only of minor impact. Most often, in hatcheries, defective rearing conditions such as high larval density, excess or poor food quality, are associated with larval

mortalities by favoring contamination with either heterotrophic bacteria or with pathogenic bacterial strains.

Different bacterial genera are known to induce mortalities in hatcheries, namely *Pseudomonas* (Colwell and Sparks, 1967), *Aeromonas* (Riquelme et al., 1996), but *Vibrio* undoubtedly represent the most harmful pathogenic bacteria for both mollusc and crustacean larvae and juveniles. Bacteria of the genus *Vibrio* can cause serious production losses in ponds cultured with *P. monodon*. Though many bacterial diseases due to vibrios have been reported, the association between the bacteria and the invertebrates remains largely unknown, which strongly complicates the differentiation of strains as probiotic, commensal versus opportunistic or pathogenic. In shrimp, larval mortalities associated with the presence of *V. harveyi*, *V. vulnificus* and *V. splendidus* have been reported in *P. monodon* and *L. vannamei* (Baticados et al., 1990; Song and Lee, 1993; Robertson et al., 1998). In other cases, *Vibrio* species are responsible for disease outbreaks in shrimp nursery or grow-out ponds, such as *V. damsela* (Song et al., 1993), *V. alginolyticus*, *V. parahaemolyticus* (Lightner, 1992) or *V. penaeicida* in *L. stylirostris* (Costa et al., 1998).

### *1.3.1.1. Viral diseases*

Viruses are considered to be the most important pathogens in shrimp. More than 20 viruses have been reported as pathogenic to shrimp. Table 1.1. gives an overview of the most important viral diseases.

**Table 1.1. Viral pathogens of penaeid shrimp**

| Family | Virus |
| --- | --- |
| **DNA virus** | |
| *Parvoviridae* | Infectious hypodermal and hematopoeitic necrosis virus (IHHNV) |
| | Hepatopancreatic parvovirus (HPV) |
| | Spawner-isolated mortality virus (SMV) |

| | |
|---|---|
| | Lymphoidal parvo-like virus (LPV) |
| *Baculoviridae* | Baculovirus penaei (BP) |
| | Monodon baculovirus (MBV) |
| | Baculovirus midgut gland necrosis virus (BMNV) |
| | Type C baculovirus of *Penaeus monodon* |
| | Haemocyte infecting non-occluded baculo-like virus |
| *Iridovidae* | Shrimp iridovirus (IRIDO) |
| *Nimaviridae* | White spot syndrome virus (WSSV) |
| **RNA Virus** | |
| *Picornaviridae* | Taura syndrome virus (TSV) |
| *Roniviridae* | Yellow head virus (YHV) |
| | Gill associated virus (GAV) |
| | Lymphoid organ virus (LOV) |
| *Reoviridae* | Reo-like virus (REO) type II and IV |
| *Rhabdoviridae* | Rhabdovirus of penaeid shrimp (RPS) |
| *Togaviridae* | Lymphoid organ vacuolization virus (LOVV) |
| *Totiviridae* | Infectious myonecrosis virus (IMNV) |
| *Bunyaviridae* | Mourilyan virus (MOV) |
| Unclassified | Monodon slow growth syndrome (MSGS) |

## *1.4. WSSV*

White spot disease (WSD caused by WSSV) emerged in East Asia in 1992-93 and it was quickly dispersed with infected seed and broodstock across the Asian continent to SE Asia and India where it caused a major pandemic, and continues to cause significant losses in some regions. WSSV is considered as the most serious pathogen affecting shrimps. WSSV outbreaks have been reported from shrimp producing countries of Asia -Bangladesh,

Cambodia, China, India, Indonesia, Iran, Japan, Korea, Malaysia, Myanmar, Philippines, Sri Lanka, Taiwan, Thailand, Vietnam- and North, Central and South America -Belize, Brazil, Columbia, Ecuador, Guatemala, Hondurus, Mexico, Nicaragua, Panama, Peru and United states- (Lightner, 1996; WB/NACA/WWF/FAO, 2001; FAO/NACA, 2003; Bondad-Reantaso et al., 2005). WSSV outbreaks in China in 1992, Thailand in 1995 to 1997 (Flegel, 2006) and Ecuador in 1999 (Rodriguez et al., 2003) have caused losses in production with thousands of metric tons, jobs (thousands) and export with millions of dollars. However, WSSV has not been reported in Australia. WSSV causes mortalities in *P. vannamei, P. stylirostris*, *P. aztecus, P. duorarum* and *P. setiferus* when experimentally infected (Lightner, 1996). The wild penaeids *Parapenaeopsis* spp., *P. semisulcatus*, *Metapenaeus* spp. and *Macrobrachium* spp. (a caridean not a penaeid*)* from Taiwan developed disease following experimental infection with WSSV (Chang et al., 1996). Most mortality occurs in young juvenile shrimps weighing 3-5 g (Takahashi et al., 1994). Resistance to WSSV has not been reported for any penaeid species (Lightner, 1996).

WSSV was first named as penaeid rod-shaped DNA virus (PRDV) or rod-shaped nuclear virus of *M. japonicus* (RV-PJ). Later other names viz. hypodermal and hematopoietic necrosis baculovirus (HHNBV), white spot baculovirus (WSSV) and systemic ectodermal and mesodermal baculovirus (SEMBV) were also applied. WSSV has even reached shrimp farms in southeastern Europe (1997) and the Middle East (1999) via live shrimp movements, and Australia and Spain with introductions of frozen infected shrimp that were used as fresh food for broodstock (OIE, 2003).

WSSV is an enveloped, double stranded DNA virus, ovoid to bacilliform in shape with a tail like extension at one end (Wang et al., 1995; van Hulten et al., 2001a; Yang et al., 2001). The average virion size for baculoviruses from the WSSV complex is 70-150 nm x 250-380 nm (Wongteerasupaya et al., 1995). Replication appears to occur in the nucleus

and protective occlusion bodies are not formed. The virus is the only member of the family *Nimaviridae*, genus *Whispovirus* (Mayo et al., 2002a, 2002b). WSSV was recently assigned to its own new genus, *Whispovirus*, and family, *Nimaviridae* (Mayo, 2002a, 2002b). Virions are large (80-120 x 250-380 nm), rod-shaped to elliptical, and with a trilaminar envelope (Inouye et al., 1994, 1996; Wang et al., 1995; van Hulten et al., 2001). The size of the WSSV genome has been differently reported for different isolates: 305107 bp (GenBank Accession No. **AF332093**), 292967 bp (GenBank Accession No. **AF369029**) and 307287 bp (GenBank Accession No. **AF440570**). In accordance with a genome size of ~300 kb, a total of 531 putative open reading frames (ORFs) were identified by sequence analysis, among which 181 ORFs are likely to encode functional proteins (OIE, 2003).

### 1.4.1. Pathogenesis

Under experimental conditions, intramuscular or oral inoculation of the virus, immersion in viral suspension, feeding of infected tissue or cohabitation with infected animals cause infection in shrimp at PL stage onwards (Yoganandhan et al., 2003a; Escobedo-Bonilla et al., 2006). WSSV infection could not be induced in the early larval stages of *P. monodon* (nauplii, zoea, mysis) by immersion and oral challenge (Yoganandhan et al., 2003a). Apparently, shrimp become susceptible to infection from PL 6, PL 10 or PL 30 onwards (Venegas et al., 1999; Flegel, 2007).

### 1.4.2. Pathology

WSSV infections of penaeid shrimp are characterized by a rapid mortality accompanied by gross signs in moribund shrimp. Natural outbreaks of WSSV are categorized into pre-acute, acute to sub-acute and chronic forms, where mortality occurs within 2-3 days, 7-10 days and 15-28 days, respectively (Sudha et al., 1998). Disease and mortality are mainly observed in juvenile shrimp (1-5 g). The signs observed in infected shrimp are white deposits of calcium of 0.5-2.0mm diameter observed on the cuticle

of the shrimp cephalothorax or carapace (Fig.1.3). Abnormal deposits of calcium, the accumulation of vacuoles and lysed debris and the necrosis of cuticular pore canals produce white spots on the cuticular epidermis (Wang et al., 1995; Lightner, 1996). Not all shrimps infected with WSSV display white spots on the carapace (Lightner, 1996). Affected animals also display expanded chromatophores that show a pink-red to reddish-brown coloration in the cephalothorax cuticle (Inouye et al., 1996). Diseased shrimp exhibit a reduction in feeding and increased lethargy. Infected shrimp swim slowly near the pond surface and eventually sink to the bottom and die (Chang et al., 1996, 1998; Lightner, 1996; Wang et al., 1999).

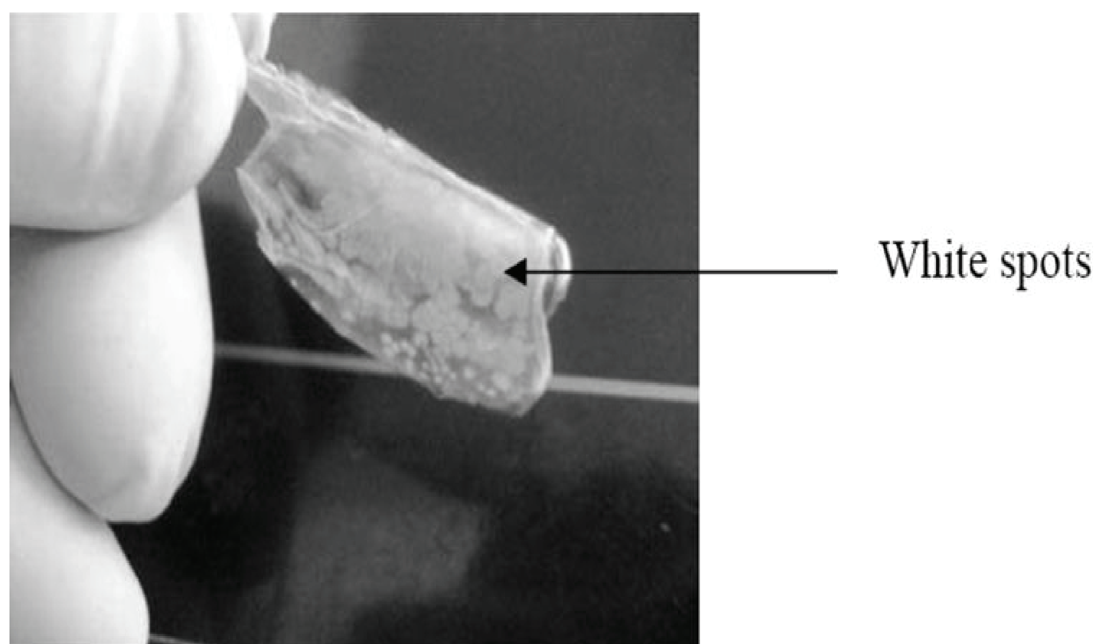



**Fig. 1.3. The clinical sign of white spot disease in an infected shrimp**

WSSV can infect cells of mesodermal and ectodermal origin, such as the subcuticular epithelium, the lymphoid organ, haemocytes, haematopoietic tissue, stomach cuticular epidermis and connective tissue (Momoyama et al., 1995; Lightner, 1996). Infected tissues display widespread focal necrosis (Wongteerasupaya et al., 1995). Degenerate cells are characterised by hypertrophied nuclei with marginated chromatin and eosinophilic to basophilic intranuclear inclusions (Chou et al., 1995; Wongteerasupaya et al., 1995). Haemocytic encapsulation of necrotic cells as

small brown masses in the stomach may be associated with infection (Momoyama et al., 1995). Other tissues and cells that may also show signs of disease include the antennal gland epithelium, the lymphoid organ sheath cells, haematopoietic cells and the fixed phagocytes of the heart (Momoyama et al., 1995; Wang et al., 1995). The lymphoid organ of diseased shrimp may be distended, swollen or shrunken (Takahashi et al., 1994) and haemolymph infiltration in the enlarged haemal sinuses and interstitial spaces may result in a hypertrophied yellowish hepatopancreas (Wang et al., 1995). Cumulative mortalities in infected populations may reach 100% within 2 to 10 days of the onset of clinical signs (Chou et al., 1995; Lightner, 1996).

### *1.4.3. Host species*

WSSV is highly infective for marine penaeid shrimp, although it can also infect many other crustaceans and marine crabs (Rodriguez et al., 2003). WSSV has a wide host range among decapod crustaceans and is found to be pathogenic to at least 78 species, mainly to decapod crustaceans including marine and freshwater shrimp, crab, crayfish and lobsters (Lightner, 1996; Lo et al., 1996a; Flegel, 1997, 2006; Flegel and Alday-Sanz, 1998). There are reports that freshwater species such as the crayfish *Pacifastacus leniusculus* (Jiravanichpaisal et al., 2001) and *Macrobrachium rosenbergii* (Chakraborty et al., 2002) are also susceptible to infection. WSSV genomic DNA can be vertically transmitted to Artemia cysts, but it is lost during hatching.

### *1.4.4. Environmental factors and WSSV infection*

Environmental factors may play a key role on the severity of disease outbreaks of aquatic animals (Snieszko, 1974). Water temperature, salinity, dissolved oxygen, ammonia, pH and toxins derived from pesticides might be associated with mass mortalities due to WSSV (Fegan and Clifford, 2001). A significant effect of water temperatures, salinity and ammonia on the disease and mortality of WSSV infected animal was reported in several studies

(Guan et al., 2003; Withyachumnarnkul et al., 2003; Rodriguez et al., 2003; Jiravanichpaisal et al., 2004).

### *1.4.5. Transmission and Potential carriers*

Recent experiments and surveys using diagnostic polymerase chain reaction (PCR) have shown that approximately forty arthropods, including penaeids, crabs, lobsters, *Macrobrachium* spp, and possibly copepods and insects can act as carriers (Chou et al., 1996; Lo et al., 1996a; Flegel, 1997; Maeda et al., 1997). Many of these arthropods, such as the wild crab, *Portunus pelagicus*, and wild krill, *Acetes* sp., are common in shrimp culture areas and may transmit the virus to penaeid culture systems with the in-take water (Supamattaya et al., 1998). Infections by WSSV have been reported in many species of cultured penaeids including: *P. monodon*, *L. vannamei, L. stylirostris*, *M. japonicus*, *F. chinensis, M. rosenbergii* and *Procambarus clarkii* (Chang et al., 1998; Lightner and Redman, 1998; Lo et al., 1999; Wang et al., 1999). Rapid transmission of WSSV on culture systems may occur from infected shrimp, through the water and by cannibalism of moribund shrimp; however, the major source of infection for shrimp farms is from infected spawners and PL.

Crustacean carriers which enter shrimp ponds may transmit WSSV when they die and are eaten by shrimps. Potential sources for WSSV transmission include human activities; seabirds or other animals (insects, fishes, copepods) 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 (Chou et al., 1996; Lo et al., 1996; Flegel, 1997, 1998; Maeda et al., 1997; Lightner et al., 1997). Other vectors can enter shrimp ponds through pumped water, favoring management strategies that reduce water exchange rates or rely in closed cycles and recirculation.

## 1.4.6. Natural epidemics

Dead, infected shrimps are considered to be the source of subsequent infection for WSSV. This is mainly due to a higher opportunity of horizontal transmission of the virus through cannibalism and the waterborne route (Wu et al., 2001). Natural epidemics of this disease have been reported throughout the world, especially in Asia. In China, high mortalities in cultivated *F. chinensis* have been reported and in Korea, among the penaeid shrimp *P. orientalis*. Since 1994, WSSV has been detected in cultured *P. monodon* in peninsular Malaysia (Wang et al., 1999), while the Indian subcontinent has been affected by WSSV epizootics, with outbreaks occurring in PL (Manivannan et al., 2002) and cultured *P. monodon* (Selvin and Lipton, 2003). In Thailand, epidemics with WSSV show a seasonal fluctuation (Withyachumnarnkul et al., 2003). In the Americas, mortalities of cultured *L. vannamei* (Boone), induced by WSSV, have occurred in Ecuador since 1999 (Rodriguez et al., 2003). In Mexico, the first cases of this disease were also detected in 1999 (Galaviz-Silva et al., 2004), when WSSV caused severe damage to the shrimp industries of both Central and South America.

## 1.4.7. Diagnostic methods for WSSV

Several methods including microscopic observation under light, dark field, phase contrast microscope, bioassay, transmission electron microscopy, immunological, molecular and histopathological methods were developed to detect WSSV infection (Lightner and Redman, 1998).

***Histopathology***: In the early stage of WSSV infection in *P. vannamei*, Cowdry A type inclusions are present, which are characterized by marginated chromatin separated from nucleoplasm. At more advanced stages of infection, basophilic inclusion bodies and at a chronic stage of infection, pyknosis and karyorrhexis were observed (Lightner, 1996). This method is less sensitive and specific than molecular and immunological methods and requires highly trained personnel.

***Field methods*:** Diagnosis of white spot syndrome depends mainly on the demonstration of eosinophilic to basophilic inclusion bodies (IB) in stained fresh squashes or impression smears of ectodermal and mesodermal tissues. Feulgen-positive intranuclear IB may be identified in cuticular epithelial cells and connective tissue cells. The gills and epithelium under the carapace are excised, stained with haemotoxylin and eosin, mounted and then viewed as squash preparations (Flegel and Sriuriairatana, 1993). WSSV infection may be confirmed by the demonstration of rod-shaped, non-occluded virions in the intranuclear IB of affected cells using electron microscopy (Lightner, 1996). There are not many field tests available for the detection of WSSV. A reverse passive latex agglutination assay (RPLA) has been proposed as a useful method for virus detection in the culture shrimp pond (Okumura et al., 2004). Other cost-effective diagnostic assays using fluoresceinated microspheres and latex beads coated with anti-WSSV serum have proved to detect the virus as early as 24 h post infection in shrimps (Sathish et al., 2004).

***Serological methods*:** These methods are 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, immunohistochemistry (Escobedo-Bonilla et al., 2005, 2007), immunoblot assays (You et al., 2002), immunochromatographic test strips (Sithigorngul et al., 2006), enzyme linked immunosorbent assay (ELISA), and western blotting (Nadala et al., 1997; Yoganandhan et al., 2004).

Monoclonal antibodies (MAbs) have been produced against WSSV. A 28 kDa envelop protein of WSSV, encoded by the VP28 gene has been extensively used for the preparation of MAbs. These MAbs developed against WSSV are the basis of a number of detection methods. MAbs have also been used to develop an antigen-capture ELISA (Ac-ELISA) test, which

can differentiate WSSV-infected shrimp from uninfected shrimp. Antiserum developed against the VP28 is able to neutralize WSSV infections of *P. monodon* in a concentration- dependent manner on intramuscular injection. Furthermore, the antiserum can detect WSSV in various organs such as the eyestalks, head muscle, gill tissue, heart tissue, haemolymph, tail tissue and appendages in experimentally infected *P. monodon* and *F. indicus*, as early as 12 and 24 h post-infection (Yoganandhan et al., 2004).

***Molecular methods*:** These include PCR, in situ hybridization and dot blot hybridization.

***PCR*:** These methods are based on primers designed against a specific part of the genome sequence of WSSV. PCR methods include one step PCR (Lightner, 1996; Lo et al., 1996), semi nested PCR (Kiatpathomchai et al., 2001), two step PCR (Tapay et al., 1999; Hossain et al., 2004), quantitative competitive PCR (Tang and Lightner, 2000) and real time PCR (Durand and Lightner, 2002). PCR has been used in cultured and captured crustaceans (Vaseeharan et al., 2006) to estimate the prevalence of the virus in shrimp PL at the time of stocking in shrimp farms, and to identify reservoirs for the virus in shrimp ponds (Thakur et al., 2002). Furthermore, PCR can identify at least four geographic isolates of WSSV from both experimentally and naturally infected shrimp (Tapay et al., 1999). A new protocol called in situ PCR can detect light infections in tissues at early stage of infection (Jian et al., 2005). Another method named loop mediated isothermal amplification (LAMP) is claimed to be more sensitivity than other PCR protocols. It can detect up to 1 femtogram (fg) of virus (Kono et al., 2004).

***In situ hybridization*:** This method detects viral DNA in the host tissues through hybridization with a DNA probe (Wongteerasupaya et al., 1996). It is useful to detect infected cells in tissues, but it is less sensitive than PCR and requires histopathology facilities.

***Dot blot hybridization***: This procedure detects a fragment of viral DNA by hybridization with a DNA probe (Dupuy et al., 2004). In dot blot hybridization, a mixture containing the molecule to be detected is applied directly on a membrane as a dot, which is then followed by detection by either nucleotide probes or antibodies. This technique is also less sensitive than PCR.

## *1.5. Approaches for the control of diseases in shrimp aquaculture*

Diseases, as formulated by Snieszko (1973), result from disturbances of the equilibrium existing between the animals, the environment and the pathogens, naturally present or introduced. Thus, today, the aquaculture industry needs to be reconsidered; control of diseases and adapted health management appear to be a priority and must be looked into in a multidisciplinary approach. Ecological and environmental aspects, together with zootechnical and nutritional concepts, must be considered; their impact on the animals must be better understood and monitored. In short, much more basic knowledge on physiology and genetics of the cultured species is required.

### *1.5.1. Prevention, Treatments and Health management*

Prevention and control of diseases lie first in the detection of the pathogens, and important progress has been made with the development of reliable diagnostic methods based on specific molecular probe-detection techniques (Mialhe et al., 1995; Walker and Subasinghe, 2000). So far, an extensive range of chemicals such as disinfectants, vitamins and antibiotics has been intensively used to treat water as preventive and curative measures (Barg and Lavilla-Pitogo, 1996). Such practices are now questioned because regular use of antibiotics has led to the appearance of drug-resistant bacteria and can also result in environmental imbalances (Kautsky et al., 2000).

#### 1.5.1.1. Probiotics

Alternative prophylactic methods are now proposed for the control of disease in larviculture like the use of probiotic bacteria. This approach is currently investigated in aquaculture for the beneficial role of non-pathogenic bacteria to protect larvae and live food against infections (Gibson et al., 1998; Verschuere et al., 2000). Data suggest that probiotics might be useful in controlling microbial infections through competition with harmful microorganisms, production of inhibitory compounds or through the stimulation of the immune system of the animal (Antony and Philip, 2008).

#### 1.5.1.2. Immunostimulants

Another approach already put into practice by shrimp producers deals with the use of immunostimulants, in particular to fight against infectious diseases. Commercial products derived from β-glucans, polysaccharides, yeast or bacterial components have been shown to increase the immune reactions of the animals and therefore their capability to eliminate pathogens (Vici et al., 2000). However, further fundamental research is necessary to establish the real effect of such products, which remains empirical in the present knowledge. Moreover, for optimal usage of potential immunostimulants, it is necessary to know more about the immune capacities of shrimp at different stages of development.

#### 1.5.1.3. Vaccines

Vaccination is a term that is applied only when the purpose is to confer long lasting protection through immunological memory. It requires primary challenge with antigen and is dependent upon clonally derived lymphocyte sub-sets to be implemented. Vaccination protocols often include the use of adjuvants (e.g. killed mycobacterial cells, aluminum salts or mineral oil) to provide a 'depot' effect and enhance the antibody response. These usually act on one or more non-specific innate components of the immune system, such as cytokines and antigen presentation. In essence they

are acting as immunostimulants to maximize a particular specific response (Smith et al., 2003).

### *1.5.2. Control of WSSV*

WSSV is being controlled by the use of closed and semi-closed systems (Limsuwan, 1996) involving the pre-treatment of water with formalin or chlorine and storage of any water to be exchanged. It is likely that effectiveness of this treatment would depend upon the quantity of virus present. The elimination of fresh feed from the diet, the exclusion of potential carriers from shrimp culture ponds and PCR screening of PL prior to stocking are also recommended as control measures (Flegel et al., 1996).

There are no adequate treatments available against WSSV (Witteveldt et al., 2004a, 2004b), which, once introduced, spreads rapidly and uncontrollably (Yi et al, 2003); therefore, control strategies have focused on exclusion of the virus from culture ponds. Some of the strategies used for WSSV control are described below.

***Chemotherapy*****:** Studies that address the use of chemotherapy as control for WSSV are few. However, studies with STEL water, a disinfectant, show that when used continuously for the disinfection of seawater, it may be effective for preventing WSSV infection in shrimp (Park et al., 2004).

***Vaccines*****:** Although invertebrates lack a true adaptive immune response, it has been shown that kuruma shrimp, *M. japonicus*, exposed to WSSV become resistant to subsequent WSSV challenge (Wu and Muroga, 2004). Intramuscular immunization of the WSSV envelope proteins VP19 and VP28 in *P. monodon* resulted in an increased survival as compared with controls (Witteveldt et al., 2004b). Recombinant rVP28 induced a high resistance, suggesting the possibility of shrimp ‘vaccination’ with recombinant proteins against WSSV (Namikoshi et al., 2004). Oral vaccination with subunit vaccines consisting of WSSV envelope proteins VP19 and VP28 has been

tried in *P. monodon*, resulting in a significantly lower cumulative mortality (Witteveldt et al., 2004b).

***Other control methods*:** The effect of water temperature on the development of WSSV has been studied, and low temperatures affect the WSSV pathogenicity and inhibit mortality in crayfish and shrimp (Dupuy et al., 2004; Jiravanichpaisal et al., 2004). Hyperthermia also increases the survival of infected shrimp, possibly through the facilitation of apoptosis in WSSV-infected *L. vannamei* (Granja et al., 2003). Variations in salinity can cause changes in the immune response of *M. japonicus*, which were more susceptible to WSSV under salinity stress (Yu et al., 2003). Some antiviral genes from *P. monodon* can aid in shrimp viral disease control (Luo et al., 2003). The peptides derived from these genes, e.g. 2E6 specifically bind to WSSV and block virus infection, suggesting that these peptides have the potential to be exploited as an antiviral peptide drug (Yi et al., 2003).

Mytilin, a cysteine-rich cationic antimicrobial peptide (AMP) abundant in mussels haemocytes, interacts at the virus level, preventing replication of the viral DNA (Dupuy et al., 2004), which could be exploited as a control mechanism for the virus. Another product useful in the control of WSSV is β-1,3-glucan, whose addition to the shrimp feed effectively improves the immunity and survival of *P. monodon* (Chang et al., 2003). Oral administration of crude fucoidan extracted from *Sargassum polycystum* can reduce the impact of WSSV infection in *P. monodon* (Chotigeat et al., 2004).

Several products have been experimentally tested for the control of viral diseases on shrimp due to their potential to stimulate the invertebrate non-specific immune system. Among these products are *Bifidobacterium* sp., *Brevibacterium* sp. and *Bacillus* sp. peptidoglycans (PG), which have been successfully tested against WSSV, showing that shrimp previously exposed to PG exhibit a higher survival rate than non-exposed shrimp (Itami et al., 1998; Lee et al., 2004). Other elements such as sulphated polysaccharides, fucoidan or microalgae cell walls have been used as immunostimulants for

shrimp. These products have been somewhat successful against pathogens like WSSV (Chotigeat et al., 2004); however, their mechanisms of action are not well understood and further research with these products is necessary. Also, some products such as *Dunaliella* extract and probiotics show a positive effect on resistance of shrimp to WSSV infection (Itami et al., 1998; Supamattaya et al., 2005).

## *1.6. Crustacean immune system*

Shrimp use different mechanisms against undesired environmental (infectious) influences. During an infection, first the proPO system is induced which will lead to melanization and generation factors which are involved in immune reactions such as peroxinectin. Next, a cellular immune response that involves different types of haemocytes, which participates in pathogen clearance by phogocytosing microorganisms or trapping them in haemocyte aggregates or nodules, or by encapsulation of larger microorganisms and cytotoxic reactions are also triggered. Nodules and encapsulated materials are often observed to become melanized, through the action of phenoloxidase (PO). Finally during systemic infection, a wide range of inducible effector molecules, such as AMPs and factors required for opsonization are produced (Cerenius and Soderhall, 2004).

Microbial infection activates multiple cellular and humoral responses. For example septic injury rapidly triggers proteolytic cascades that lead to localized blood coagulation and melanization. The clotting system is an important reaction in both vertebrates and invertebrates to prevent blood loss through wounds. Clotting of haemolymph in crustaceans is considered as an important defense system (Soderhall et al., 1988; Smith and Chisholm, 1992; Soderhall and Cerenius, 1992) as it minimizes haemolymph loss on injury, seals wounds against microbial invasion and entraps any opportunistic micro-organism that may have gained access to the body (Kopacek et al., 1993; Yeh et al., 1998).

The first and essential internal defense process is the recognition of invading micro-organisms, which is mediated by the haemocytes and plasma proteins (Vargas-Albores and Yepiz-Plascencia, 2000). The invertebrate immune system presumably recognizes large groups of pathogens, represented by fixed common molecular patterns, rather than fine structures, specific for particular microbes (Soderhall et al., 1996). Several types of recognition proteins have been described and are called pattern recognition proteins (PRPs). The PRPs recognize carbohydrate moieties of cell wall components of micro-organisms, like lipopolysaccharides (LPS) or PG from bacteria, or β-1,3-glucans from fungi (Soderhall et al., 1996; Vargas-Albores et al., 1996, 1997). Some of the PRPs are lectins and can work directly as agglutinins or opsonins (Kopacek et al., 1993; Soderhall et al., 1996). After binding of the PRP ligand with the microbial component, a second site becomes active for cellular binding. Haemocyte activation is generated after this second binding step (Vargas-Albores and Yepiz-Plascencia, 2000). After detection of foreign material, haemocytes migrate to the site of invasion by a process of chemotaxis that results in inflammation. The open circulatory system demands a rapid and efficient defense, in which the proteoloytic cascades play an important role (Sritunyalucksana and Soderhall, 2000).

Upon activation and degranulation of the haemocytes, the inactive proPO is converted to the active PO by ppA. The PO enzyme catalyses the stepwise oxidation of phenols to quinones, followed by several intermediate steps that lead to the formation of melanin. During this formation also antimicrobial factors are formed (Soderhall et al., 1996; Soderhall and Cerenius, 1998). An important factor that is associated with the proPO system is peroxinectin (Sritunyalucksana et al., 2001). Peroxinectin has two different functions: cell-adhesion and peroxidase activity. Transmembrane receptors of the integrin family on the haemocytes play an important role in the cell adhesion function of peroxinectin (Johansson, 1999). The cell-

adhesion is involved in attachment, spreading, phagocytosis, encapsulation, nodule formation and agglutination (aggregation), while the antimicrobial properties of the peroxidase activity of the protein might help to kill invading micro-organisms (Johansson and Soderhall, 1988, 1989; Kobayashi et al., 1990; Thornqvist et al., 1994). After ingestion, shrimp haemocytes use cytotoxic oxygen radicals to kill the foreign material (Song and Hsieh, 1994; Munoz et al., 2000).

If large amounts of particles enter the body or if they are too large to be internalized, several haemocytes will cooperate to seal off the pathogens, these phenomena are called nodule formation and encapsulation, respectively (Soderhall et al., 1996). Enzyme inhibitors (Kazal and Serpin families), also produced by the haemocytes, are necessary to regulate the proteinase cascades and prevent over-activation and damage to the host tissue. Also α-2-macroglobulin, which serves as a broad spectrum protease-binding protein is stored in the haemocyte granules. In addition, haemocytes play an important role in the production and discharge of agglutinins (e.g. lectins) (Kopacek et al., 1993), of antibacterial peptides (Destoumieux et al., 1997, 2000a, 2000b) and of cytotoxic molecules such as lysosomal enzymes (lysozyme, esterases, phosphatases, phospholipases, peroxidases and proteases) (Millar and Ratcliffe, 1994). For an efficient immune defense, all different components of the immune system must work together.

Immune systems have developed to protect multicellular organisms from foreign substances. During evolution, two types of immune systems have developed to detect foreign substances, namely innate (natural) immunity and adaptive (acquired) immunity. The adaptive immune response of vertebrates has developed more sophisticated and complicated mechanisms including an immunological memory with generation of a large repertoire of antigen recognition receptors and innate immune systems such as phagocytosis, natural killer cells and complement system for both recognizing and eliminating foreign invaders (Hoffmann et al., 1996; Carroll,

1998). The innate immunity is an ancient form of protective mechanisms that appeared early in the evolution of multicellular organisms, whereas adaptive immunity is younger in origin (Kimbrell and Beutler, 2001). While adaptive immunity occurs only in vertebrates, invertebrates have a rapid and efficient innate system to recognize and destroy non-self material, including pathogens. The innate immune system is further divided into humoral and cellular defense responses (Fearon and Locksley, 1996; Fearon 1997; Medzhitov and Janeway, 1997).

Invertebrates have an open or semi-open circulatory system and aquatic invertebrates live in continuous contact with potential pathogens (Auffret and Oubella, 1997; Canesi et al., 2002). In the open circulatory systems (e.g. echinoderms), blood is called coelomic fluid and the blood cells are called coelomocytes. In the semi open circulatory systems (e.g. arthropods), the blood is called haemolymph and the blood cells, haemocytes. Although invertebrates lack the complexity of the adaptive immune system in which memory is the hallmark and rely solely on innate immunity their amazing diversity, abundance and evolutionary success argue for a highly efficient defense system against infections.

The external cuticle is a first line of defense to provide an effective physical and chemical barrier against the attachment and prevention of pathogens. The digestive tract, which is the main route of invasion, is partly lined with chitinous membranes and its hostile environment of acids and enzymes is able to inactivate and digest many viruses and bacteria. In most cases the cuticular defense are sufficient to protect against even quite virulent pathogens, which often only produce disease when the integument has been physically damaged. Once pathogens gain entry into the hemocoel of the host, they encounter a complex system of innate defense mechanisms involving cellular and humoral responses.

Humoral defenses include the production of AMPs, reactive intermediates of oxygen or nitrogen, and the complex enzymatic cascade that

regulate clotting or melanization of haemolymph. In contrast, a cellular immune response that involves different types of haemocytes, which participate in pathogen clearance by phagocytosing microorganisms or trapping them in haemocyte aggregates or nodules or by encapsulation of larger microorganisms and cytotoxic reactions are also triggered. In fact, there is an overlap between humoral and cellular defense, since many humoral factors affect haemocyte function and haemocytes are an important source of many humoral molecules (Elrod-Erickson et al., 2000; Lavine and Strand, 2002). An illustration of the innate immune reactions in crustaceans is shown in Fig. 1.4. The main components of the innate immunity of crustaceans are summarized below.

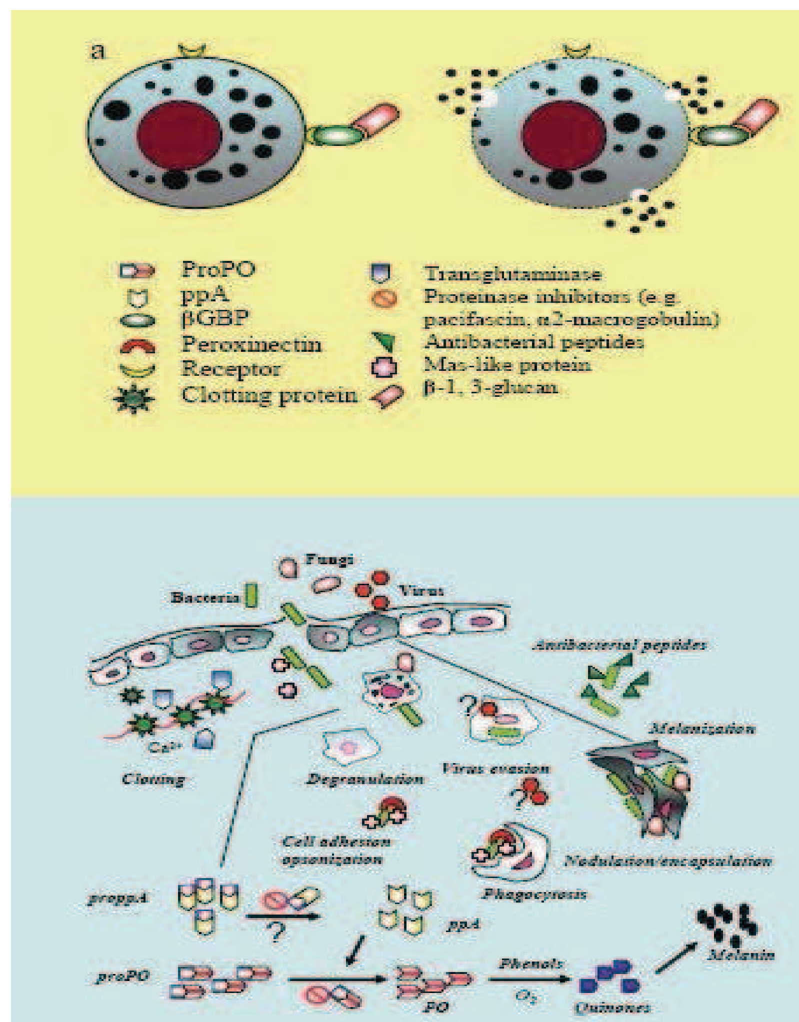



**Fig. 1.4. Schematic view of innate immune responses in crustaceans (Adapted from Jiravanichpaisal, 2005)**

### 1.6.1 Haemocytes

The haemocytes play an important and central role in the internal defense. In invertebrates, the most important role of the circulating haemocyte is the protection of the animal against invading microorganisms by participating in recognition, phagocytosis, melanization and cytotoxicity (Tzou et al., 2002; Cerenius and Soderhall, 2004). Three types of circulating haemocytes are morphologically recognized in crustaceans (Soderhall and Smith, 1983; Hose et al., 1987; Tsing et al., 1989; Johansson et al., 2000) but their actual relationship in terms of lineage remains an open question. In general, the hyaline cell is the smallest cell type with a high nucleus/cytoplasm ratio and no or few cytoplasmic granules (Soderhall and Smith, 1983). The granular cell (GC) is the largest cell type with a relatively smaller nucleus and fully packed with granules (Smith and Soderhall, 1983; Kobayashi et al., 1990). The semigranular cells (SGC), which contain small granules is an intermediate between the hyaline and the granular cell (Bauchau, 1981; Soderhall and Cerenius, 1992). The hyaline cells are considered as phagocytes (Soderhall et al., 1986). The SGC display some phagocytic capacities and are specialized in particle encapsulation (Persson et al., 1987). Whereas both the SG and the GC are found to degranulate spontaneously in vitro (Johansson and Soderhall, 1985) and both are found to participate in the proPO system which is an important component of the cellular defense reactions (Soderhall and Smith, 1983; Johansson et al., 1995).

### 1.6.2 Haematopoiesis

Haematopoiesis is the process by which haemocytes mature and subsequently enter the circulation. A proposed model for haemocyte production and maturation in *P. monodon* is shown in Fig. 1.5. This process involves proliferation, commitment and differentiation from undifferentiated hematopoietic cells (Medvinsky and Dzierzak, 1999; Barreda and Belosevic, 2001). Haematopoiesis provides a mechanism by which haemocytes that

have expired or are damaged can be replaced by newly synthesized cells (Medwinsky and Dzierzak, 1999; Barreda and Belosevic, 2001). Haemocytes are constantly produced in crustaceans, although the rate by which this process occurs can be altered rapidly under the influence of different micro-environmental factors. The hematopoietic tissue (hpt) has been detected and studied in several crustaceans, but the mechanisms behind the release of blood cells into circulation in most invertebrates are still unknown. Hpt is believed to contain precursor cells of the different haemocyte types.

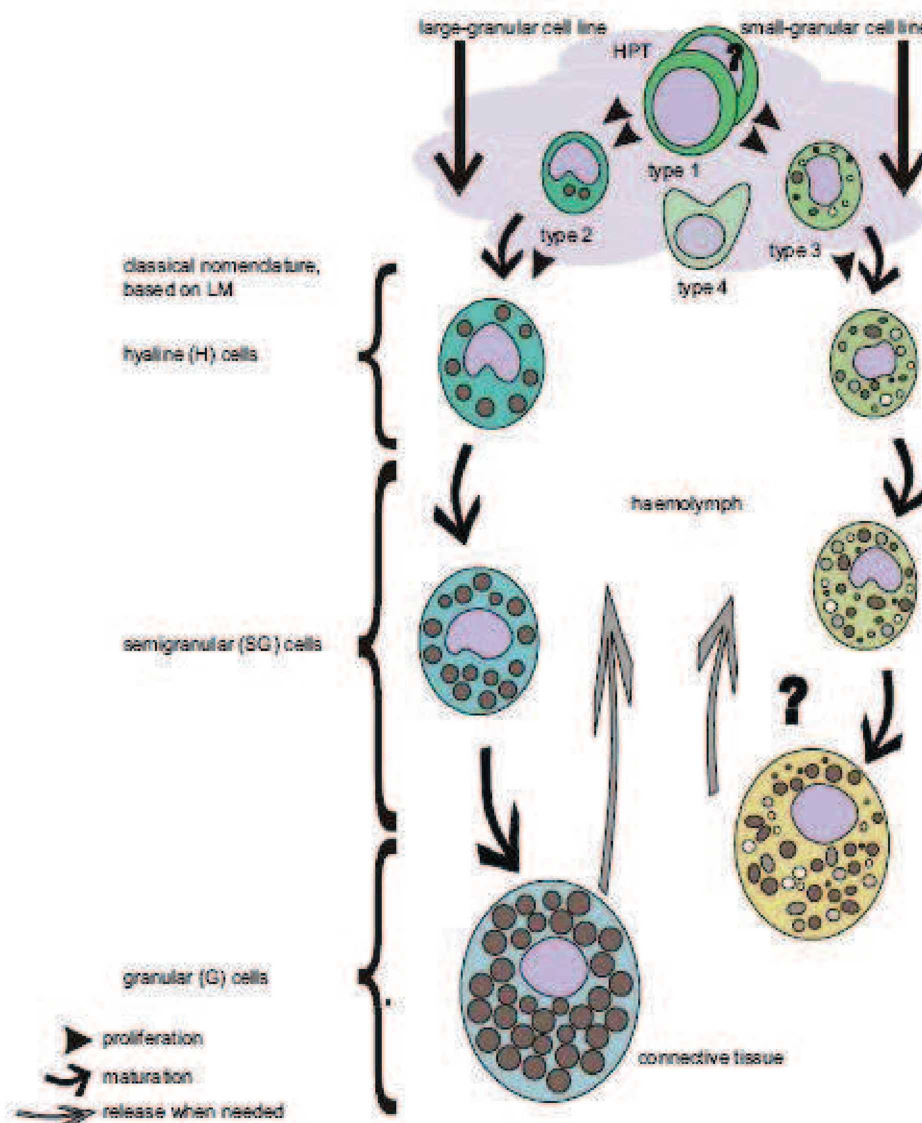



**Fig. 1.5. A proposed model for haemocyte production and maturation in *Penaeus monodon* (Adapted from van de Braak, 2002).**

***H = Hyaline cells; SG = Semigranular cells; G = Granular cells; HPT = Hepatopancreas***

In crustaceans, the sheet-like hpt is located dorsally on the stomach and is particularly abundant in the hollow between cardiac and pyloric stomach. The hpt comprises small lobules containing differentiating and maturing haemocytes, is surrounded by a thin sheath of collagenous connective tissue and is in close contact with the blood sinuses (Johansson et al., 2000). The hpt has different cell types based on their morphology. Hpt has at least five different types of cells which might correspond to developmental stages of GCs and SGCs. Type 1 was considered the least differentiated precursor cell while Types 2, 3 and 4 were speculated to be different stages of GCs development due to the presence of granules. Type 5, cells differ to Types 2–4 and was suggested to be a precursor of SGCs. It is believed that the haemocytes are mature when they are released to the circulation (Chaga et al., 1995).

## *1.6.3 Cellular immune response*

The main defense responses involving haemocytes against foreign invaders are phagocytosis, nodulation and encapsulation.

### *1.6.3.1 Phagocytosis*

Phagocytosis is one of the earlier and complex immune responses. It refers to the engulfment of entities by an individual cell and is based on recognition, engulfment, and intracellular destruction of invading microorganisms. Haemocytes phagocytose various biotic targets such as bacteria, yeast, and apoptotic cells or abiotic targets like synthetic beads or India ink particles (Yokoo et al., 1995). Phagocytosis is initiated by recognition and binding of a target particle to the phagocytic cell, followed by uptake through cytoskeleton modification and intracellular vesicular transport to phagosomes where the engulfed target is destroyed (Ramet et al., 2002; Pearson et al., 2003). Phagocytosis is critical for host defense against invading pathogens and for the removing of apoptotic cells produced during development (Aderem and Underhill, 1999).

### 1.6.3.2 Nodulation

Nodulation is a cellular immune response that is restricted to invertebrates in defense against foreign bodies too large for phagocytosis by individual haemocytes. Nodule formation is usually followed by encapsulation against a massive bacteria infection like vibrios. Peroxinectin was shown to be involved in encapsulation and nodule formation processes (Johansson et al., 1995; Lee and Soderhall, 2001). In most crustaceans melanization participates in nodule and capsule formation.

### 1.6.3.3 Encapsulation

Encapsulation refers to the binding of haemocytes to larger targets that are too large to be phagocytosed by individual haemocytes. Unlike phagocytosis, the formation of nodulation and encapsulation results in a mutilayered, overlapping sheath, of haemocytes around the invader. A process that is ultimately accompanied by blackening of the capsule due to melanization, and finally the parasite is killed within the encapsulated capsules. Several factors including asphyxiation, the local production of cytotoxic quinones or semiquinones via the proPO activation cascade, free radicals, and antibacterial peptides have been suggested to function as killing agents (Gillespie et al., 1997). At the ultrastructural level the formation of nodulation and encapsulation looks mostly identical, suggesting that they are essentially the same process, although against different targets (Ratcliffe and Gagen, 1976).

## 1.6.4 Humoral Immune Responses

### 1.6.4.1 The coagulation system

Invertebrates have efficient mechanisms that quickly prevent blood loss and also help in the trapping of microbes from entering and spreading throughout the haemocoel. Haemolymph clotting is an important part of the innate immune system which overlaps the humoral/cellular boundary and involves a combination of soluble and cell-derived factors (Johansson, 1999;

Theopold et al., 2002). Clotting is important in reducing haemolymph loss and for initiating wound healing in crustaceans. It is also a critical immune defense to form a secondary barrier to infection, immobilized bacteria/fungi and hence promote their killing.

In shrimps, coagulation is generally very strong and rapid. The clotting system represents an important component in host defense. The activation of the clotting cascade in response to LPS or β-l,3-glucans results in the formation of a clot which entraps invading microorganisms. In crustaceans, clotting occurs through the polymerization of the clotting protein catalyzed by a $Ca^{2+}$ ion dependent transglutaminase (TGase), which is released from haemocytes upon the stimulation by foreign organisms or tissue damage (Hall et al., 1999; Wang et al., 2001). This process is also linked, in crustaceans, to the triggering of the proPO activating system (Soderhall and Cerenius, 1992) and, in the horseshoe crab, to the release of antimicrobial substances from the amoebocytes (Iwanaga, 1993).

#### *1.6.4.2 The prophenoloxidase activating system (proPO system)*

The proPO system is an efficient part of the innate immune response and consists of a complex cascade of several proteins that are involved in melanization, cytotoxic reactions, cell adhesion encapsulation, and phagocytosis (Cannon et al., 2004). The proPO system is triggered by the presence of minute amounts of microbial components, such as LPS and PG from bacteria and β-1, 3 glucans from fungi (Soderhall and Cerenius, 1998; Sritunyalucksana et al., 1999, 2001; Sritunyalucksana and Soderhall, 2000). This ensures that the system will become active in the presence of potential pathogens. This system is composed of PRPs including lipopolysaccharide and β-1,3 glucan binding protein (LGBP), β-1,3 glucan binding protein (BGBP), and peptidoglycan binding protein (PGBP), several serine protease and their zymogens, proPO as well as proteinase inhibitors, which are important regulatory factors to avoid activation of the system where it is not

appropriate (Cerenius and Soderhall, 2004). The enzyme involved in melanin formation is phenoloxidase. Crustacean proPO is synthesized in the blood cells (Aspan et al., 1995). This enzyme catalysis the oxidation of phenols to quinones, which then will polymerize non-enzymatically to melanin (Soderhall et al., 1996). ProPO is activated by a prophenoloxidase-activating enzyme (ppA), which is a serine protease previously activated in turn by microbial cell walls. Two protease inhibitors, α-2-macroglobulin and a trypsin inhibitor, can block ppA. About fourteen proteins of the proPO activating system and associated factors in both insects and crustaceans have been purified and characterized (Soderhall and Cerenius, 1998).

### 1.6.4.3 Cytokines

Cytokines and chemokines are secreted proteins with growth, differentiation, and activation functions that regulate the nature of immune responses. They interact with stem and progenitor cells through specific receptors and regulate haematopoiesis (Beschin et al., 2001). In invertebrates, cytokine-like molecules having lectin-like activities have been found (Raftos, 1996). In crayfish, *P. leniusculus*, a PK protein, Astakine, was characterized as a cytokine (Soderhall et al., 2005). Astakine induces a strong haematopoiesis in in-vitro hpt cell cutures and in live animals.

### 1.6.4.4 Prokineticin

The PKs are small (80-90 aa), cysteine rich, and secreted proteins that regulate diverse biological processes.

### 1.6.4.5 Pattern recognition proteins (PRPs)

Recognition of microbial components, which are distinct from those of host cells, is the first step to trigger the innate immunity. PRPs are the triggering molecules of the proPO system, since they bind microbial components and then induce activation of proteinases in the proPO system. For instance, lectin-like proteins can bind to carbohydrates present on microbial cell walls followed by activation of immune response to

kill/remove the invading microbes (Medzhitov and Janeway, 1997). Current studies have emphasized the important roles of non-self recognition molecules both in the vertebrate and the invertebrate immune systems (Lee and Soderhall, 2002).

### *1.6.4.6 Masquerade-like protein*

The masquerade-like (mas-like) protein was identified as a PRP in haemocytes. The crustacean mas-like protein can recognize microbes and following recognition of and binding to bacteria or yeast cell walls, the mas-like protein is processed by a proteolytic enzyme (Lee and Soderhall, 2001). It has binding activity to LPS, β-1,3-glucans, gram-negative bacteria, and yeast. Besides, it has an opsonic and cell adhesive activity (Huang et al., 2000; Lee and Soderhall, 2001) and is a multifunctional protein. It exists usually in an intact form and is processed by an unknown proteolytic enzyme when binding to microbes.

### *1.6.4.7 Toll-like receptors*

Besides, Toll/Toll-like receptors are an evolutionary ancient family of pattern recognition receptors playing important roles in innate immune responses. Most of these studies were carried out in insects (Leulier and Lemaitre, 2008). A novel Toll receptor gene was identified from *P. monodon* (Arts et al., 2007) and Chinese shrimp *F. chinensis* (Yang et al., 2008). However, the mechanism for how this Toll homolog is regulated by microbial challenge is still unknown.

### *1.6.4.8 Agglutinin and Lectin*

Lectins/agglutinins are glycoproteins usually without catalytic activity that have the ability to bind to specific carbohydrates and they are present in almost all living organisms. Various biological activities such as the cellular and tissue transport of carbohydrates and glycoproteins (Vasta, 1992), cell adhesion (Vasta et al., 1999), opsonization (Cerenius et al., 1994) and nodule formation (Koizumi et al., 1999) involve the interaction between

lectins and carbohydrates. Some lectins such as BGBP and LGBP have been suggested to enhance activation of the proPO-system in invertebrates (Cerenius et al., 1994). BGBP also interacts with an extracellular superoxide dismutase (SOD) involved in binding with peroxinectin (a cell adhesive and opsonic peroxidase) to the cell surface. Several agglutinins or lectins have been detected and characterised in different crustaceans (Soderhall and Cerenius, 1992; Smith and Chisholm, 1992). However, all the physiologic roles of lectins in invertebrates remain unclear but it appears that these molecules are involved in recognition and defense mechanisms against pathogens and may act as opsonins (Vasta, 1990).

### *1.6.4.9 Proteinase inhibitors*

Many serine proteinase inhibitors have been characterized both in vertebrates and invertebrates. Most of them fall into well-established families of proteinase inhibitors such as Kasal, Kunitz, serpins, α-macroglobulins and metalloproteinase inhibitors (Kanost, 1999). All known serine proteinase inhibitors in arthropods can be grouped in three mechanistically different groups: canonical inhibitors (standard mechanism), serpin family and α-macroglobulin. They are involved in a variety of fundamental physiological processes, such as blood clotting, innate immune response and embryogenesis (vertebrates), moulting, metamorphosis and proPO cascades (invertebrates) mainly to prevent or regulate proteolysis (Simonet et al., 2002).

### *1.6.4.10 Alpha-2-Macroglobulin-Like ( -2-M) Protein*

Shrimp α-2-M protein has been shown to be localized in the membrane of all haemocyte types and in large vesicular inclusions of the hyaline and semi-granular cells and possess a key role in mechanisms for regulation of defense and inflammatory processes (Armstrong et al., 1994). Although a major and ubiquitous component of blood, the functions of the α-2-Ms remain largely unresolved. They are known as proteinase inhibitors,

forming complexes with a wide variety of endoproteinases via an internal thioester (van Leuven, 1982).

### *1.6.4.11 Reactive oxygen compounds*

Many oxygen compounds are toxic to living cells. In order to protect itself from toxic oxygen compounds formed in the oxygen metabolism, every cell must contain systems that deal with the undesired and potentially dangerous products formed. The most important reactive oxygen species (ROS) can be said to be the superoxide ion radical hydrogen peroxide and the hydroxyl radical OH. The superoxide anion can easily form in reactions where molecular oxygen is present, and is scavenged by the enzyme superoxide dismutase (SOD) to form hydrogen peroxide and $O_2$. Hydrogen peroxide is scavenged by catalase to form water and oxygen and by peroxidase in the presence of a reducing agent. The most reactive oxygen species is the hydroxyl radical, OH. The reactive properties of oxygen compounds are not simply harmful; if directed at the right target, they can also be used in the destruction of foreign material. It has long been accepted that a so-called oxidative burst with raised oxygen consumption occurs in reaction towards foreign material, in human as well as in plants (Wojtaszek, 1997). The membrane-bound enzyme complex, NADPH oxidase, will assemble after binding of the cell to foreign material and reduce molecular oxygen to superoxide (Chanock et al., 1994).

### *1.6.4.12 Superoxide dismutases*

Superoxide dismutases have been described of three major groups, depending on their metal ion content. There are MnSOD found in mitochondria, FeSOD found in bacteria, and CuZnSOD found in eukaryotes.

### *1.6.4.13 Haemocyte adhesion molecules*

Cell adhesion, essential for the function of multicellular organisms, is not only involved in many physiological processes such as development,

wound healing and haemostasis, but also in pathological conditions, for examples metastasis of cancer cells and inflammatory disease (Hynes, 1992).

### *1.6.4.14 Peroxinectin*

Peroxinectin is a multifunctional molecule, in addition to attachment and spreading, it also contains different biological functions shown in vitro, as an inducer of degranulation (Johansson and Soderhall, 1989), encapsulation enhancer (Kobayashi et al., 1990), an opsonin (Thornqvist et al., 1994) as well as exhibiting peroxidase activity (Johansson et al., 1995). In crustaceans, cell adhesion protein, peroxinectin, appears to bind the surface of the blood cells. Peroxinectin is released by exocytosis from the haemocytes. It has two different functions, one is as a peroxidase and the other is a cell-adhesion function. The likely production of antimicrobial substances by the peroxidase activity of the protein might help to kill invading microorganisms, while the cell-adhesion activity can lead to phagocytosis, degranulation, and capsule formation.

### *1.6.4.15 Integrins*

Integrins are a family of integral membrane protein dimers that serve as receptors for extracellular matrix and act in cell–cell adhesion and as receptors in transmembrane signaling (Hynes, 1992). Peroxinectin, as well as BGBP, contains putative integrin binding sites.

### *1.6.4.16 Lysozyme*

Lysozyme is a widely distributed enzyme, located in many tissues of invertebrates and vertebrates. It catalyzes the hydrolysis of bacterial cell walls and acts in non-specific innate immunity against the invasion of bacterial pathogens (Jolles and Jolles, 1984).

# 1.7 Antimicrobial Peptides (AMPs)

## 1.7.1. Background of the AMPs

Antimicrobial peptides (AMPs) are small, basic, single gene-encoded peptides that are generally synthesized as preproproteins and are activated as part of the host defense systems in plants, insects, fish, amphibia, birds, and mammals. AMPs are conventionally defined as polypeptide antimicrobial substances, encoded by genes and synthesized by ribosomes, with fewer than 100 amino acid residues. AMPs are evolutional weapons, which are widely used by animals and plants in their innate immune systems to fend off invading microbes (Boman et al., 2003; Martin et al., 1995; Ganz 1999; Zasloff, 2002) thereby representing an ancient host defence mechanism (Maxwell et al., 2003). AMPs are typically relatively short (12 to 100 amino acids), positively charged (net charge of +2 to +9), amphiphilic peptides present in single-celled microorganisms, insects and other invertebrates, plants, amphibians, birds, fish, and mammals, including humans (Martin et al., 1995; Bachere, 2003; Wang and Wang, 2004). Some AMPs are produced constitutively whilst others are synthesized in response to microbial attack (Gallo et al., 2002) at rates, which are up to one hundred fold faster than those used for protein synthesis by the adaptive immune system (Boman, 2003). This ready availability of AMPs form a crucial component of innate immune systems making it a highly effective first line of defense in animals (Ganz, 2003) and there is evidence that some AMPs may play wider roles by also acting as immunomodulatory signals and attenuating antimicrobial responses of the adaptive immune system (Yang et al., 2002). Hundreds of AMPs have been isolated so far, and irrespective of their origin, spectrum of activity and structure, most of these peptides share several common properties. The expression of these AMPs can be constitutive or can be inducible by infectious and/or inflammatory stimuli, such as proinflammatory cytokines, bacteria, or bacterial molecules that

induce innate immunity, e.g. LPS (Hancock, 2001; Cunliffe and Mahida, 2004). AMPs exhibit rapid killing, often within minutes *in vitro*, and a broad spectrum of activity against various targets, including gram-positive and gram-negative bacteria, fungi, parasites, enveloped viruses, and tumor cells (Baker et al., 1993; Hancock and Scott, 2000; Fernandez-Lopez et al., 2001; Zasloff, 2002). In addition, their antimicrobial activity can be enhanced by the synergy between individual cationic peptides within a host, and between the peptides and other host factors, such as lysozyme (Hancock and Scott, 2000).

#### ***1.7.1.1 Resistance to chemical antibiotics: an unsolved and growing problem***

It is widely accepted among clinicians, medical researchers, microbiologists and pharmacologists, that antibiotic resistance will, in the very near future, leave healthcare professionals without effective therapies for bacterial infections. Consequently, the priority for the next decades should be focused in the development of alternative drugs and/or the recovery of natural molecules that would allow the consistent and proper control of pathogen-caused diseases. Ideally, these molecules should be as natural as possible, with a wide range of action over several pathogens, easy to produce, and not prone to induce resistance.

The new generation of native peptide molecules, also known as Anti Microbial Peptides (AMPs) seem to fit this description. As a consequence, they have been termed "natural antibiotics", because they are active against a large spectrum of microorganisms, including bacteria and filamentous fungi, in addition to protozoan and metazoan parasites (Vizioli and Salzet, 2003). All of these molecules are key elements directly implicated in the innate immune response of their hosts, which includes the expression of fluid phase proteins that recognize pathogen-associated molecular patterns, instead of specific features of a given agent to promote their destruction. As a result,

the response is very fast, highly efficient and applicable to a wide range of infective organisms (Hoffmann and Reichhart, 2002).

### *1.7.2 Terminology*

The terminology applied to these antimicrobial substances varies in the scientific literature. Descriptive terms that have been used include “defense peptides,” reflecting their teleological or functional role in defense against microbial invasion; “lytic peptides” or “pore-forming proteins,” reflecting their probable action as membrane-permeabilizing agents; “cationic peptides,” reflecting their electrochemical structure; and “AMPs,” a more generic term describing their functional capabilities.

### *1.7.3 The nature of AMPs*

The endogenous AMPs of plants and animals are typically cationic (i.e. contain excess lysine and arginine residues) amphipathic molecules composed of 12-45 amino acid residues (Hancock and Hans-George, 2006). Hence the term ‘cationic’ AMPs is used to describe these molecules, with at least two excess positive changes due to lysine and arginine residues and around 50% hydrophobic amino acids. More than 500 such peptides have been discovered. They fit into at least four structural classes, namely β-sheet, comprising two to three β-strands stabilized by disulphide bridges, amphipathic α-helices, extended structures, and loop structures. Despite these different folding patterns, there appear to be two types of three-dimensional configurations, an amphipathic structure with opposing hydrophobic and polar cationic faces and a cationic double-wing structure with two regions of positive charge bracketing a hydrophobic core. These peptides are termed antimicrobial because they have unusually broad spectra of activity. These can include an ability to kill or neutralize Gram-negative and Gram-positive bacteria, fungi (including yeasts), parasites (including planaria and nematodes), cancer cells, and even enveloped viruses like HIV and herpes simplex virus. Nevertheless, many peptides are

quite selective for microbes over eukaryotic cells. Not all peptides have all of the above activities. However, a single 13-aa peptide, indolicidin, for example, is able to kill bacteria, fungi, and HIV.

## *1.7.4 Distribution of AMPs*

AMPs are produced ubiquitously throughout nature. AMPs are a universal feature of the defense systems of virtually all forms of life, with representatives found in organisms ranging from bacteria to plants and invertebrate and vertebrate species, including mammals. They form part of the ancient, nonspecific innate immune system, which is the principal defense system for the majority of living organisms. In many cases, their primary role is in the killing of invading pathogenic organisms, however, it is increasingly recognized that they may also function as modulators of the innate immune response in higher organisms (Scott and Hancock, 2000; Yang et al., 2004; Zanetti, 2004; Bowdish et al., 2005a, 2005b). In multicellular animals, they may be expressed systemically (for example, in insect haemolymph or vertebrate immune cells) and/or localized to specific cell or tissue types in the body most susceptible to infection, such as mucosal epithelia and the skin.

AMPs tend to be found in those parts of animals that are most likely to come into contact with pathogens from the environment. Thus, they are found on the skin, ear, and eye, on epithelial surfaces, including the tongue, trachea, lungs, and gut, and in the bone marrow and testes. They are also the most prevalent protein species of neutrophils, being associated with azurophilic granules and comprising a major non-oxidative killing mechanism of these dedicated antimicrobial phagocytes (Hancock, 2006).

### *1.7.4.1 Prokaryotes*

Bacterial AMPs, also called bacteriocins, produced by bacteria were among the first to be isolated and characterized and are generally extremely potent compared with most of their eukaryotic counterparts (Mattick and

Hirsch, 1947). While they do not protect against infection in the classical sense, they contribute to survival of individual bacterial cells by killing other bacteria that might compete for nutrients in the same environment. Their activities may be either narrow or broad spectrum, capable of targeting bacteria within the same species or from different genera. The bacteriocins constitute a structurally diverse group of peptides, and it was recently proposed that they be classified into two broad categories: lanthionine containing (lantibiotics) and non-lanthionine containing (Cotter et al., 2005). The most extensively studied lantibiotic is nisin, produced by *Lactococcus lactis*, which has been commonly used for nearly 50 years as a food preservative without significant development of resistance. Mersacidin, a tetracyclic peptide that is produced by *Bacillus* spp. (Chatterjee et al., 1992a, 1992b), displays bactericidal activity against methicillin-resistant *S. aureus* that is comparable to that of vancomycin, but without the development of cross-resistance (Kruszewska et al., 2004).

### *1.7.4.2 Plants*

In plants, it is widely believed that AMPs play an important and fundamental role in defense against infection by bacteria and fungi. So far, only peptides with a β-sheet globular structure have been identified in plants, with the two major and best-studied groups being thionins and defensins (Garcia-Olmedo et al., 1998). Physiologically relevant concentrations of thionins are active against bacteria and fungi in vitro, and studies utilizing transgenic plants have shown that heterologous expression of thionins can confer protection against bacterial challenge (Chang et al., 2005; Epple et al., 1997). Plant defensins display antibacterial and antifungal activities in vitro (Terras et al., 1992). Consistent with a defensive role, they are found in leaves, flowers, seeds, and tubers.

### 1.7.4.3 *Invertebrates*

Since invertebrates lack the adaptive immune system found in vertebrate species, they are reliant solely upon their innate immune systems to counteract invading pathogens. Considering the extraordinary evolutionary success of this group of organisms, it is evident that invertebrate innate immune mechanisms are extremely effective. Numerous AMPs have now been identified in invertebrates, and they are recognized as playing a key role in protection from pathogenic organisms. AMPs are found in the haemolymph (plasma and haemocytes), in phagocytic cells, and in certain epithelial cells of invertebrates. They can be expressed constitutively, for example, in the haemocytes of marine arthropods such as shrimp, oyster, and horseshoe crab (Bachere et al., 2004; Iwanaga and Kawabata, 1998), or induced in response to pathogen recognition, such as antifungal peptides in *Drosophila* (Lemaitre et al., 1996). Among some of the prototypic invertebrate AMPs are the α-helical cecropins (fly haemolymph) and melittin (bee venom) as well as the β-hairpin-like peptides tachyplesin and polyphemusin (horseshoe crab). The horseshoe crab-derived peptides possess some of the most potent antibacterial and antifungal activities observed. Interestingly, polyphemusin also displays activity against human immunodeficiency virus (HIV) (Masuda et al., 1992). However, the most abundant group of AMPs in invertebrates is the defensins, which are open-ended cyclic peptides with three or four disulfide bridges. The activities of invertebrate defensins can be divided according to whether their principal biological activity is directed toward bacteria or fungi (Bulet et al., 2004).

### 1.7.4.4 *Vertebrates*

AMPs have been isolated from a wide range of vertebrate species, including fish, amphibians, and mammals, indicating that, even in the presence of an adaptive immune response, these peptides have an important role in host defense. Direct microbicidal activity is associated with vertebrate

AMPs to various degrees under physiological conditions, and these activities likely contribute to the first line of defense, especially where they are found in very high concentrations, such as in the granules of phagocytic cells or the crypts of the small intestine (Scott and Hancock, 2000; Yang et al., 2002, 2004; Bowdish et al., 2005a, 2005b). However, it is increasingly recognized that in addition to direct microbicidal activity, small cationic peptides perform critical immunomodulatory functions and may be involved in the control of inflammation, which serves to recruit a variety of other microbicidal mechanisms (Yang et al., 2002, 2004; Bowdish et al., 2005a). Consistent with their role in direct and indirect antimicrobial defenses, AMPs in vertebrates are found at sites that routinely encounter pathogens, such as mucosal surfaces and the skin, as well as within the granules of immune cells (Yang et al., 2002, 2004; Bowdish, et al., 2005a).

Amphibian skin glands have proven to be a rich source of AMPs, with approximately 500 having been described to date as originating from this source (Rinaldi, 2002; http://www.bbcm.univ.trieste.it/_tossi/pag1.html). The α-helical magainins (Zasloff, 1987) are the prototypic amphibian AMPs, with strong membrane-permeabilizing activity towards gram-positive and gram-negative bacteria, fungi, yeasts, and viruses. Structure-function relationships and the mechanism of action of magainin have been extensively studied, and these peptides have subsequently served as the template for development of the first clinical antibacterial peptide treatment (Lamb and Wiseman, 1998; Ge et al., 1999). The broad antibacterial and antifungal activities of dermaseptins, isolated from the skin of South American frogs, have also been widely studied. In addition to their presence in the skin, amphibian AMPs are produced in the mucosa of the stomach, indicating a role in protection from ingested pathogens. The best-characterized examples are the Asian toad peptides buforin and buforin II, which are generated by cleavage of the nucleosome protein histone 2A. A

number of excellent reviews have covered this large group of AMPs (Simmaco et al., 1998; Rinaldi, 2002; Bulet et al., 2004).

Cathelicidins are a large and diverse group of vertebrate AMPs. Most cathelicidins are stored in an inactive propeptide state, mostly within granules of circulating immune cells. Neutrophil secretory granules are the predominant source of cathelicidins, but they may also be expressed in mucosal surfaces in the mouth, lung, and genitourinary tract and in skin keratinocytes in inflammatory disorders (Frohm et al., 1997). Cathelicidins have been isolated from many mammalian species, such as mice, rabbits, sheep, horses, and humans.

One of the best characterized bovine AMPs is BMAP-28, an α-helical peptide which rapidly permeabilizes the membranes of a broad spectrum of bacteria and fungi at moderate concentrations in vitro (Skerlavaj et al., 1996). In contrast, only one cathelicidin is expressed in humans: LL-37 (hCAP18). In addition to direct microbicidal activity, LL-37 has important additional roles in host defense, including chemotactic properties and modulation of inflammatory responses (Zanetti, 2004; Bowdish et al., 2005b). A second prominent group of mammalian AMPs is the defensins (Ganz, 2003) cyclic peptides which are categorized into three subfamilies on the basis of the disulfide pairings between their six conserved cysteine residues or their macrocyclic nature. As with cathelicidins, vertebrate defensins are synthesized as prepeptides which require proteolytic processing to their active peptide forms (Tang et al., 1999). Depending on the species, defensins are found in the granules of neutrophils, macrophages, NK cells, intestinal Paneth cells and epithelial tissues, the skin, certain mucosal surfaces such as the respiratory passage and urinogenital tract, and many bodily fluids (Fang et al., 2003).

## 1.7.5 Biological activity

The AMPs display a broad spectrum of activity. Many AMPs not only kill bacteria, they are cytotoxic also to fungi (Fehlbaum et al., 1996; Kieffer et al., 2003), protozoa (Arrighi et al., 2002), malignant cells (Cruciani et al., 1991; Baker et al., 1993; Lindholm et al., 2002), and even enveloped viruses like HIV, herpes simplex virus, and vesicular stomatitis virus (Tamamura et al., 1998). AMPs exhibit selectivity against different microorganisms, of which the molecular basis is not completely understood.

On the one hand, many AMPs display broad-spectrum activity against gram-negative bacteria, gram-positive bacteria, and fungi (Miyasaki and Lehrer, 1998). On the other hand, some peptides, e.g. andropin (Samakovlis et al., 1991) and insect defensins (Meister et al., 1997) preferentially kill gram-positive bacteria, while others preferentially kill gram-negative bacteria, e.g. apidaecin (Casteels and Tempst, 1994), drosocin (Bulet et al., 1996), and cecropin P1 (Boman et al., 1991). Peptides that preferentially kill fungi have also been described, e.g. drosomycin (Meister et al., 1997) and plant AMPs (Tailor et al., 1997). Normal human cells are relatively resistant, but it should be mentioned that certain cationic AMPs, such as melittin from bees (Perez-Paya et al., 1994), mastoparan from wasps (Delatorre et al., 2001), charybdotoxin from scorpions (Tenenholz et al., 2000) and temporin L from frogs (Rinaldi et al., 2002) are potent toxins.

The AMPs, magainins and their analogs have been found to be able to lyse haematopoietic tumor and solid tumor cells with little toxic effect on normal blood lymphocytes (Cruciani et al., 1991; Baker et al., 1993). The mechanism of antitumor activity of magainins was suggested to target cell membrane by a non-receptor pathway (Baker et al., 1993). Tachyplesin, an AMP present in leukocytes of the horse crab, *Tachypleus tridentatus* (Hirakura et al., 2002) showed that it inhibited the proliferation of both cultured tumor and endothelia cells by disrupting their membranes and inducing apoptosis.

In contrast, temporin L, a 13 amino acid long peptide isolated from the skin of the european red frog, *Rana temporaria,* induced necrosis of tumor cells (Rinaldi et al., 2001, 2002). Cyclotides, members of macrocyclic cystine-knotted peptides exhibit antimicrobial, anticancer, and antiviral activities (Tam et al., 1999; Lindholm et al., 2002). Other AMPs, such as defensins (Kagan et al., 1990), cecropin (Moore et al., 1994), lactoferricin (Vogel et al., 2002) and lactoferrin possess antitumor activity.

Several cationic amphipathic peptides also display antiviral activity in vitro. Defensins are able to neutralize herpes simplex virus (HSV), vesicular stomatitis virus and influenza virus (Daher et al., 1986; Ganz and Lehrer, 1995). Tachyplesins and polyphemusins are active against vesicular stomatitis virus, influenza A virus and HIV (Tamamura et al., 1996). Melittins (Wachinger et al., 1998), cecropins (Wachinger et al., 1998) and indolicidin also display anti-HIV activity. Melittin and cecropins have been found to inhibit the replication of HIV-1 by suppression of the long terminal repeat gene (Wachinger et al., 1998).

### 1.7.6 Diversity of AMPs

More than 1000 AMPs have been discovered so far from animals as well as plants (http://www.bbcm.univ.trieste.it/~tossi/pag1.htm). The diversity of sequences is such that the same peptide sequence is rarely recovered from two different species of animals, even those closely related. However, both within the AMPs from a single species, and even between certain classes of different peptides from diverse species, significant conservation of amino-acid sequences can be recognized in the preproregion of the precursor molecule. The design suggests that constrains exist on the sequences involved in the translation, secretion or intracellular trafficking of this class of membrane-disruptive peptides (Zanetti et al., 2000).

The diversity of AMPs probably reflects the species' adaption to the unique microbial environments that characterize the niche occupied,

including the microbes associated with acceptable food sources (Simmaco et al., 1998; Boman, 2000). It appears reasonable to speculate that an individual could find itself in the midst of microbes against which the peptides of its species were ineffective, although the individual might suffer, the species itself could survive through emergence of individuals expressing beneficial mutations.

A common property of AMPs is their tendency to fold into amphipathic structures. The diversity of the peptide primary structures is due to the necessity of the host immunity to successfully adapt to different environments by retaining its efficacy against specific microbial pathogens. Hence, the conservation of antimicrobial functions is highly dependent on the amphipathic properties of AMPs and not necessarily on the retention of primary sequence homology. Yet amphipathicity is highly influenced by the amino acid composition and arrangement in the primary sequence, which affects the specificity of antimicrobial activity in a given environment (Bals et al., 1998a, 1998b)

One of the surprising features of AMPs is the conservation of antimicrobial functions despite their structural diversity. As illustrated in Fig. 1.6, there is a signal sequence that serves to guide the protein to secretory vesicles. The active form of the peptide is released after protease-specific digestion (Harwig et al., 1992; Valore and Ganz, 1992; Pestonjamasp et al., 2001; Shinnar et al., 2003; Murakami et al., 2004). The propeptide is sometimes used as a basis for classification.

**Fig. 1.6 The Gene structure of a typical Antimicrobial Peptide (Adapted from Zaiou and Gallo, 2002)**

### 1.7.6.1 AMPs in marine invertebrates

The ocean covers 71% of the surface of the earth and contains approximately half of the total global biodiversity, with estimates ranging between 3 and 500 x $10^6$ different species (Haug et al., 2002). Marine macrofauna alone comprise 0.5 to 10 x $10^6$ species (de Vries and Hall, 1994). Therefore, the marine environment, especially marine invertebrates that rely solely on innate immune mechanisms for host defense, is a spectacular resource for the development of new antimicrobial compounds. Table 1.2 gives an overview of AMPs isolated and characterized from the marine invertebrates covering the phyla Porifera, Cnidaria, Annelida, Mollusca, Arthropoda, Echinodermata and Urochordata.

**Table 1.2. Major AMPs/AMP families isolated from marine invertebrates**

| SOURCE ORGANISM | | | AMP |
|---|---|---|---|
| **Phylum** | **Group** | **Species** | |
| Porifera | Sponges | *Discodermia kiiensis* | Discodermin |
| Cnidaria | Jelly Fish | *Aurelia aurita* | Aurelin |
| Annelida | Polychaetes | *Arenicola marina* | Arenicin |
| | | *Nereis diversicolor* | Hedistin |
| | | *Perinereis aibuhitensis* | Perinerin |
| Mollusca | Bivalves | *Mytilus edulis* | Defensins A and B |
| | | | Myticin |
| | | | Mytilin A and B |
| | | | Mytimicin |
| | | *Crassostrea virginica* | Defensin |
| | | *Mytilus galloprovincialis* | Myticin A and B |
| Arthropoda | Limulus | *Limulus polyphemus* | Defensins 1 and 2 |
| | | | Mytilin B, C, D, and G1 |
| | | | Polyphemusin |
| | | | Tachyplesin II |
| | | *Tachyplesus gigas* | Tachyplesin III |
| | | | Tachycitin |
| | | *Tachypleus tridentatus* | Big defensin |
| | | | Tachystatin |

| | | | |
|---|---|---|---|
| | | | Tachyplesin I |
| | Crustacea | *Pacifastacus leniusculus* | Astacidin, Pl crustin, ALF |
| | | *Carcinus maenas* | Carcinin, ALF |
| | | *Callinectes sapidus* | Callinectin, ALF |
| | | *Hyas araneus* | Hyasin, arasin |
| | | *Scylla paramanosian* | ALF, crustin |
| | | *Scylla serrata* | ALF, crustin |
| | | *Eriocheir sinensis* | ALF |
| | | *Panulirus argus* | Crustin, PET-15 |
| | | *Homarus americanus* | Crustin, Homarin |
| | | *Homarus gammarus* | Crustin |
| | | *Litopenaeus vannamei* | Penaeidin-2, -3, -4, crustin |
| | | *Litopenaeus setiferus* | Penaeidin-2, -3, -4, crustin, ALF |
| | | *Litopenaeus stylirostris* | Penaeidin-2, -3 |
| | | *Farfantepenaeus paulensis* | Penaeidin-2 |
| | | *Litopenaeus schmiitti* | Penaeidin-2, -4 |
| | | *Penaeus monodon* | Penaeidin-3, -5, crustin, ALF |
| | | *Fenneropenaeus chinensis* | Penaeidin-3, crustin, ALF |
| | | *Penaeus semisulcatus* | Penaeidin-3 |
| | | *Marsupenaeus japonicus* | Crustin, ALF |
| Echinodermata | Sea Urchin | *Strongylocentrotus droebachiensis* | Strongylocin |
| Urochordata | Tunicates | *Styela clava* | Styelin, Clavanin, Clavaspirin |
| | | *Halocynthia aurantium* | Dicynthaurin, Halocidin |
| | | *Halocynthia papillosa* | Halocyamine |
| | | *Halocynthia papillosa* | Papillosin |
| | | *Styela plicata* | Plicatamide |

On the basis of the charge of the AMP molecules they are classified as follows (Table 1.3).

**Table 1.3 Various Classes of AMPs based on its charge / nature**

| Nature of AMP | AMP | Source organism |
|---|---|---|
| Anionic peptides | Maximin H5 | *Bombina maxima* (Amphibian) |
| | Dermcidin | Humans |
| Linear cationic α-helical peptides | Cecropin-A | *Hyalophora cecropia* (Insect) |
| | Andropin | *Drosophila melanogaster* (Insect) |
| | Moricin | *Bombyx mori* (Insect) |
| | Ceratotoxin | *Ceratitis capitata* (Insect) |
| | Melittin | *Apis indica* (Insect) |
| | Cecropin P1 | *Ascaris* (Nematode) |
| | Magainin | *Xenopus laevis* (Amphibian) |
| | Dermaseptin | *Phyllomedusa sp.* (Amphibian) |
| | Bombinin | *Bombina variagata* (Amphibian ) |
| | Brevinin-1 | *Rana brevipoda porsa* (Amphibian) |
| | Esculentins | *Rana esculenta* (Amphibian) |
| | Buforin | *Bufo bufo garagriozans* (Amphibian) |
| | Pleurocidin | *Pleuronectes americanus* (Winter flounder) |
| | Cathelicidins, Seminalplasmin, ovispirin | Cattle, Sheep and Pig |
| | Cap18 | Rabbit |
| | Ll37 | Human |
| Cationic peptides enriched for specific amino acids | Abaecin, apidaecins, hymenoptaecin | *Apis mellifera* (Insect) |
| | Drosocin | *Drosophila* (Insect) |
| | Pyrrhocoricin | European sap-sucking bug |

| | | |
|---|---|---|
| | Bactenecins | Cattle, sheep, goat |
| | PR-39, Prophenin | Pig |
| | Coleoptericin, holotricin | Beetle |
| | Indolicidin | Cattle |
| | Histatins | Humans |
| Anionic and cationic peptides that contain cysteine and form disulphide bonds | Brevinins | *Rana palustris* (Amphibian) |
| | Protegrin, β-defensins | Pigs |
| | Tachyplesins | Horseshoe crab |
| | HNP-1, -2, β-defensins | Humans |
| | Np-1 | Rabbit, rat |
| | β-defensins | Cattle, goat and poultry |
| | β-defensins | Mice, rat |
| | θ-defensin | Rhesus monkey |
| | Defensin A | *Aedes aegypti* (Insect) |
| | Drosomycin | *Drosophila melanogaster*(Insect) |
| | Defensins | Plant |
| Anionic and cationic peptide fragments of larger proteins | Lactoferricin | Lactoferrin |
| | Casocidin I | Human casein, Bovine milk |
| | Antimicrobial domains | Bovine α-lactalbumin |
| | Antimicrobial domains | Human haemoglobin |
| | Antimicrobial domains | Human lysozyme |
| | Antimicrobial domains | Human ovalbumin |

### 1.7.6.2 AMPs based on charge /nature

#### Anionic AMPs

Anionic AMPs are small (7.2–8.2 kDa) peptides present in surfactant extracts, broncho-alveolar lavage fluid and airway epithelial cells (Brogden et al., 1996, 1998, 1999). They are produced in mM concentrations, require zinc as a co-factor for antimicrobial activity and are active against both gram-positive and gram-negative bacteria. They are similar to the charge neutralizing pro-peptides of larger zymogens, which also have antimicrobial activity when synthesized alone (Brogden et al., 1997).

#### Cationic AMPs

A second subgroup contains ~290 cationic peptides, which are short (contain <40 amino acid residues), lack cysteine residues and sometimes have a hinge or 'kink' in the middle (Tossi et al., 2000; Gennaro et al., 2002). In aqueous solutions many of these peptides are disordered, but in the presence of trifluoroethanol, sodium dodecyl sulphate (SDS) micelles, phospholipid vesicles and liposomes, or Lipid A, all or part of the molecule is converted to an α-helix (Gennaro and Zanetti, 2000). As observed in LL-37, the extent of α-helicity correlates with the antibacterial activity against both gram-positive and gram-negative bacteria and the increased α-helical content correlates with stronger antimicrobial activities (Park et al., 2000).

#### Cationic AMPs rich in aminoacids

A third subgroup contains ~44 cationic peptides that are rich in certain amino acids (Otvos, 2002). This group includes the bactenecins and PR-39, which are rich in proline (33–49%) and arginine (13–33%) residues; prophenin, which is rich in proline (57%) and phenylalanine (19%) residues; and indolicidin, which is rich in tryptophan residues (Gennaro and Zanetti, 2000; Otvos, 2002). These peptides lack cysteine residues and are linear, although some can form extended coils.

### *Anionic and Cationic AMPs with cysteine residues*

A fourth subgroup of anionic and cationic peptides (~380 members), contain cysteine residues and form disulphide bonds and stable β-sheets. This subgroup includes protegrin from porcine leukocytes, and a diverse family of defensins. There are ~55 α-defensins, which include human neutrophil peptides (HNPs) and cryptdins and comprise 29–35 amino acid residues, including six cysteines that are linked by three intramolecular disulphide bonds (Lehrer et al., 1993). There are ~90 β-defensins from both humans (HBDs) and animals that comprise 36–42 amino acid residues including six cysteines that are linked by three intramolecular disulphide bonds (Ganz et al., 1990; Ganz, 2002; Schutte and McCray, 2002). In addition, there are ~54 arthropod (insect) defensins, ~58 plant defensins and a rhesus δ-defensin (RTD-1), which is an 18-residue peptide that forms a circular molecule cross-linked by three disulphide bonds (Weiss et al., 2002).

### *AMPs that are fragments of larger proteins*

Finally, there are anionic and cationic peptides that are fragments of larger proteins. These fragments have antimicrobial activity and are similar in composition and structure to the AMPs described above. The diversity of AMPs discovered is so great that it is difficult to categorize them except broadly on the basis of their secondary structure (Epand and Vogel, 1999; van't Hof et al., 2001).

### *1.7.6.3 AMPs based on structure*

The fundamental structural principle is the ability of a peptide to adopt a shape in which clusters of hydrophobic and cationic amino acids are spatially organized in discrete parts of the molecule. Based on their secondary structure AMPs are classified into four major groups (van't Hof et al., 2001). The various structural classes of AMPs are shown in Table 1.4 and Fig.1.7.

**Table 1.4.Various classes of AMPs based on its structure**

| STRUCTURE | AMP | SOURCE ORGANISM |
|---|---|---|
| α-helix | Cecropins, Mellitin | Insect |
| | Magainin, LL-37, PGLa, Brevinin-1, Temporin | Amphibian |
| | PMAP-23 | Porcine |
| β-Sheet | α and β-Defensin | Human, Rabbit |
| | Dermaseptin | Frog |
| | Tachyplesin,Polyphemusin | Horseshoe crab |
| | Protegrin | Human, Porcine |
| | Androctonin | Scorpion |
| | Pn-AMP 1 | Plant |
| Cyclic β-sheet | θ-Defensin | Primate, Human |
| β-Turn | Lactoferricin | Bovine , Human |
| Linear with repeating motifs | Bactenecins 5 and 7, PR-39, Indolicidin | Mammals |
| | Diptericin, Apidaecin | Insects |
| Extended | Indolicidin, PR-39 | Bovine, Porcine |
| Extended turn | Tenecin-3 | Insect |
| α-helix / extended | Buforin II | Toad |
| Looped peptide | Bactenecin 1 | Bovine |
| | Ranalexin | Frog |
| | Thanatin | Insect |
| Mixed structure (4-disulfide α-helix 1β-sheet ) | Defensins | Plants |
| Mixed structure (4-disulfide α-helix 1 β-sheet) | Drosomycin | Arthropods |
| Mixed structure (3-disulfide β-sheet rich) | α- and β-defensins | Mammals |
| Mixed structure (3-disulfide α-helix 1 β-sheet) | Defensins | Arthropods, Molluscs |
| Mixed structure (3-disulfide 2α-helices 1β-sheet) | γ-thionins | Plants |

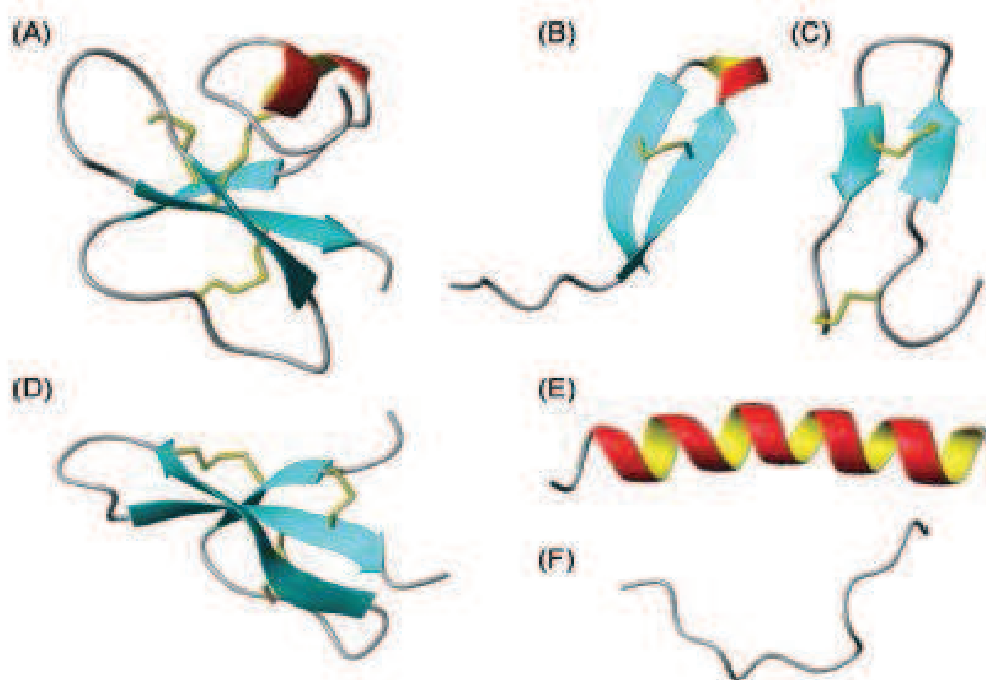



**Fig. 1.7. Structural classes of Antimicrobial Peptides (Adapted from Jenssen et al., 2006)**

***(A) Mixed structure (β-defensin); (B) Looped (thanatin); (C) β-sheeted (polyphemusin); (D) β-sheeted (defensin-1); (E) α-helical (magainin-2); (F) Extended (indolicidin). The disulfide bonds are indicated in yellow***

## *Group I: Linear peptides with an α-helical structure*

One of the larger and better studied classes of AMPs is those forming cationic amphipathic helices, e.g. magainin, cecropin A and temporins (Boman, 1995; Mangoni et al., 2000). These peptides adopt disordered structures in aqueous solution while fold into an α-helical conformation upon interaction with hydrophobic solvents or lipid surfaces. α-helical peptides are often found to be amphipathic and can either absorb onto the membrane surface or insert into the membrane as a cluster of helical bundles. The majority of the cytotoxic amphipathic helical peptides are cationic and they do exhibit selective toxicity for microbes. One of the most studied of the cationic, antimicrobial, amphipathic helical peptides is magainin (Zasloff et al., 1987). There are also hydrophobic or slightly anionic α-helical peptides. Peptides that are not cationic exhibit less selectivity towards microbes compared with mammalian cells. An example of a well-studied hydrophobic

and negatively charged cytotoxic peptide is alamethicin (Duclohier and Wroblewski, 2001; Kikukawa and Araiso, 2002).

### *Group II: Cysteine rich AMPs possessing β-pleated structure*

In contrast to the linear α-helical peptides, β-sheet peptides are cyclic peptides constrained either by disulfide bonds, as in the case of human β-defensin-2 (Hancock, 2001), tachyplesins (Matsuzaki, 1999), protegrins (Harwig et al., 1995), and lactoferricin (Jones et al., 1994) or by cyclization of the peptide backbone, as in the case of gramicidin S (Prenner et al., 1999), polymyxin B (Zaltash et al., 2000), and tyrocidines (Bu et al., 2002). They largely exist in the β-sheet conformation in aqueous solution that may be further stabilized upon interactions with lipid surfaces.

Defensins are among the most characterized β-sheet-forming AMPs. Studies aimed at understanding the importance of the disulfide bridges for their activity have been carried out. Detailed studies have indicated that replacement of the cysteine residues by certain amino acids like alanine, aspargine, and leucine led to inactivation, whereas analogs with aromatic residues phenyl alanine, tyrosine, and hydrophobic amino acids like leucine, methionine, and valine retained broad spectrum antimicrobial activity (Tamamura et al., 1998). Studies with tachyplesin analogs suggested that the cyclic structure was essential for antimicrobial activity (Matsuzaki et al., 1997; Tamamura et al., 1998; Rao, 1999). A study of the orientation of protegrin in membranes using oriented circular dichroism has demonstrated two different states of the peptide to insert into a membrane, depending on the peptide concentration, the nature of the lipid, and the extent of hydration (Heller et al., 1998). The peptide tachyplesin permeabilizes both bacterial and artificial lipid membranes (Matsuzaki et al., 1997), suggesting a similar mode of action as the α-helical magainin.

### *Group III: Linear peptides with an extended structure, characterized by over representation of one or more amino acids*

Certain AMPs have an unusual amino acid composition, having a sequence that is rich in one or more specific amino acids. For example, the peptide histatin, which is produced in saliva, is highly rich in histide residues (Brewer et al., 1998; Tsai and Bobek, 1998; Helmerhorst et al., 1999). This peptide translocates across the yeast membrane and targets to the mitochondria, suggesting an unusual antifungal mechanism (Helmerhorst et al., 1999). The peptides produced by porcine neutrophils belonging to the cathelicidin family are very rich in proline and arginine or proline and phenylalanine (Zhao et al., 1995; Linde et al., 2001). Tryptophan is of particular interest with regard to the partitioning of peptides into membranes because of its propensity to position itself near the membrane/water interface (Yau et al., 1998; Persson et al., 1998). Examples of AMPs that are rich in tryptophan include tripticin (Lawyer et al., 1996) and indolicidin (Selsted et al., 1992). Indolicidin was reported to adopt a turn conformation which greatly enhances its membrane activity (Ladokhin et al., 1999). Indolicidin has been shown to permeabilize the outer membrane of *E. coli* (Falla et al., 1996) to form channels, as revealed by conductance measurements with planar bilayers (Falla et al., 1996; Wu et al., 1999). The unusual amino acid composition has led to studies directed towards delineation of the role of multiple tryptophan residues in its biological activity as well as interactions with model membranes (Falla et al., 1996; Subbalakshmi et al., 1996).

### *Group IV: Peptides containing a looped structure*

In contrast to other AMPs, proline-arginine-rich peptides cannot form amphipathic structures due to the incompatibility of high concentration of proline residues in such structures and have been proposed to adopt a polyproline helical type-II structure (Boman et al., 1993; Cabiaux et al., 1994).

Lantibiotics contain small ring structures enclosed by a thioether bond and their structure and properties have recently been reviewed (Montville and Chen, 1998). One of the lantibiotics, nisin, is currently used as an antimicrobial agent for food preservation and this peptide has relatively high activity against Gram-positive bacteria due to its specific high affinity with Lipid II, a precursor in the bacterial cell wall synthesis (Breukink and de Kruijff, 1999; Breukink et al., 1999). Recently synthesized six and eight residue cyclic D,L-α-peptides have been found to exhibit high efficacy to kill bacteria with low haemolytic activity (Fernadez-Lopez et al., 2001). Upon binding to lipid membranes the cyclic peptides can stack to form hollow, β-sheet-like tubular structures increasing membrane permeability. With short size, easy to synthesize and being proteolytically stable, this class of peptides holds considerable potential in fighting existing and emerging infectious diseases.

### *1.7.7 Induction and Regulation of AMP expression*

AMPs in multicellular organisms are found on external surfaces such as the skin or the lungs or they are sequestered in granules of neutrophils, from where they can be released to kill microbes. Some AMPs are synthesized constitutively, for instance the histatins (Tsai and Bobek, 1998) and human β- defensin-1 (Yang et al., 2002). Others are often induced in response of an infection (Hoffmann et al., 1999) and thus can be considered as acute-phase proteins, e.g. LL- 37 (Frohm et al., 1997) and human β-defensin-2 (Harder et al., 2000; Schutte and McCray, 2002; Yang et al., 2002).

The large majority of AMPs synthesized by multicellular organisms are encoded by the genome. Insects and mammals typically express multiple AMPs. For example, at least ten sheep genes encode AMPs, including eight cathelicidins and two β-defensins (Huttner et al., 1998). The bovine genome contains genes for at least eleven cathelicidins (Scocchi et al., 1997) and over twenty β-defensins (Ryan et al., 1998). The AMPs are produced mainly

through regular processes of gene transcription and ribosomal translation, often followed by further proteolytic processing. However, there are some AMPs that are produced non-ribosomally (Jack and Jung, 2000; Moffitt and Neilan, 2000). Magainins are synthesized as long preproproteins containing six copies of the peptide. Proteolytic processing leads to the release of the individual magainin peptides (Ketchem et al., 1993). The 35-37 residue insect cecropins are synthesized as preproproteins of 62 amino acids and processing involves several protease activities (Gudmundsson et al., 1991). Interestingly, in many cationic AMPs such as the temporins, the negative charge of the carboxyl terminus is removed by an amidation process (Simmaco et al., 1996).

Recently, it was found that several AMPs are released by cleavage of intact proteins that may have no or limited antibacterial activity themselves. This has been demonstrated first for the milk protein lactoferrin (Bellamy et al., 1992). Proteolytic cleavage of the intact bovine protein by pepsin under acidic conditions releases a 25 residue peptide, lactoferrin B, which shows a significantly increased bacteriostatic potency compared to the intact protein (Bellamy et al., 1992).

AMPs can neutralize host responses to conserved bacterial signaling molecules such as endotoxic LPS from gram-negative bacteria, or LTA from gram-positive bacteria (O'Leary and Wilkinson, 1988). Such molecules interact with Toll-like receptors on the surface of host cells to trigger signaling cascades and cause upregulation of cytokines, such as tumor necrosis factor (TNF) and interleukin 6, chemokines, and dozens of other gene products (Medzhitov, 2001). Although many AMPs are induced by bacterial molecules or up-regulated by inflammatory stimuli involving a Toll-related IL-1 receptor (Williams et al., 1997; Tauszig et al., 2000), only a few genes encoding such inducible peptides were found.

Drosomycin gene expression is exclusively regulated via NF-kB/I-kB signaling pathway; diptericin and drosocin gene expressions are regulated

by the independent, not yet well defined, *imd* pathway, whereas gene induction for cecropin, insect defensin and the antimicrobial protein attacin (Khush et al., 2001) requires contributions from both signaling pathways. These pathways explain the differential response of *Drosophila* to fungi, gram-negative and gram-positive bacteria. In plants, the production of inducible antimicrobial proteins is up-regulated via similar signaling pathways. For instance, microbial products induce an intracellular proteolytic cascade in potato cells leading to binding of nuclear factors PBF-1 and PBR-2 to an elicitor-response element (ERE) 26 on the DNA (Subramaniam et al., 1997).

Again, differential responses against different types of microbes are observed (Gomez-Gomez et al., 1999; Keller et al., 1999; Cardinale et al., 2000). Like animal cells, plant cells are able to communicate microbial infection to neighbor cells using plant hormones as functional analogues of interleukins. In plants two different hormone-dependent signaling pathways have been identified that lead to differential responses to distinct microorganisms. The salicylic acid signaling pathway induces antibacterial responses while the cis-jasmonic acid/ethylene signaling pathway induces antifungal responses (Thomma et al., 1998; Pieterse and Van Loon, 1999).

### *1.7.8 Regulation of synthesis and release of AMPs*

Many of the plant and invertebrate peptides (e.g., insect defensins and plant defensins) structurally and functionally resemble their vertebrate counterparts but a comprehensive evolutionary lineage has not yet been established. Both plants and invertebrates induce the synthesis of AMPs in response to infection. In vertebrates, AMP synthesis is either constitutive or inducible by microbial macromolecules and/or cytokines. The epithelial β-defensin of the bovine trachea, the tracheal AMP (TAP), is synthesized in the airway epithelia when these are exposed to inhaled bacteria or LPS (Diamond et al., 1996). This response is initiated by LPS receptors that

ultimately signal to transcriptional regulators including the NF-kB complex, acting on NF-kB binding motifs in the promoter of the TAP gene. In addition to transcriptional regulation of synthesis, stimulus-dependent degranulation provides an additional level of responsiveness and specificity. Thus the granulocytes of many vertebrates contain antimicrobial defensin peptides in their phagocytic granules and another class of AMPs, cathelicidins, in granules destined for extracellular secretion (Rice et al., 1987; Sorensen et al., 1997). Intestinal Paneth cells, positioned at the bottom of narrow crypts in the small intestine, release their defensin-rich granules (Ouellette and Selsted, 1996) upon stimulation by cholinergic or bacterial stimuli, both of which are associated with food ingestion (Qu et al., 1996).

All known AMPs are synthesized as larger precursors, containing one or multiple copies of the active peptide segment which are released by proteolytic processing. In the simplest cases the co-translational removal of an N-terminal signal peptide frees the active moiety but more commonly one or more anionic propieces are also removed during processing (Zasloff, 1987; Terry et al., 1988; Valore and Ganz, 1992). Perhaps the most intriguing and as yet unexplained processing pattern is seen with cathelicidins, a group of peptides with a conserved 100 amino acid domain that is frequently proteolytically cleaved from the highly variable C-terminal antimicrobial domain (Zanetti et al., 1995). In phagocytes, the cathelicidins are commonly stored as inactive precursors in secretory granules. In many cases, the processing enzyme is neutrophil elastase contained in a separate set of storage granules. During phagocytosis, this binary system combines to generate active AMPs. The function of the highly conserved cathelin domain is not yet known.

### *1.7.9 Localization of AMPs*

The highest concentrations of AMPs are found in animal tissues exposed to microbes or cell types that are involved in host defense. Epithelial

surfaces secrete AMPs from both barrier epithelia and glandular structures (Zasloff, 1987; Diamond et al., 1991; Jones and Bevins, 1992; Ouellette and Selsted, 1996). Phagocytic cells contain several types of storage organelles (granules) for microbicidal substances and digestive enzymes (Levy, 1996; Ganz and Lehrer, 1997). In the process of phagocytosis, granules fuse to phagocytic vacuoles that contain ingested microbes, thereby exposing the microbes to very high concentrations of microbicidal and digestive substances. Other granules are secreted into the extracellular fluid where their contents kill microbes or inhibit their multiplication. Both types of granules contain abundant AMPs (Selsted et al., 1984; Ganz et al., 1985; Cowland et al., 1995). In invertebrates, the fluid portion of blood (hemolymph) as well as the granules of phagocytic cells (haemocytes) contains AMPs (Boman et al., 1991; Iwanaga et al., 1994). Secretion of AMPs from the fat body (equivalent to the liver in vertebrates) into haemolymph appears to be the dominant mechanism in injured or infected insects, while haemocytes may be the more important source in horseshoe crabs. Like in vertebrates, insect epithelia, most prominently the gut, secrete tissue-specific AMPs (Richman and Kafatos, 1996; Richman et al., 1997), a response which is likely to be important in insect resistance to intestinal parasites.

### *1.7.10 Mechanism of action*

Many AMPs bind in a similar manner to negatively charged membranes and permeate them, resulting in the formation of a pathway for ions and solutes (McElhaney et al., 1999). Before reaching the phospholipid membrane peptides must transverse the negatively charged outer wall of Gram-negative bacteria containing LPS or through the outer cell wall of Gram-positive bacteria containing acidic polysaccharides. Hancock and coworkers described this process as a 'self-promoted uptake' with respect to Gram-negative microorganisms (Hancock, 1997). In this mechanism, the peptides initially interact with the surface LPS, competitively displacing the

divalent polyanionic cations and partly neutralize LPS. This causes disruption of the outer membrane and peptides pass through the disrupted outer membrane and reach the negatively charged phospholipid cytoplasmic membrane. The membrane-active properties of such peptides have been extensively studied using model membranes (McElhaney et al., 1999). The amphipathic peptides can partition into cytoplasmic membrane through hydrophobic and electrostatic interactions, causing stress in the lipid bilayer. When the unfavorable energy reaches a threshold, the membrane barrier property is lost, which is the basis of the antimicrobial action of these peptides. Peptide-mediated cell killing can be rapid. Some linear α-helical peptides kill bacteria so quickly that Boman (1995) reported that it is technically challenging to characterize the steps (if there are any) preceding cell death. Other peptides, such as magainin 2 (Zasloff, 1987), cecropin P1 (Boman et al., 1993), PR-39 (Boman et al., 1993) and SMAP29 (Kalfa et al., 2001), kill bacteria in 15–90 minutes. Regardless of the time required, or the specific antimicrobial mechanism, specific steps must occur to induce bacterial killing (Matsuzaki et al., 1995).

### *1.7.10.1 Attraction*

AMPs must first be attracted to bacterial surfaces, and one obvious mechanism is electrostatic bonding between anionic or cationic peptides and structures on the bacterial surface. Studies show that peptides like magainin 2 and cecropin A, for example, readily insert into monolayers, large unilamellar vesicles and liposomes that contain acidic phospholipids (Silvestro et al., 1997; Zhao et al., 2001). However, gram-negative and gram-positive bacteria are much more complex than model membranes and cationic AMPs are likely to first be attracted to the net negative charges that exist on the outer envelope of gram-negative bacteria e.g. anionic phospholipids and phosphate groups on LPS and to the teichoic acids on the surface of gram-positive bacteria.

### 1.7.10.2 Attachment

Once close to the microbial surface, peptides must traverse capsular polysaccharides before they can interact with the outer membrane, which contains LPS in gram-negative bacteria, and traverse capsular polysaccharides, teichoic acids and lipoteichoic acids before they can interact with the cytoplasmic membrane in gram-positive bacteria. This concept is important but is rarely addressed in mechanistic studies. Once peptides have gained access to the cytoplasmic membrane they can interact with lipid bilayers. In vitro studies of AMPs incubated with single or mixed lipids in membranes or vesicles show that peptides bind in two physically distinct states (Huang, 2000). At low peptide/lipid ratios, α-helical peptides, β-sheet peptides and defensins adsorb and embed into the lipid head group region in a functionally inactive state (referred to as the surface or S state) that stretches the membrane (Chen et al., 2003). The extent of membrane thinning is specific to the peptide and directly proportional to the peptide concentration.

### 1.7.10.3 Peptide insertion and membrane permeability

At low peptide/lipid ratios, peptides are bound parallel to a lipid bilayer (Yang et al., 2001). As the peptide/lipid ratio increases, peptides begin to orientate perpendicular to the membrane. At high peptide/lipid ratios, peptide molecules are orientated perpendicularly and insert into the bilayer, forming transmembrane pores (referred to as the I state). The I state peptide/lipid ratio varies with both the peptide and target lipid composition (Lee et al., 2004), and a number of models have been proposed to explain membrane permeabilization.

#### Barrel-stave model

In the 'barrel-stave model' (Fig. 1.8), peptide helices form a bundle in the membrane with a central lumen, much like a barrel composed of helical peptides as the staves (Ehrenstein and Lecar, 1997; Yang et al., 2001). This

type of transmembrane pore is unique and is induced by alamethicin. The hydrophobic peptide regions align with the lipid core region of the bilayer and the hydrophilic peptide regions form the interior region of the pore. The alamethicin-induced transmembrane pores can contain 3–11 parallel helical molecules, and the inner and outer diameters have been calculated as ~1.8 nm and ~4.0 nm, respectively (He et al., 1998; Spaar et al., 2004). The walls of the channel are ~1.1 nm, which is approximately the diameter of the alamethicin helix and is consistent with eight alamethicin monomers arranged according to the barrelstave model (Yang et al., 2001). However, changes in bilayer lipid composition can modulate peptide aggregation equilibria and the number of peptides in the aggregate (Cantor, 2004).

***Carpet model***

In the 'carpet model' (Fig. 1.9), peptides accumulate on the bilayer surface (Pouny et al., 1992). This model explains the activity of AMPs such as ovispirin (Yamaguchi et al., 2001) that orientate parallel ('in-plane') to the membrane surface (Bechinger, 1999). Peptides are electrostatically attracted to the anionic phospholipid head groups at numerous sites covering the surface of the membrane in a carpet-like manner. At high peptide concentrations, surface-oriented peptides are thought to disrupt the bilayer in a detergent-like manner, eventually leading to the formation of micelles (Shai, 1999; Ladokhin et al., 2001). At a critical threshold concentration, the peptides form toroidal transient holes in the membrane, allowing additional peptides to access the membrane. Finally, the membrane disintegrates and forms micelles after disruption of the bilayer curvature (Oren and Shai, 1998; Bechinger, 1999).

***Toroidal-pore model***

In the 'toroidal-pore model' (Fig. 1.10), AMP helices insert into the membrane and induce the lipid monolayers to bend continuously through the pore so that the water core is lined by both the inserted peptides and the

lipid head groups (Matsuzaki, et al., 1996). This type of transmembrane pore is induced by magainins, protegrins and melittin (Matsuzaki et al., 1996; Yang et al., 2001; Hallock et al., 2003). In forming a toroidal pore, the polar faces of the peptides associate with the polar head groups of the lipids (Yamaguchi et al., 2002). The lipids in these openings then tilt from the lamellar normal and connect the two leaflets of the membrane, forming a continuous bend from the top to the bottom in the fashion of a toroidal hole; the pore is lined by both the peptides and the lipid head groups, which are likely to screen and mask cationic peptide charges (Yang et al., 2001). The toroidal model differs from the barrel-stave model as the peptides are always associated with the lipid head groups even when they are perpendicularly inserted in the lipid bilayer (Yang et al., 2001). Magainin-induced toroidal pores are larger and have a more variable pore size than alamethicin-induced pores (Yang et al., 2001). They have an inner diameter of 3.0–5.0 nm and an outer diameter of ~7.0–8.4 nm, and each pore is thought to contain only 4–7 magainin monomers and ~90 lipid molecules (Matsuzaki et al., 1997, 1998; Yang et al., 2001). The mechanisms of defensins are not as well defined (Zasloff, 2002; Lehrer, 2004) but they also permeabilize membrane bilayers containing negatively charged phospholipids (Fujii et al., 1993; Zasloff, 2002; Lehrer, 2004). Although descriptions of membrane damage seem to vary, they are likely to be related. It has been suggested that ion channels, transmembrane pores and extensive membrane rupture do not represent three completely different modes of action, but instead are a continuous gradation between them (Dathe and Wieprecht, 1999).

#### *1.7.10.4 Models of intracellular killing*

Although the formation of ion channels, transmembrane pores and extensive membrane rupture eventually leads to the lysis of microbial cells, there is increasing speculation that these effects are not the only mechanisms of microbial killing. There is increasing evidence to indicate that AMPs have

other intracellular targets (Fig. 1.11). Some early observations revealed that there are alternate sites of AMP activity — for example, Bac7 fragments 1–16, 1–23 and 1–35 did not permeabilize *E. coli* but caused a 2–5 log reduction in the number of organisms (Gennaro and Zanetti, 2000). Non-membrane external targets such as autolysins and phospholipases are activated by AMPs.

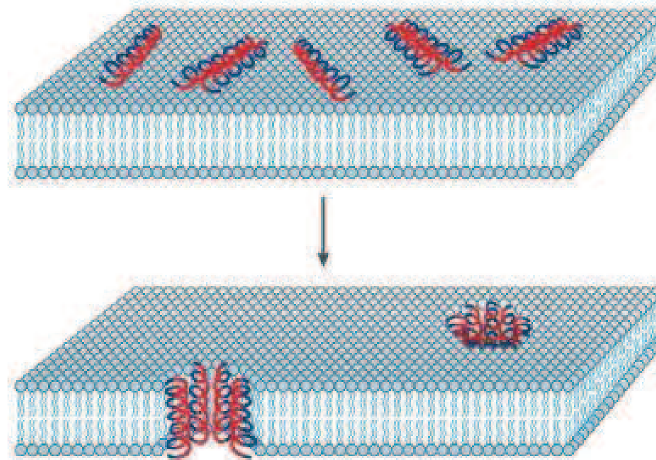


**Fig. 1.8. The barrel-stave model of antimicrobial-peptide-induced killing (Adapted from Brogden, 2005)**

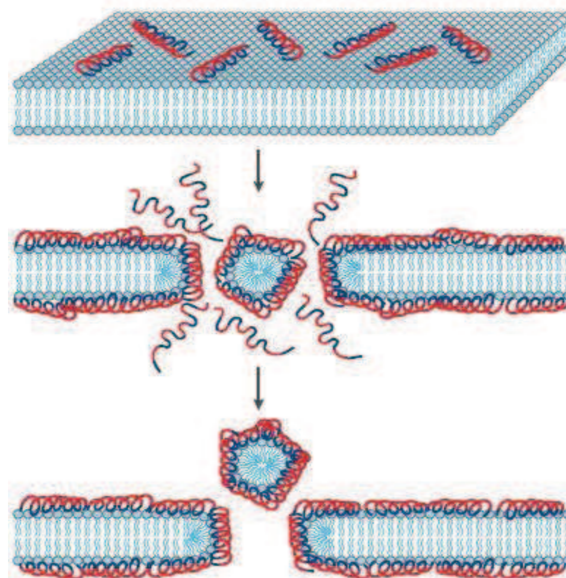


**Fig. 1.9. The carpet model of antimicrobial-induced killing (Adapted from Brogden, 2005)**

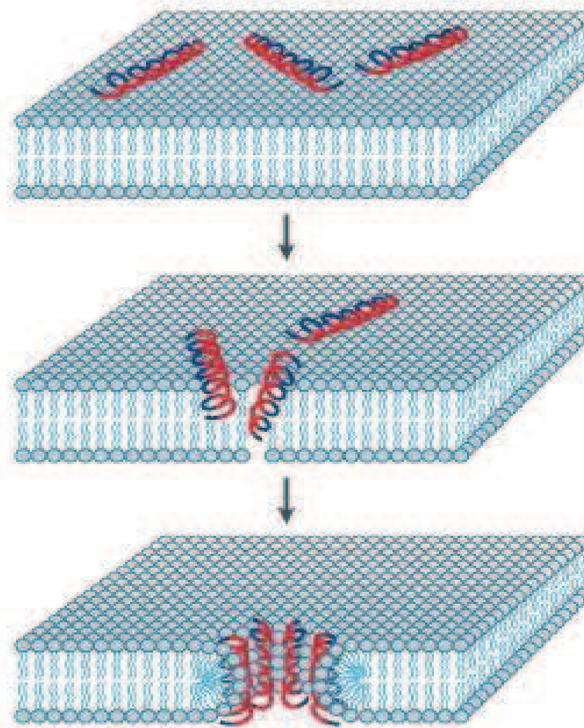


**Fig. 1.10. The toroidal-pore model of antimicrobial peptide induced killing (Adapted from Brogden, 2005)**

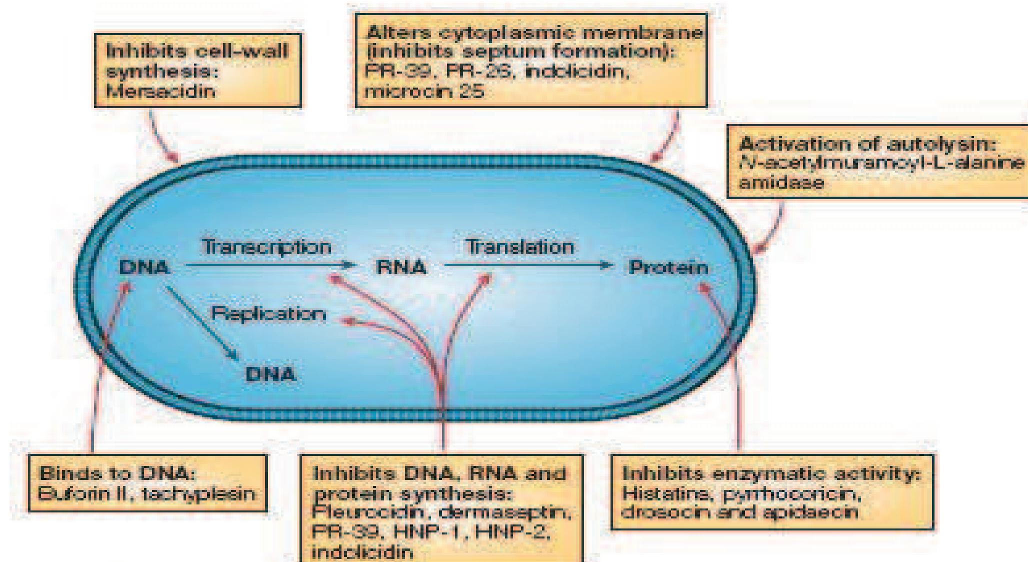



**Fig. 1.11. Mode of action for intracellular antimicrobial peptide activity (Adapted from Brogden, 2005). In this figure *Escherichia coli* is shown as the target microorganism.**

Apidaecin, a short, proline-rich antibacterial peptide, is translocated by a permease/transporter-mediated mechanism (Casteels et al., 1993). Once in the cytoplasm, translocated peptides can alter the cytoplasmic membrane septum formation, inhibit cell-wall synthesis, inhibit nucleic-acid synthesis, inhibit protein synthesis or inhibit enzymatic activity. PR-39, which is a proline–arginine-rich neutrophil peptide, and its N-terminal 1–26 fragment, PR-26, induce filamentation of *Salmonella enterica* serovar Typhimurium (*S. typhimurium*), and indolicidin induces filamentation of *E. coli* (Shi et al., 1996; Subbalakshmi and Sitaram, 1998). Cells exposed to these peptides have an extremely elongated morphology, which indicates that the peptide-treated cells are unable to undergo cell division. It is not known whether cell filamentation is due to the blocking of DNA replication or the inhibition of membrane proteins that are involved in septum formation.

Lantibiotics are AMPs from gram-positive bacteria that contain the thioether amino acid lanthionine. The lantibiotic mersacidin inhibits PG biosynthesis by interfering with membrane associated transglycosylation (Brotz et al., 1998). Buforin II binds to *E. coli* DNA and RNA and alters their electrophoretic mobilities in 1% agarose gels (Park et al., 1998), and tachyplesin binds in the DNA minor groove (Yonezawa et al., 1992). α-helical peptides (pleurocidin and dermaseptin), proline and arginine rich peptides (PR-39 and indolicidin) and defensins (HNP-1) inhibit DNA, RNA and protein synthesis in *E. coli* (Lehrer et al., 1989; Boman et al., 1993, Subbalakshmi and Sitaram, 1998; Patrzykat et al., 2002). At their minimal inhibitory concentrations, pleurocidin and dermaseptin both inhibit nucleic acid and protein synthesis without damaging the *E. coli* cytoplasmic membrane (Patrzykat et al., 2002). PR-39 stops protein synthesis and induces degradation of some proteins that are required for DNA replication (Boman et al., 1993). HNP-1 and -2 can reduce DNA, RNA and protein synthesis, and HNP-1 also inhibits the synthesis of periplasmic β-galactosidase (Lehrer et al., 1989). Indolicidin completely inhibits DNA and RNA synthesis in *E. coli*

but does not have any effect on protein synthesis (Subbalakshmi and Sitaram, 1998).

Histatins bind to a receptor on the fungal cell membrane, enter the cytoplasm and induce the nonlytic loss of ATP from actively respiring cells (Kavanagh and Dowd, 2004). Their action can also disrupt the cell cycle and lead to the generation of reactive oxygen species (Andreu and Rivas, 1998). Short, proline-rich antibacterial peptides also have several different effects. Pyrrhocoricin, drosocin and apidaecin bind specifically to DnaK, a 70-kDa heatshock protein (Otvos et al., 2000), and nonspecifically to GroEL, a 60-kDa bacterial chaperone (Otvos et al., 2000). Pyrrhocoricin reduces the ATPase activity of recombinant DnaK (Kragol et al., 2001) and pyrrhocoricin and drosocin alter the refolding of misfolded proteins indicating that drosocin and pyrrhocoricin binding prevents the frequent opening and closing of the multi-helical lid over the peptide-binding pocket of DnaK, permanently closes the cavity and inhibits chaperone-assisted protein folding (Kragol et al., 2001).

### *1.7.10.5 The basis of peptide activity and specificity*

There is extensive information about the characteristics and traits of effective AMPs, the locations at which they are produced and their ranges of activities (Boman, 1995; Gennaro and Zanetti, 2000; Tossi et al., 2000; Otvos, 2002; Ramanathan et al., 2002). Although many relationships between peptide structure and antibacterial activity have been described (Gennaro and Zanetti, 2000; Tossi et al., 2000; Powers and Hancock, 2003), little is known about the molecular basis of the marked differences in peptide activity and specificity. For example, the differences in the susceptibility of a single microorganism to a panel of AMPs indicate that the size, the sequence, the degree of structuring (for example, helical content), the charge, the overall hydrophobicity, the amphipathicity and the respective widths of the hydrophobic and hydrophilic faces of the helix are all important (Boman and

Hultmark, 1987; Gennaro and Zanetti, 2000). Several additional traits are also found to be correlated with antimicrobial activity (Yount and Yeaman, 2004). Altering any of the above parameters, including the hydrophobic moment and the angle subtended by charged residues, can modify antimicrobial and haemolytic activity of peptides (Dathe et al., 2002). Most of these parameters are closely related and altering one parameter can result in significant changes in the others (Tossi et al., 2000). This indicates that there are limits to the amount of 'tweaking' that can be done to develop the ideal AMP.

### *1.7.10.6 Antibacterial peptides*

By far the best-studied classes of cationic AMPs are those with antibacterial activity (Faber et al., 2005). It is well understood that regardless of their actual target of action, all antibacterial cationic peptides must interact with the bacterial cytoplasmic membrane (Hancock and Rozek, 2002). The driving physical forces behind antibacterial activity include net positive charge (enhancing interaction with anionic lipids and other bacterial targets), hydrophobicity (required for membrane insertion and often driven by this process), and flexibility (permitting the peptide to transition from its solution conformation to its membrane-interacting conformation). Each of these characteristics can vary substantially over a particular range but are essential for the function of the peptides as antimicrobial agents and allow them to interact with bacterial membranes, which is critical to their exertion of antimicrobial effects.

#### *Structural requirements for antibacterial peptides*

As mentioned above, cationic AMPs are generally categorized into four structural classes, i.e., α-helical, β-sheet, loop, or extended structures (Boman, 1995; Hancock, 1997); however, there are many peptides that do not fit into this simplified classification scheme. For many peptides these secondary structures are observed only when the peptides interact with membranes (Rozek et al., 2000). The plasticity of the secondary structure of

indolicidin has been suggested to permit different interactions with different molecules, including DNA and membranes (Hsu et al., 2005).

One approach to increase the antibacterial activity of cationic peptides has been to alter their flexible secondary structures. By changing the membrane-associated shape of indolicidin so that the N and C termini were drawn closer together, the activity against gram-negative bacteria was increased. This shape could also be stabilized by adding a cysteine residue to each end and creating a disulfide bridge (Rozek et al., 2003). Similar attempts to stabilize specific structural elements have been made with a cecropin-melittin hybrid peptide, in which the α-helical structure in solution was stabilized by the introduction of a covalent lactam bond between two residues four amino acids apart, resulting in improved activity of some constructs (Houston et al., 1998). Alternatively, introduction of a disulfide bond within the C-terminal α-helix of sakacin P to increase the amount of α-helical structure led to a broadening of the spectrum of activity (Uteng et al., 2003). Thus, pre-conditioning peptides to adopt structures related to their final membrane-associated ones can occasionally be advantageous while giving rise to other assets such as protease stability.

The antibacterial activity of cationic peptides can also be modulated through alteration of the peptide's hydrophobicity or net charge. Studies have demonstrated that high levels of hydrophobicity can decrease selectivity between the desired bacterial targets and host cells (Kustanovich et al., 2002; Zelezetsky et al., 2005). Similarly, incorporation of charged residues above a certain maximum (varying with each peptide) does not lead to an increase in activity (Dathe and Wieprecht, 1999). Thus, this balance of charge and hydrophobicity can be delicate and must be empirically determined for each series of peptides.

Consequently, the inclusion of a particular peptide into a structural group does not give an indication as to its mode of action or its spectrum of activity. In fact, some peptides with very similar secondary structures have

quite opposite characteristics with respect to antibacterial activity. Indeed, different peptides may be membrane permeabilizing at their minimal effective concentrations or at concentrations well above or well below these concentrations. Nonetheless, antibacterial peptides seem largely able to affect their antimicrobial activity because of their amphipathicity or amphiphilicity and because of the presence of regions within the folded structure with high concentrations of positively charged residues (Powers et al., 2004).

***Mechanism of antibacterial activity***

As described in section 1.1.3.9.

### *1.7.10.7 Antifungal peptides*

Knowledge of antifungal peptides has accelerated in recent years. Their mode of action was first described as involving either fungal cell lysis or interference with fungal cell wall synthesis (de Lucca and Walsh, 1999). However, as the numbers of known antifungal peptides increase, new modes of action are being identified. It is intriguing to note that peptides with primarily antifungal activity, such as many of those isolated from plants, tend to be relatively rich in polar and neutral amino acids, suggesting a unique structure-activity relationship (Lustig et al., 1996).

***Structural requirements for antifungal peptides***

Studies have shown that antifungal peptides vary substantially in sequence and structure, and peptides as structurally diverse as eucommia (Huang et al., 2004), the α-helical P18 (Lee et al., 2004), and the extended peptide indolicidin (Lee et al., 2003), as well as plant defensins and a coleopteran β-sheet peptide from *Acrocinus longimanus* (Barbault et al., 2003), have all shown antifungal activity. Thus, like for antibacterial peptides, there are no obvious conserved structural domains that give rise to antifungal activity. Modification of ineffective AMPs has revealed that relatively modest changes often result in antifungal activity. Although no conserved sequences are evident for the antifungal peptides, several have been

demonstrated to possess specific biochemical characteristics, such as chitin (Fujimura et al., 2004; Huang et al., 2004) or heparin (Shimazaki et al., 1998; Andersson et al., 2004) binding abilities. Structure-activity relationship studies on three synthetic bovine lactoferricin (amino acids 17 to 30) -derived peptides revealed a significant positive correlation between the pI values of peptides and their candidicidal activity (Nikawa et al., 2004). Another study also showed a direct correlation between the ability of peptides to form complexes with lipid mixtures and their antifungal activity (Lopez-Garcia et al., 2004).

***Mode of action of antifungal peptides***

It was demonstrated that the rabbit-defensin NP-2 resulted in permeabilization of *C. albicans (*Patterson-Delafield et al., 1980; Lehrer et al., 1985). Moerman et al. (2002) subsequently demonstrated that α-helical pore-forming peptides isolated from scorpion venom had antifungal activity. Using the organic compound SYTOX green, it was demonstrated that the peptide permeabilizes fungi (Roth et al., 1997). Lee et al. (2002, 2003) have shown that indolicidin exerts its fungicidal activity by disrupting the structure of the fungal cell membrane, in a salt-dependent and energy-independent fashion, via direct interactions with the lipid bilayer. This contrasts to the situation for bacteria, in which indolicidin, although membrane active, appears to penetrate cells and act on macromolecular synthesis (Falla et al., 1996; Subbalakshmi and Sitaram, 1998).

Bovine lactoferricin have shown to result in profound ultrastructural damage to the cell wall of *C. albicans* (Bellamy et al., 1993; Park et al., 2004). The mechanism of action of certain antifungal peptides is still a matter of debate. The formation of reactive oxygen species has been suggested to be the crucial step in the fungicidal mechanism of a number of AMPs (Narasimhan et al., 2001), including histatin 5 and lactoferrin-derived peptides (Helmerhorst et al., 2001; Lupetti et al., 2002). Helmerhorst et al. (1999) found that exposure to the cationic peptide histatin 5 caused a

depletion of mitochondria in *C. albicans*. Thus, it is reasonable to hypothesize that AMPs may interact with mitochondria in a manner very similar to some of their actions on bacteria.

#### *1.7.10.8 Antiparasitic peptides*

Several AMPs possess an antiprotozoan mode of action that indicates parallels with their antibacterial, antiviral, or antifungal modes of action. Magainin 2 was one of the first antimicrobial host defense peptides demonstrated to display antiprotozoan activity, leading to swelling and eventual bursting of *Paramecium caudatum* (Zasloff, 1987). The anti-nematodal effect of the porcine cathelicidin PMAP-23 has been demonstrated against both the eggs and worms of *Caenorhabditis elegans*. Studies have indicated that this effect is exerted through disruption of the cell membrane via pore formation or via direct interaction with the lipid bilayers (Park et al., 2004), resembling the antifungal mode of action. Analogous studies on magainin 2 analogues revealed that short stretches of hydrophobic amino acids were important for leishmanicidal activity (Guerrero et al., 2004). It seems likely that antiprotozoan activity may be dependent on peptide motifs fundamentally different from those required for bacterial, viral, and fungal activities.

#### *1.7.10.9 Antiviral peptides*

Representatives from all four structural classes of the cationic host defense peptides have shown the ability to inhibit viral infection. The spectra of viruses that are affected comprise primarily enveloped RNA and DNA viruses. The antiviral activity of AMPs often appears to be related to the viral adsorption and entry process (Belaid et al., 2002) or is a result of a direct effect on the viral envelope (Aboudy et al., 1994; Robinson, et al., 1998). However, it appears to be impossible to predict antiviral activity based primarily on secondary structures of peptides. For example α-helical peptides such as cecropins, clavanins, and the cathelicidin LL-37 have been

shown to cause minimal or no herpes simplex virus (HSV) inactivation (Ourth et al., 1994; Yasin, et al., 2000; Benincasa et al., 2003), while α-helical magainins, dermaseptin, and melittin have shown quite potent anti-HSV activity (Aboudy et al., 1994; Yasin et al., 2000; Belaid et al., 2002; Matanic and Castilla, 2004). Conversely, β-sheet peptides such as defensins, tachyplesin, and protegrins as well as the β-turn peptide lactoferricin have all shown high activity towards HSV (Lehrer et al., 1985; Daher et al., 1986; Yasin et al., 2000, 2004; Andersen et al., 2003; Sinha et al., 2003; Jenssen et al., 2004; Chen et al., 2008a). It should be noted that within the different peptide subclasses, activity may vary considerably (Yasin et al., 2000).

***Structural requirements for antiviral peptides***

Synthetic analogues of several naturally occurring AMPs have been made in an attempt to identify important structural features contributing to the antiviral activity. Different strategies for design of such peptides have been pursued. Several groups have looked at the importance of charged and aromatic amino acids, since antiviral peptides are often highly cationic and amphiphilic (Daher et al., 1986; Tamamura et al., 1994; Yasin et al., 2000; Jenssen et al., 2004).

However, the spatial positioning of the charged amino acids seemed to be more important for antiviral activity than the actual net charge (Jenssen et al., 2004). For lactoferricin the nature of the aromatic amino acid appeared to be of minor importance for the antiviral activity, although its contribution to the secondary structure and thereby presentation of the charged residues might be crucial (Jenssen et al., 2005). Detailed studies on the influence of secondary structure domains on anti-HSV activity illustrated that the α-helicity of a peptide could not explain its antiviral activity (Jenssen et al., 2006), thus implying that the presentation of the charged residues is of greatest importance with respect to anti-HSV activity (Jenssen et al., 2004). The presence of hydrophobic and positively charged residues is critical but not sufficient for antiviral activity, and this may relate to different

conformations adopted by these peptides in the context of the native protein (Giansanti et al., 2005).

Lactoferricin and polyphemusin have β-structures stabilized by one and two internal disulfide bridges, respectively. These disulfide bridges have been shown to be crucial for the antiviral activity of the peptides (Tamamura et al., 1994; Andersen et al., 2001; Jenssen et al., 2004b). Despite their diverse structures, many peptides possess analogous antiviral modes of action (Jenssen et al., 2004a, 2004b), indicating that these peptides are able to interact with their targets, despite large structural differences. Interestingly, although the viral target of the AMPs appears to vary, the demonstrated antiviral effects are quite similar.

### *Mode of action of antiviral peptides*

***Blocking of viral entry by heparan sulfate interaction*:** Heparan sulfate is the most important glycosaminoglycan molecule with respect to viral attachment (Spillmann, 2001; Mettenleiter, 2002); consequently, blocking of heparan sulfate can reduce the viral infection (WuDunn and Spear, 1989; Shieh et al., 1992). The importance of heparan sulfate for different viral infections varies considerably. Enzymatic removal of cellular heparan sulfate and chondroitin sulfate has led to the observation that these proteoglycan molecules have minor influences on HIV attachment to host cells. However, it has been demonstrated that they are of major importance for HIV entry and replication (Argyris et al., 2003). The large number of positively charged residues in AMPs enable them to interact electrostatically with negatively charged cell surface molecules, including heparan sulfate. Human α-defensin, LL-37, and magainin have all been shown to interact with different glycosaminoglycan molecules (James et al., 1994; Schmidtchen et al., 2001, 2002). The peptides exhibited different antiviral effects for HSV type 1 and 2, an observation attributed to the combined effects of the amino acid content and the structures of the peptides (Andersen et al., 2003; Jenssen et al., 2004a,

2004b, 2006). Interestingly, differences in antiviral effects against HSV-1 and HSV-2 have also been reported for other polycationic and even polyanionic compounds (Hutton et al., 1973; Langeland et al., 1988). These differences may reflect the viral specificity of particular receptor molecules and the differential ability of peptides to interact with the different viral receptors. Bovine lactoferricin also exhibited anti-HIV activity, and this might be related to heparin sulfate binding. Binding of HIV to the CD4 surface receptor is known to induce conformational changes in gp120 in the viral envelope, resulting in increased affinity for heparan sulfate. This finding implies that heparan sulfate is important at a later stage of the virus-cell attachment process (Vives et al., 2005).

***Blocking of cell-to-cell spread*:** The effect of antiviral peptides is also related to their ability to inhibit the spread of virus from one cell to a neighboring cell across tight junctions (cell-to-cell spread) or inhibition of giant cell (syncytium) formation. Rabbit α-defensin NP-1 has been reported to inhibit both primary entry and cell-to-cell spread of HSV (Sinha et al., 2003). Similar anti-HSV activity has been indicated for bovine lactoferricin (Andersen et al., 2004; Jenssen et al., 2004), while the polyphemusin analogue T22 and tachyplesin I have been demonstrated to inhibit syncytium formation in cocultures of persistently HIV type 1 (HIV-1)-infected cells (Morimoto et al., 1991; Nakashima et al., 1992).

***Blocking of viral entry by interaction with specific cellular receptors*:** AMPs might interact directly with specific viral receptors on the host cell (Tamamura et al., 1996; Cole et al., 2002), influencing viral attachment, entry, or intracellular shuttling (Murakami et al., 1997; Tamamura et al., 1998). Recently a new HSV entry receptor was described (Perez et al., 2005); it is effectively blocked by binding of an α-helical peptide in a coiled-coil formation (Perez-Romero et al., 2005). There is certain evidence that this

receptor may be a potential target for several α-helical cationic peptides (Jenssen et al., 2004).

***Blocking of viral entry by interaction with viral glycoproteins*:** AMP interactions with glycoproteins in the viral envelope have been proposed to influence the viral entry process. The closely related retrocyclin-1 binds HIV gp120 with high affinity, as long as the envelope protein is glycosylated, probably resulting in an anti-HIV activity (Wang et al., 2003). The polyphemusin analogue T22 has been demonstrated to inhibit fusion between the HIV envelope and the host cell membrane (Nakashima et al., 1992) through specific binding of the viral envelope protein gp120 and the T-cell surface protein CD4 (Tamamura et al., 1996).

***Membrane or viral envelope interaction***

***Viral envelope interaction*:** AMPs are known for their ability to interact with lipid membranes, resulting in destabilization, translocation, pore formation, or lysis (Dathe and Wieprecht, 1999; Sitaram and Nagaraj, 1999). This makes the viral envelope a potential target for direct interaction. Indolicidin causes a direct inactivation of the HIV-1 particle, indicating a membrane-mediated antiviral mechanism (Robinson et al., 1998). Dermaseptin also exerts an anti-HIV activity prior to viral entry by direct interaction with the viral particle, disturbing its organization and disrupting the viral membrane (Lorin et al., 2005). In contrast, the anti-HSV activity of dermaseptin is proposed to result from blocking of viral entry by interaction with viral or cellular surface molecules involved in the attachment/adsorption/fusion phase of HSV (Belaid et al., 2002). Electron micrographs of the tachyplesin-1-treated VSV particle showed naked and damaged virions, confirming the direct effect of peptides on the viral envelope (Murakami et al., 1991).

***Cellular membrane interaction:*** Host cell membranes are involved in several stages of viral interaction, and due to the ability of peptides to

interact with and permeabilize membranes, this must be considered as a potential target. Similar permeabilization of the host cell membrane appears to occur (Lau et al., 2005), and the resulting alteration of host membranes could affect the efficiency of viral entry.

***Intracellular targets and host cell stimulation*: **It is known that antimicrobial host defense peptides such as PR39 and LL-37 are able to cross lipid membranes, including the plasma and nuclear membranes of host cells, while others are constitutively located as precursors inside host cell vacuoles (Haukland et al., 2001; Andersen et al., 2004; Lau et al., 2005). Cellular internalization of these AMPs can result in gene/protein stimulation, influencing host cell antiviral mechanisms (Bowdish et al., 2004) or might block viral gene/protein expression (Wachinger et al., 1998). Because of the known ability of peptides to interact with DNA (Park et al., 1998; Kanyshkova et al., 1999; Hsu et al., 2005; Song et al., 2005), it is speculated that they can directly influence viral nucleic acid synthesis, as shown for polyphemusin T22 and lactoferrin. Conversely, peptides are known to have immunomodulatory activities which include the upregulation of interferons and chemokines (Scott et al., 2002; Bowdish et al., 2005c; Chang et al., 2005), and thus peptides might exert their antiviral activities in part by stimulating the antiviral immune system of the host cell. Direct effects of peptides on the intracellular steps of viral infection have been demonstrated (Wachinger et al., 1998; Matanic and Castilla, 2004). Early steps in the HIV-1 replication cycle are inhibited by protegrin-1 (Steinstraesser et al., 2005), while HIV-1 integrase is effectively inhibited by indolicidin (Krajewski et al., 2004).

### *1.7.11. Mechanisms of selective toxicity*

An essential property of a safe antimicrobial factor is toxicity against its microbial targets without harming the host. The ubiquity of AMPs suggests that their antimicrobial property need not be associated with host toxicity. Most of the AMPs are synthesized as inactive proteins prior to

exposure to their targets, and AMP activation by post-translational processing ensures that the cellular membrane structures are protected from any contact with the active peptides prior to reaching their microbial targets. For the peptides that are secreted in specific extracellular milieu, the peptide concentration may be effective against specific pathogens but sublethal to host cells. In addition to environmental influence, there are some fundamental structural properties that may generally render microbial organisms more susceptible than the host to AMPs. This antimicrobial selectivity, although not completely understood for several AMPs, is partially explained by differences in lipid composition of eukaryotic and bacterial cell membranes (Bechinger et al., 1992; Biggin and Sansom, 1999). Eukaryotic membranes are likely to form hydrophobic interactions with AMPs due to the lower proportion of negative lipids and the presence of cholesterol in their membrane outer leaflet.

AMPs are known to form stronger interactions with bacterial endotoxins and negatively charged surface phospholipids (e.g. LPS, lipotheic acids, phosphatidyl glycerol) than with zwitterionic and cholesterol-containing liposomes (Gough et al., 1996; Zhang et al., 2000; Heinzelmann et al., 2001; Dathe et al., 2002) Similarly, higher negative:zwitterionic phospholipid ratios enhance AMP-induced leakage of fluorescent molecules (e.g. calcein) from model membranes (Matsuzaki, et al., 1991, 1995; Cabiaux et al., 1994; Giffard et al., 1997; Zhang et al., 2001). Thus, electrostatic interactions between AMPs and negative lipids on the bacterial surface may provide the energy that drives AMP insertion in microbial membranes.

Another key modulator of AMP-membrane interactions is hydrophobicity. While cationicity is the primary determinant of antibacterial selectivity, hydrophobicity probably enhances activity against gram-positive organisms and may compromise antimicrobial selectivity if raised beyond an optimal level. Hence, the amphipathic nature of AMPs, or the combined

structural contributions of hydrophobicity and cationicity are critical to antibacterial selectivity (Kang et al., 1998; Dathe et al., 1999, 2001; Giangaspero et al., 2001; Guerrero et al., 2004).

## *1.7.12 Characteristics that affect antimicrobial activity and specificity*

### *1.7.12.1 Amphiphilicity as a general strategy in the antimicrobial action of α-AMPs*

AMPs are usually grouped according to their secondary structures (Martin et al., 1995; Hancock, 1997) and within these structures, three distinct forms of amphiphilicity can be identified (Phoenix et al., 2002). Indolicidin (Staubitz et al., 2001) and tritrpticin (Yang et al., 2001) for example exhibit primary amphiphilicity with a core segment composed of a short central apolar sequence that is flanked at each end by cationic residues (Staubitz et al., 2001; Yang et al., 2001; Phoenix, et al., 2002). Studies on both AMPs have suggested this core segment is essential for membrane interaction and partitions into membranes such that its central apolar sequence resides within the membrane hydrophobic core region and both sets of flanking cationic residues interact with the lipid head-group region of the same leaflet (Rozek et al., 2000; Yang et al., 2003). Evidence implies that the distribution of hydrophobicity along these highly symmetrical sequences is important for peptide orientation during the antimicrobial action of these AMPs (Staubitz et al., 2001). In contrast to the above, cysteine rich β-sheet AMPs, such as defensins (Raj and Dentino, 2002), and other conformationally restrained AMPs (Marshall and Arenas, 2003), form a major group of peptides that invade microbial membranes though the use of tertiary amphiphilicity. Defensins may be taken as representative of tertiary amphiphiles and the sequences and folding patterns of these AMPs varies widely across mammals, insects and plants (Raj and Dentino 2002; Brogden et al., 2003),

generating a rich spectrum of amphiphilic structures. In this manner, tertiary amphiphilicity has been tailored by AMPs to facilitate microbial membrane invasion and the antimicrobial actions of a structurally diverse range of peptides that are appropriate to the defense needs of an equally diverse range of host organisms (Raj and Dentino 2002; Marshall and Arenas, 2003; Brogden et al., 2003). The final group includes α-helical AMPs (e.g. magainin) which possess secondary amphiphilicity (Phoenix et al., 2002, 2003). As for tertiary amphiphilicity, it is well established that variations in the secondary amphiphilicity of α-AMPs can lead to the use of different mechanisms of microbial membrane invasion and antimicrobial action.

#### *1.7.12.2 The influence of amino acid composition on the antimicrobial action of amphiphilic α-AMPs*

Peptides often contain the basic amino acid residues lysine or arginine, the hydrophobic residues alanine, leucine, phenylalanine or tryptophan, and other residues such as isoleucine, tyrosine and valine. Ratios of hydrophobic to charged residues can vary from 1:1 to 2:1. Amphipathic α-helical peptides are often more active than peptides with less-defined secondary structures. Clearly, a major role for strongly basic, anionic and polar residues on the one hand and apolar residues on the other hand, is to facilitate the formation of amphiphilic structure. Glycine, which is an efficient N-capping agent (Ladokhin and White, 1999), was also found to be abundant in α-AMPs and the propensity of peptides to form α-helical structure is known to be influenced by capping effects (Tossi et al., 2000). Furthermore, this propensity is strongly favoured by the C-terminal amidation of peptides, providing an additional hydrogen bond for α- helix-stabilization (White and Wimley, 1999). Also, residues such as leucine, alanine and lysine, which have a strong propensity to stabilize α-helical structure, are particularly well represented in α-AMPs.

#### 1.7.12.3 The influence of sequence length and molecular mass on the antimicrobial action of amphiphilic α-AMPs

The size of AMPs vary from 6 amino acid residues for anionic peptides to greater than 59 amino acid residues for Bac7. Even di- and tripeptides with antimicrobial activity have been reported. Currently, the shortest known α-AMPs include anoplin, a 10 residue wasp venom peptide, whilst the longest include the prolevitide precursor, a 68 residue amphibian extudate (Dennison et al., 2003). Most recently, the α-AMPs, active against gram-negative bacteria, exhibited the narrowest range of sequence lengths, 19 to 27 amino acids, and molecular masses, 1.7 to 2.5 kDa; whilst the peptides, active against both gram-positive and gram-negative bacteria, exhibited the widest variation in these parameter with sequence lengths between 10 and 68 residues and molecular masses between 1.1 and 7.4 kDa (Dennison et al., 2003). It is suggested that sequence length and molecular mass, per se, are not important to either the antimicrobial efficacy or specificity of the α-AMPs analyzed.

However, the vast majority of α-AMPs are <4.5 kDa and <45 residues in length (Dennison et al., 2003) and the relative invariance of these factors across a diverse range of species implies biological relevance to the role of these peptides as defense agents. This importance may derive from the fact that short, low molecular weight peptides are metabolically economical to the host and can be more easily stored in large amounts (Gallo et al., 2002), thereby increasing the efficiency of host response to microbial attack.

#### 1.7.12.4 The influence of pI, net charge and charged residues on the antimicrobial action of amphiphilic α-AMPs

Anionic peptides that are complexed with zinc, or highly cationic peptides, are often more active than neutral peptides or those with a lower charge. α-AMPs exhibit a wide range in isoelectric points (pI), varying from 12.7, shown by horse myeloid cathelicidin (Scocchi et al., 1999), to 4.2 shown

by enkelytin (Goumon et al., 1996) and the Amoeba poreforming peptide isoform A (Leippe et al., 1991). It is shown that there exhibit no discernable correlation between pI values and MICs of AMPs (Dennison et al., 2003).

The membranes of both fungi and gram-negative bacteria are generally more effective barriers to the action of AMPs than those of gram-positive bacteria (Phoenix et al., 2003) and these findings suggested that the unique specificity of gram-positive α-AMPs for the more susceptible membranes of gram-positive bacteria might be related to reduced cationicity. Studies on individual α-AMPs have shown that net charge can modulate the antimicrobial specificity, and efficacy, of these peptides (Tossi et al., 2000). Whilst a direct correlation between magnitude of positive charge and efficacy could therefore not be demonstrated, it is well established that the net positive charge of α-AMPs enables these peptides to target the anionic membranes of microbes and that this positive charge primarily derives from the enrichment of these peptides with the strongly basic residues, lysine and arginine (Papagianni, 2003).

## *1.7.13 Potential applications of AMPs*

### *1.7.13.1 Development of AMPs for clinical applications*

There is no question that, with the increasing antibiotic resistance problem, there is a need to develop new classes of antibiotics (Bonomo, 2000). In this respect, AMPs are on the brink of a breakthrough. Cationic AMPs have many of the desirable features of a novel antibiotic class (Hancock, 1998). In particular, they have a broad spectrum of activity, kill bacteria rapidly, are unaffected by classical antibiotic resistance mutations, do not easily select antibiotic resistant variants, show synergy with classical antibiotics, neutralize endotoxin, and are active in animal models. The intriguing idea of developing AMPs as innovative antibiotics has been followed up by several biotechnological companies. With the use of protein-biochemical methods and recombinant DNA technology, the structures of

naturally occurring peptides serve as starting points for the development of new drugs. Several derivatives of AMPs have been through the pharmaceutical process, including human phase I–III studies. The use of human AMPs as drug is restricted so far by the still unknown biological function of these molecules and the high cost for the generation of sufficient amount (Bals, 2000).

Most clinical trials proposed or underway involve topical therapy. Such treatments are likely to be effective and safe because the more toxic cationic peptides and lipopeptides, including gramicidin S and polymyxin B, are already in skin creams. Cationic peptides with excellent activity against a broad range of bacteria would be especially indicated where there is a risk from seriously resistant pathogens or where current treatments are ineffective. A more advanced form of topical treatment would be aerosol therapy into the lung, an organ especially prone to problems with resistant microorganisms. Aerosol treatment is already used successfully for the polycationic trisaccharides gentamicin and tobramycin and the polycationic lipopeptide colistin in treatment of *P. aeruginosa* lung infections in patients with cystic fibrosis. These antibiotics are usually given in liposomal formulation and show little toxicity and reasonable efficacy long term. Oral therapy may be possible for gastrointestinal infections; nisin is being developed through to clinical trial in *Helicobacter pylori* infection (Hancock, 1997). Various applications of AMP are given in Table 1.5.

**Table 1.5 Applications of AMP**

| AMP | Source | Application |
|---|---|---|
| alfAFP | Plant (Alfalfa) | Antifungal effect in transgenic potato |
| Cecropin B | Insect | Antibacterial effect in transgenic rice, antiviral effect against important viral pathogens of fish |
| CEMA | Synthetic (Cecropin-melittin) | Antifungal effect in transgenic tobacco, antibacterial effect against bacterial pathogen in fish |

| Lactoferricin | Cow | Mastitis control in goat |
|---|---|---|
| LzP | Hen (Lysozyme) | Food preservative |
| MSI-99 | Synthetic (Magainin analog) | Antifungal and antibacterial effect in transgenic tobacco, tomato, grapevine, banana |
| Nisin | *Lactococcus lactis* | Food preservative |
| Polyoxins | *Streptomyces* | Fungicide, insecticide |
| Pn-AMP2 | Plant (Morning glory) | Antifungal effect in transgenic tobacco |
| MSI-78 | Synthetic (Magainin analog) | Wound healing (Phase III clinical trial) |
| IB367 | Pig protegrin analogue | Against polymicrobic infections in oral mucasitis |
| NEUPREX1 | Synthetic (Cationic peptide fragment) | For endotoxin mediated complications of meningococcal disease (Phase III clinical trial) |
| MX-226 / CPI-226 | Bovine indolicidin-based | Prevention of contamination of central venous catheters (Phase III clinical trial) |
| MX594AN | Indolicidin-based | Against mild to moderate acne (Phase II clinical trial) |
| Polymyxin B & E | *Bacillus polymyxa* | Food preservative, against multi-drug resistant *Pseudomonas*, Topical application |
| Gramicidin S | *Bacillus brevis* | Food preservative, Topical application |
| hLF1-11 | Human lactoferricin-based | For the prevention of infections in patients undergoing allogeneic stem cell transplantation |
| Daptomycin | *Streptomyces roseosporus* | FDA approved (2003) - for the treatment of skin infections caused by *S. aureus* |
| Colomycin | *Bacillus polymyxa* | Systemic application |
| Pexiganan | Magainin analog | For the treatment of diabetic foot ulcer infections (Phase III clinical trial) |
| MX-226 / CPI-226 | Cationic peptide | For the prevention of catheter-related infections (Phase III clinical trial) |
| MX594AN | Indolicidin-based | For the treatment of acne (Phase II clinical trial) |

### 1.7.13.2 Introduction of peptide antibiotics to the market

Due to the increasing interests of AMPs, many companies are making efforts to introduce the AMP products to the market. Natural AMPs have potential application in food preservation as they specifically kill microbial cells by destroying their unique membranes. LAB bacteriocins have well-documented lethal activity against food-borne pathogens and spoilage microorganisms (Cleveland et al., 2001), and can play a vital role in the design and application of food preservation technology (Leistner and Gorris, 1995; Montville and Winkowski, 1997). Currently, nisin is approved as a food preservative in more than 40 countries worldwide (Delves- Broughton, 1990) and the use of pediocin PA-1 is covered by several European and US patents (Vandenbergh et al., 1989). Both nisin and pediocin PA-1 have applications in dairy and canned products (Leisner et al., 1996; Montville and Winkowski, 1997).

Magainin Pharmaceuticals have taken the α-helical magainin variant peptide MSI-78 into phase-III clinical trials in studies of efficacy against polymicrobic foot-ulcer infections in diabetes. Applied Microbiology has initiated a trial testing the efficacy of the bacterial lantibiotic peptide nisin against *Helicobacter pylori* stomach ulcers. Iseganan (IB-367, Intrabiotics, Mountain View, CA, USA), a protegrin derivative (Mosca et al., 2000), has passed phase II clinical trials for application against oral mucositis successfully and the company has announced plans to launch Phase II/III clinical study to investigate iseganan HCl (Giles et al., 2002) in the prevention of ventilator-associated pneumonia (VAP). Another formulation of this company, iseganan HCl solution for inhalation, has completed phase I clinical trials in cystic fibrosis patients. Other companies, for examples, Periodontix Inc. (Watertown, MA, USA) has entered phase I clinical trials for the application of a histatin-derived peptide against oral candidiasis and Trimeris (Durham, NC, USA) has successfully completed a phase II clinical

trial, in which peptide T-20 (Su et al., 1999; Cohen et al., 2002; Wei et al., 2002) reduced the viral load of HIV-infected patients with up to 97%. Also Demegen (Pittsburgh, PA, USA) has successfully completed animal studies with peptide D2A21 (Robertson et al., 1998) as therapeutic for several types of cancer and has been developing this peptide gel formulation as a wound healing product to treat infected burns and wounds. Demegen's P113L (histatin 5 fragment) Oral Rinse exhibits significant binding to oral mucosal membranes and has an excellent human safety profile in over 400 treated patients. Another product of Demegen, P113D derived from histatins (Sajjan et al., 2001), had been granted orphan drug status for the treatment of cystic fibrosis infections. Interestingly, a number of evidence has shown efficacy of some AMPs against systemic infections, including α-helical-peptide (SMAP29) efficacy against *P. aeruginosa* peritoneal infections, β-sheet-protegrin efficacy against methicillin-resistant *S. aureus* (MASA), vancomycin-resistant *Enterococcus faecalis* (VRE) *and P. aeruginosa* infections, and indolicidin in liposomal formulation against *Aspergillus* fungal infections (Ahmad et al., 1995; Steinberg et al., 1997; Saiman et al., 2001). Entomed (Illkirch, France)'s product, heliomicin (Lamberty et al., 2001) for systemic antifungal treatment is under preclinical stage. Human lactoferricin (AM Pharma, Bunnik, Netherland) and bactericidal/permeability-increasing protein (Xoma, Berkeley, CA, USA, Gray et al., 1989) have also been proved to have potential for systemic applications. Indeed, Neuprex™, a systemic formulation of the recombinant BPI-derived peptide rBPI 21 (Xoma Corp., Berkeley, CA, USA, Horwitz et al., 1996), has proven to be very effective in treatment of meningococcal sepsis in phase II/III clinical trials and more than 1000 patients have received NEUPREX in clinical studies without any safety concerns. Other major companies marketing AMPs include Novozyme (http://www.novozymes.com/en) and Micrologix (Migenix; http://www.migenix.com).

### *1.7.13.3 Limitations as therapeutics*

The considerations that will determine any clinical use of cationic peptides include toxicity, stability, immunogenicity, route of application, and formulation, and very little information on these questions has been published. The future of AMPs as antibiotics appears to be great and as mentioned above. Despite this, many issues remain to be solved. For example, these peptides have relatively high molecular weights compared with most antibiotics and will have to be produced recombinantly to keep prices down (Hancock and Lehrer, 1998). Although several processes for doing this have been described, they have not yet been successfully performed on an industrial scale. Another issue is toxicity. AMPs are generally considered to be highly selective antimicrobial agents. However, peptides do not discriminate absolutely between eukaryotes and prokaryotes, although the former are less sensitive. Some cationic AMPs are very toxic for mammalian cells (e.g. bee venom melittin), whereas others show little or no acute cytotoxicity.

Several studies show that simple eukaryotes, such as yeasts, fungi, parasites (Jaynes et al., 1988; Ahmad et al., 1995), even as large ones as planaria (Zasloff, 2002), are effectively killed by cationic peptides. In addition, their unique pharmacological properties have limited their application to topical use. Another issue would be their lability to proteases in the body. In this regard, there are strategies for protecting the peptides from proteases, including liposomal incorporation or chemical modification. Recent publications describe also changes in bacterial cell wall components induced by environmental conditions, which may be involved in bacterial resistance towards AMPs (Groisman et al., 1997; Wosten and Groisman, 1999). Technical difficulties and high production costs have made the pharmaceutical industry reluctant to invest much effort in the development of antibiotic peptide therapeutics so far. The AMPs are too expensive or with

a too limited spectrum to be used on a large, commercially interesting scale. The biggest challenge of the near future will be to overcome the pharmacological limitations of these interesting molecules and to develop them into therapeutics.

#### *1.7.13.4 AMP resistance*

Bacterial pathogens have developed the means to curtail the effect of AMPs. Direct degradation of AMPs and modification of cell surface properties are two major strategies used by Gram-negative bacteria to resist the bactericidal activity of AMPs. The former strategy is dependent on the production of outer membrane-associated proteases, which cleave AMPs outside the cells and enable bacteria to evade killing. Because AMPs act on bacterial membranes, Gram-negative bacteria can also protect themselves from attack by AMPs via modification of their cell surface properties to prevent the binding of AMPs to the outer membrane or decrease the permeability of the outer membrane (Guo et al., 1997, 1998; Ernst et al., 1999). Furthermore, synergistic action of multiple resistance strategies can greatly decrease the bactericidal activity of AMPs. Another feature of peptides that may interfere with their use as therapeutics is immunogenicity. To this end, as this paragraph illustrate, the pharmacology and pharmacokinetics of AMPs are still unknown and many studies on these topics are required before the feasibility of peptide therapeutics will be generally accepted by the pharmaceutical industry.

#### *1.7.13.5 Possible explanations for the continued success of AMPs*

The dearth of reported cases of bacterial resistance has led to the suggestion that resistance to these immune peptides is unable to develop (Hancock and Scott, 2000). Nevertheless, bacteria have been exposed to the actions of AMPs for many millions of years and infections can still be rapidly cleared (Hurst et al., 2003); this contrasts dramatically with synthetic antibiotics. There must therefore, be some aspect of AMPs or the manner in

which they are administered which results in their continued success, despite the selective advantage that resistance would hold for bacteria. Although recognition proteins are thought to change under directional selection, AMPs have been found to be highly conserved; this suggests that co-evolution with pathogens is not occurring (Jiggins and Kim, 2005). The specific characteristics of the antimicrobial immune response limit the development of bacterial resistance or the selective enrichment of resistant mutants once they occur. It has been proposed that this may be due to the specific mode of action of AMPs. Many commonly used synthetic antibiotics harm bacteria by blocking the action of an essential protein (Gura, 2001). In contrast, AMPs generally attack the bacterial cell membrane (Merrifield et al., 1994; Lowenberger, 2001; Papagianni, 2003; Levashina, 2004). It has been suggested that because the AMPs target such a basic physical property, bacteria are unlikely to develop resistance (Hancock and Scott 2000; Gura, 2001; Zasloff, 2002). AMPs are effective against a range of pathogens including bacteria, fungi, and some enveloped viruses (Dang et al., 2006). Another possible explanation for the continued success of insect AMPs is that they are capable of influencing the bacterial mutation rate. It is thought that cationic peptides might induce genomic responses in bacteria treated with AMPs, in addition to any lethal effect on the bacterial membrane (Hong et al., 2003). The proposal that antibody multi specificity can be mediated by conformational diversity of pre existing isomers to increase the effective size of the antibody repertoire (James et al., 2003), is perfectly applicable to understand diversity of existing AMPs as well as the potential of those derived from multiple and heterogeneous type of precursors. Only time will verify these assumptions.

## *1.8 Relevance of the present study*

Despite significant efforts in the identification and characterization of a large number of pathogens affecting shrimp production and an increasing

number of research groups studying crustacean immunity, host defense mechanisms in shrimps remains relatively poorly understood. Although during recent years knowledge at the molecular level has been gathered on the ways in which invertebrates defend themselves against bacteria and fungi, little is known about the defense mechanisms elicited by virus. Also, in comparison with the vertebrates in commercial animal production, there are practically no criteria for specific evaluation of the health status of cultured shrimps. A better understanding of the haemolymph defense system will facilitate a further development of health parameters. Therefore, the aim of the present research was to contribute to the knowledge on the functioning of the haemolymph defense system of shrimp in terms of AMP gene expression. During the last decade, although, intensive efforts have been undertaken for characterization of the structural and a few non-structural protein genes of WSSV, little is known about the molecular mechanisms underlying the WSSV life cycle and mode of infection. In contrast to the great amount of literature on antibacterial and, to a lesser extent, on antifungal and antiprotozoan activities, few studies have focused on the effect and mode of action of AMPs on viruses. Although the exact mechanism is still not yet completely understood, several immunostimulants and probiotics are being applied in invertebrate culture systems, to induce and build up protection against a wide range of diseases. However, nothing is known about the relationship between AMP gene expression and administration of immunostimulants/probiotics. In order to develop effective intervention strategies for disease control in shrimp culture, a scientific basis for the health modulators in shrimp is required. Therefore, the present study also aims to obtain a better understanding of the functioning of the defence system of shrimps in terms of AMP gene expression in response to the administration of immunostimulants, probiotic bacteria and WSSV challenge.

## 1.9 Objectives

The present study was undertaken with the following objectives:

- Molecular characterization and phylogenetic analysis of AMPs in *P. monodon* and *F. indicus*
- Time-course of AMP gene expression in response to WSSV challenge in *P. monodon*
- Tissue-wise expression profile of AMP genes in *P. monodon*
- Expression profile of AMP genes in response to the administration of immunostimulants in *P. monodon*
- Expression profile of AMP genes in response to the administration of probiotic bacteria in *P. monodon*

## 1.10 Outline of the thesis

The thesis is presented in seven chapters. Chapter 2 and 3 deals with the molecular characterization and phylogenetic analysis of three major families of AMPs viz. ALF, crustins and penaeidins in the giant tiger shrimp, *P. monodon* and the Indian white shrimp, *F. indicus* respectively. Chapter 4 elucidates the expression profile of AMP genes and WSSV genes in the haemocytes of *P. monodon* in response to WSSV challenge on a time-course basis. Chapter 5 and 6 elucidates the gene expression profiling of AMPs in the haemocytes and other tissues of *P. monodon* in response to the administration of immunostimulants (two strains of marine yeasts and their respective β-glucans) and probiotic bacteria (*Bacillus* MCCB101, *Micrococcus* MCCB104 and combination of *Bacillus* + *Micrococcus*) pre- and post-challenge WSSV respectively. The present research work is summarized in chapter 7 with special emphasis on salient findings of the study. Future prospects of the work are also discussed. This is followed by a list of references, GenBank accessions and publications.

# CHAPTER-2

## *Molecular Characterization and Phylogenetic Analysis of Antimicrobial Peptides in Penaeus monodon*

## 2.1. Introduction

Invertebrates have developed an efficient mechanism against microbial infection through the production of potent antimicrobial peptides (AMPs) and polypeptides. AMPs are essential effectors in the innate immune response in most organisms. AMPs act against a broad range of microbes including gram-negative, gram-positive, fungal, and some viral and protozoan species (Bachere, 2000a). During the past two decades, several hundred AMPs have been isolated and characterized from invertebrates including insects, limulids, molluscs, shrimps, and arachnids (Bulet et al., 2004). Although AMPs differ in their amino acid sequences and secondary structure, most of these molecules share some common features, such as low molecular weight (less than 15 kDa, with less than 50 amino acid residues), high positive charge residues and adopt an amphipathic structure (α–helix, hair-pin like β-sheet, β–sheet or α–helix/β–sheet mixed structures) that is believed to be essential for their antimicrobial action (Bulet et al., 2004). They are produced mainly through regular processes of gene transcription and ribosomal translation, often followed by further proteolytic processing. Studies in mammals and a few invertebrates have indicated that AMP function is regulated at multiple levels, including transcriptional and translational control, subcellular localization, post-translational modification and secretion regulation (Kaiser and Diamond, 2000).

Several studies have been carried out to seek a better understanding of the mode of action of the different classes of AMPs currently available, particularly against bacteria. Considering their structure and physicochemical characteristics, there is compelling evidence that a common step in the microbial killing mechanism consists of their electrostatic

interaction with negatively charged cell walls and/or membranes, followed by the peptide insertion and membrane permeabilization (Brogden, 2005). This mode of action could explain why AMPs fail to induce resistance and are highly effective against pathogenic microorganisms resistant to conventional antibiotics (Vizioli and Salzet, 2002). The development of resistance to AMPs appears to be rather difficult, since it implies dramatic changes in the phospholipid composition of cell membranes that may result in pleiotropic effects on transport and enzymatic systems, seriously threatening microorganism survival (Alberola et al., 2004). In addition to their capacity to permeabilize membranes, recent observations have shown that some peptides can also be translocated to the microorganism cell cytoplasm and interact with intracellular targets, interfering with cell wall, nucleic acid or protein synthesis and with enzymatic activity (Brogden, 2005).

In penaeid shrimps, three main families of AMPs have been currently described and characterized from the haemocytes: penaeidins (Destoumieux et al., 1997), crustins (Bartlett et al., 2002) and anti-lipopolysaccharide factors (ALFs) (Somboonwiwat et al., 2005). Penaeidins are mainly active against gram-positive bacteria, filamentous fungi (Destoumieux et al., 1999), viruses and protozoans (Bachere, 2003). Whereas ALFs have a broader antimicrobial spectrum, including gram-negative bacteria (Somboonwiwat et al., 2005; de la Vega et al., 2008). Conversely, crustins are reported to have a more-restricted activity spectrum, affecting mainly marine gram-positive bacteria (Relf et al., 1999; Bartlett et al., 2002; Zhang et al., 2007). In shrimps, AMPs have been proved to be produced and stored in the haemocytes granules (Shigenaga et al., 1990; Destoumieux et al., 2000b; Mitta et al., 2000, Lamberty et al., 2001). AMP gene expression and distribution are regulated through haemocyte reactions, such as cell recruitment and massive release of AMPs and through haemocyte proliferation, which characterizes a system reaction (Munoz et al., 2002). Transcripts of AMP-encoding genes have also been

observed in gills, heart and intestine (Imjongirak et al., 2007; Zhang et al., 2007; Supungul et al., 2008) but as these tissues are highly vascularised, it is assumed the transcripts from these organs is due primarily to the haemocytes.

### 2.1.1. *Anti-lipopolysaccharide factor (ALF)*

Anti-lipopolysaccharide factor (ALF), a small basic protein, was initially isolated and characterized from the haemocytes of horseshoe crabs, *Tachypleus tridentatus* (TALF) and *Limulus polyphemus* (LALF) (Tanaka et al., 1982). ALFs can bind and neutralize lipopolysaccharide (LPS) and possess broad spectrum antimicrobial activities. ALF has a strong antibacterial activity, especially on the growth of gram-negative R type bacteria (Wang et al., 2002). As most of the infectious bacterial pathogens in shrimp are gram-negative bacteria, studies on ALF will be more useful in shrimp disease control. Structurally, ALF contains two conserved cysteine residues comprising a cluster of positive charged residues within a disulphide loop that has been defined as the LPS-binding domain (Hoess et al., 1993). The function of the ALF lies on the positively charged cluster within a disulfide loop in its primary structure as suggested by Aketagawa et al. (1986). From the three-dimensional structure of LALF, the amphipathic disulfide loop binds lipid A and neutralizes the biological effects of LPS (Hoess et al., 1993). The ALFs from horseshoe crabs, shrimps and crabs also contain the two highly conserved-cysteine residues and the highly conserved cluster of positively charged residues within the disulfide loop. The synthetic disulfide loops from ALFPm3 and ALFSp have been shown unequivocally to be antimicrobially active, though the actual mechanism is not known (Somboonwiwat et al., 2005; Imjongjirak et al., 2007).

They are produced and stored in the haemocyte, a prime cell in the immune system (Destoumieux et al., 2000b; Somboonwiwat et al., 2005; Burge et al., 2007). The ALF is a small basic protein that has the ability to

inhibit the endotoxin or lipopolysaccharide (LPS) mediated coagulation system, and thus exhibits strong anti-bacterial activity against the gram-negative bacteria in particular (Morita et al., 1985). It binds to LPS and neutralizes LPS-induced gelation of limulus amebocyte lysate (Alpert et al., 1992). Limulus ALF has been proved to possess strong antibacterial activity, especially on the growth of gram-negative bacteria (Wang et al., 2002). As most of the infectious bacterial pathogens in shrimp are gram-negative bacteria, studies on ALF will be more useful in shrimp disease control.

Although the anti-gram-positive bacterial activity of ALFs has been reported, its binding to the gram-positive bacteria has not been studied in depth (Somboonwiwat et al., 2005). With strong and broad spectrum antimicrobial activity, the ALFs and their derivatives are becoming potential therapeutic agents for prophylactic treatment of viral and bacterial infectious diseases, as well as for septic shocks (Vallespi et al., 2000; Pan et al., 2007).

ALFPm3, the most abundantly expressed isoform of *P. monodon*, was cloned and expressed in a recombinant system and its antimicrobial activity was investigated (Somboonwiwat et al., 2005). ALFPm3 has a potent and broad spectrum of activity against several classes of microorganisms, including gram-positive bacteria, gram-negative bacteria and filamentous fungi (Somboonwiwat et al., 2005). Moreover, ALFPm3 has also an antiviral effect against human herpes virus (HSV-1) (Carriel-Gomes et al., 2007). The ALFPm3 has been expressed in the yeast *Pichia pastoris* expression system (Somboonwiwat et al., 2005) and the recombinant ALFPm3 protein exhibited antimicrobial activity against gram-negative and gram-positive bacteria and fungi. Another possible role of the ALF as an antiviral molecule has been shown in *P. leniusculus* as it interferes with the replication of white spot syndrome virus (Liu et al., 2006).

More recently, the ALF was identified and characterized in many crustaceans including *Fenneropenaeus chinensis*; ALF*Fc* (Liu et al., 2005), *Marsupenaeus japonicus*; M-ALF (Nagoshi et al., 2006), *Penaeus monodon*;

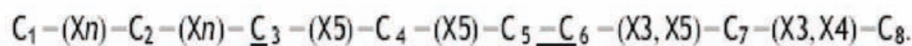
ALF*Pm*1-5 (Supungul et al., 2002; Somboonwiwat et al., 2005), *Litopenaeus setiferus* (Gross et al., 2001), *Pacifastacus leniusculus* (Liu et al., 2006), *Carcinus maenas* (Towle and Smith, 2006), *C. sapidus* (Towle and Smith, 2006), *Scylla paramamosain* (Imjongjirak et al., 2007), *Macrobrachium olfersi* (Rosa et al., 2008), *Farfantepenaeus paulensis* (Rosa et al., 2008) and *Litopenaeus schmitti* (Rosa et al., 2008).

## 2.1.2. Crustins

### 2.1.2.1. Structural features

Crustins, a widely distributed family of AMPs was first isolated from the shore crab, *C. maenas* as an 11.5 kDa peptide (Relf et al., 1999). Crustins are cationic, cysteine-rich antimicrobial AMPs having molecular weight of 7-14kDa, with an isoelectric point in the range of 7.0-8.7, present in crustaceans that contain one whey-acidic protein (WAP) domain at the carboxy terminus (Smith et al., 2008). This domain has eight cysteine residues in a conserved arrangement that forms a tightly packed structure described on PROSITE as a four-disulphide core (4DSC). The 4DSC motif is not unique to crustins. The term 'WAP' is derived from the name given to a family of proteins, originally discovered in the whey fraction of mammalian milk. Whilst all these milk proteins are characterized by possession of two WAP domains, each comprising 50 amino acids (Ranganathan et al., 1999), numerous other non-milk WAPs are now also known and these may have one or more 4DSCs. Amongst these non-milk WAPs are small secretory proteins with protease inhibitory properties or regulatory functions in growth, tissue differentiation or regulation and may sometimes be expressed in certain cancer states (Schalkwijk et al., 1999; Bouchard et al., 2006). Particularly well-known WAP domain containing proteins in mammals are anti-leukoproteinases, elafins and trappins. Analysis of numerous WAPs from vertebrates reveals a high degree of similarity between the WAP domain structures, and Ranganathan et al. (1999) have proposed that the PROSITE definition of the domain

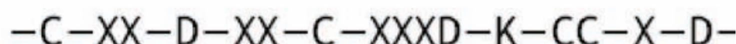
structure be modified to the following identifying motif whose positions of the eight conserved cysteines ($C_1$-$C_8$) are as follows:



X indicates any residue and Xn is a stretch of n residues. The signature motif of the central four cysteines that form the basis of the 4DSC is underlined. The definition of a crustin offered here complies with this format and thereby excludes the many cys-rich crustacean antimicrobial proteins that lack a WAP domain, for example defensins or penaeidins. Importantly, it embraces some crustacean molecules that have not so far been specifically designated as crustins or for which an antibacterial function is yet to be established. Moreover, it sets crustins within their own category of WAP-containing proteins from those found in other taxa (Schalkwijk et al., 1999; Talas-Ogras, 2004; Andrenacci et al., 2006; Nile et al., 2006) and distinguishes them from those that have a WAP domain at the N-terminus or more than one at the C-terminus, even though there may be some functional equivalence between these proteins in terms of their role in inflammation and/or antimicrobial properties (Hiemestra et al., 1996; Hiemstra, 2002; Hagiwara et al., 2003; Jimenez-Vega et al., 2007).

All crustins described to date possess a leader/signal sequence at the N-terminus and the WAP domain at the carboxyl end. The putative signal sequence at the N-terminus comprises ~16–24 amino acids, which in many species shows strong representation of valine residues. The cleavage site, which marks the end of the signal sequence, at least as predicted by software programs, is usually between alanine and glycine, although in some crustins it lies between glycine and glutamine, alanine or threonine. Analysis of genomic and recombinantly expressed carcinin has revealed that the signal and mature sections are probably not encoded by separate exons (Brockton et al., 2007). It is unclear if the signal sequence is directing trans-membrane transportation of the protein, as in insects and mammals, or if the mature

protein is released from the haemocytes through regulated exocytosis, as seems to be the case with the penaeidins (Destoumieux et al., 2000b; Munoz et al., 2002). The WAP domain, in contrast to the signal sequence, is highly conserved between species and in several crustins, especially those from shrimp, aspartic acid and lysine residues are positioned as follows:

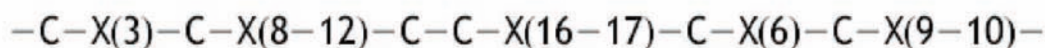



This arrangement, however, does not hold true in every case (Jiravanichpaisal et al., 2007). Other differences in the WAP domain of crustins could well come to light as more crustin-like sequences are determined for a wider range of crustacean species. Despite small differences in the sequences, the domain, itself, seems to form a tightly coiled structure enclosing two β-sheets and α-helical segment. Structural models of the WAP domain from three different crustins (Smith et al., 2008) suggest that the tertiary structure of this part of the molecule is well conserved between decapod species (Fig. 2.1, 2.2).

### *2.1.2.2. Crustin types*

The region between the signal sequence and WAP domain is variable but conforms to one of a small number of distinct structural patterns with regard to the presence or absence of other domains. The arrangements of these are largely, but not entirely, conserved within taxonomic sub-groups of arrangements within the decapoda. At least three main subgroups appear to exist and we propose that they should be designated Types I–III (Figure 2.1, 2.2) at least until future research, especially from non-decapod taxa, renders this classification redundant. The three types of crustin are distinguished on the basis of the domain structure between the signal sequence and the WAP domain. In Type I crustins, the region that lies between the signal sequence and the WAP domain is of variable length and cysteine-rich but has rarely more than six of these residues. These cannot form a full 4DSC configuration

and the region might be loosely thought of as resembling an incomplete 4DSC. A suitable consensus framework for this region might be:




These types of crustins are present mainly in crabs, lobsters and crayfish (the Pleocyemata) (Stoss et al., 2003; Hauton et al., 2006; Brockton et al., 2007; Christie et al., 2007; Jiravanichpaisal et al., 2007). Type II crustins, on the other hand, possess not only a cysteine-rich region but also a long glycine-rich domain of approximately 40–80 amino acid adjacent to the signal region (Fig. 2.1, 2.2). The number of glycines varies between species but is usually between 20 and 50, and in shrimp, it is often arranged as repeat VGGGLG motifs that vary in number from 5 to 8 (Bartlett et al., 2002; Supungul et al., 2004; Vargas-Albores et al., 2004; Rosa et al., 2007; Zhang et al., 2007; Amparyup et al., 2008; Supungul et al., 2008). Type II crustins were the second type to be described (Bartlett et al., 2002) and occur mainly in shrimp (Dendrobrachiata).

A third group of WAP domain-containing proteins from decapods resembles crustins but lack not only the gly-rich domain of the Type II molecules but also the cysteine-rich region present in both Type I and Type II (Fig. 2.1, 2.2). Only a few of these are known and so far reports of them are confined to the shrimps, *P. monodon* (Supungul et al., 2004), *L. vannamei* (Jimenez-Vega et al., 2004), *M. japonicus* (Rojtinnakorn et al., 2002) and *F. chinensis*. In the literature these types of proteins have been termed not crustins, but single-whey domain (SWD) proteins (Jimenez-Vega et al., 2004), chelonianin-like proteins (Chen et al., 2005) or anti-leukoproteinase-like proteins (Rojtinnakorn et al., 2002).

### *2.1.2.3. Crustin isoforms*

Molecular studies have revealed that some decapod species express more than one type of crustin that differ from one another only by a few amino acids, usually 1–4. The potential number of variants of the encoded

protein can thus, at least theoretically, be quite large. For example, evidence from cDNA libraries has revealed that multiple isoforms exist in *P. monodon* (Supungul et al., 2004, 2008), *L. setiferus* (Bartlett et al., 2002) and *M. japonicus* (Rattanachai et al., 2004). An interesting situation exists in *L. vannamei* for which Bartlett et al. (2002) have reported the occurrence of six possible crustin variants while Vargas-Albores et al. (2004) have discovered two. Possibly many other isoforms exist that are yet to be recorded. Depending on the amino acids involved and their location within the protein, isoforms may vary slightly in biological activity and undergo positive selection if any confer survival advantages to their host under local conditions.

The major work on different aspects of crustins include *L. vannamei* (Gross et al., 2001; Bartlett et al., 2002; Vargas-Albores et al., 2004; Jimenez-Vega et al., 2004), *L. setiferus* (Gross et al., 2001; Bartlett et al., 2002; Rosa et al., 2007), *P. monodon* (Jimenez-Vega et al., 2004; Supungul et al., 2004, 2008; Chen et al., 2004a, 2005; Jiravanichpaisal et al 2007; Zhang et al., 2007; Amparyup et al., 2008), *M. japonicus* (Rattanachai et al., 2004; Zhang et al., 2007), *L. schmitti* (Rosa et al., 2007) *F. chinensis* (Zhang et al., 2007; Amparyup et al., 2008), *F. brasiliensis* (Rosa et al., 2007), *F. paulensis* (Rosa et al., 2007) and *F. subtilis* (Rosa et al., 2007).

### *2.1.2.4. Biological properties*

Crustins are widely regarded as antimicrobial effectors, yet there have been surprisingly few studies of their antibacterial properties invitro. Carcinin, which was purified from the haemocytes of *C. maenas* on the basis of its ability to inhibit the growth of bacteria, appears to be active against gram-positive but not gram-negative bacteria (Relf et al., 1999). The crustins purified from the spider crab, *Hyas araneus* and shrimp *F. chinensis*, also seem to kill gram-positive bacteria (Haug et al., 2006; Zhang et al., 2007), but no detectable activity against gram-negative bacteria or fungi (Zhang et al., 2007). A crustin type II from *P. monodon* is the only crustin so far claimed to kill gram-negative bacteria (Amparyup et al., 2008). As yet no studies have

been made of the spectra of antibacterial activities of the Type III molecules and this is urgently required to confirm their status as members of the crustin family. It would be very interesting to see if they too kill gram-positives and have any effects at all on gram-negatives. Thus, whilst several crustins clearly have antibacterial effects primarily against gram-positive bacteria, broader specificity to deal with a wider range of pathogens can occur and seems to be achieved through sequence variations. The extent to which single nucleotide substitutions, as occurs in isoforms, alter function remains largely untested (Smith et al., 2008).

### *2.1.2.5. Mechanism of action*

Unfortunately, the mechanism of action of crustins on susceptible bacteria is unknown. As crustins all share a common WAP domain, this part of the molecule must play a key role in its antibacterial effects. It would appear that tertiary structure of the 4DSC is essential for microbicidal activity (Smith et al., 2008).

### *2.1.2.6. Synthesis and storage*

Crustins have been proved to be an important antimicrobial protein in the plasma and haemocyte granules of crustaceans and described as a component of the innate immune system (Vargas-Albores et al., 2004). These AMPs are dominantly synthesized and stored in haemocytes (Soderhall and Cerenius, 1998; Relf et al., 1999; Destoumieux et al., 2000b; Bartlett et al., 2002; Jimenez-Vega et al., 2004; Vargas-Albores et al, 2004; Hauton et al., 2006; Brockton et al., 2007; Imjongirak et al., 2007; Amaryup et al., 2008; Supungul et al., 2008) and their release from haemocytes is induced by bacterial infection (Soderhall and Cerenius, 1998; Munoz et al., 2002). Mechanisms of actions and functions of crustins are still largely unknown, although they contain a whey acidic protein (WAP) domain, common to proteinase inhibitory activities as well as antimicrobial activities (Vargas-

Albores et al., 2004). Many full-length cDNA and several ESTs of crustins have been described in a wide range of penaeid prawns.

#### *2.1.2.7. Patterns of expression*

Evidence of the role of crustins as direct antimicrobial defence effectors has been sought from studies of their expression in different tissues and following experimental infection. Certainly most crustins seem to be constitutively expressed by the blood cells (Soderhall and Cerenius 1998; Relf et al., 1999; Vargas-Albores et al., 2004; Hauton et al., 2006; Brockton et al., 2007; Amparyup et al., 2008; Supungul et al., 2008) often at very high levels (Hauton et al., 2006). The proteins seem to be synthesised in the granular haemocytes, at least from the few studies on haemocyte populations (Relf et al., 1999; Brockton et al., 2007). Transcripts of crustin-encoding genes have also been observed in gills, heart and intestine (Zhang et al., 2007; Supungul et al., 2008), but as these tissues are highly vascularised, it is not clear if the signal from these organs is due primarily to the haemocytes. Patterns of expression during development are also very poorly understood, as most studies of crustins have been performed only on late-stage postlarvae or adults. Larvae of the shrimp, *P. monodon*, have been reported to express a crustin transcript at high levels at all stages of development from nauplii stage IV through to juveniles (Jiravanichpaisal et al., 2007).

### *2.1.3. Penaeidins*

#### *2.1.3.1. Structural features*

The penaeidins, a unique antimicrobial peptide family, were first purified from haemocytes of the shrimp, *L. vannamei*, and their encoding genes were cloned from shrimp haemocyte cDNA (Destoumieux et al., 1997). Penaeidins are the well-characterized AMP family at the level of gene expression and biological activities. Penaeidins were the subjects of two review articles (Bachere et al., 2000a; Destoumieux et al., 2000a) soon after

their discovery and initial characterization, and they have since been subjects of extensive and indepth research (Bachere et al., 2004).

The penaeidins are highly cationic molecules having molecular weight of 5.5 to 6.6 kDa and pI ranging from 9.34 to 9.84. Penaeidins possess antibacterial, antifungal as well as chitin binding ability. Therefore, it is suggested that they can be involved in chitin assembly or wound healing and may be essential in shrimp protection during the moulting cycles (Destoumieux et al., 2000b). Penaeidins have several isoforms and are classified into Penaeidin-2 (PEN2), Penaeidin-3 (PEN3), Penaeidin-4 (PEN4) and Penaeidin-5 (PEN5) according to their similarity of amino acid sequences (Cuthbertson et al., 2002, 2005). The classification and characterization of penaeidin isoforms have been summarized in the database, PenBase (Guegen et al., 2006). Between three penaeidin subgroups, PEN3 is the most abundant at both levels of peptide and mRNA in *L. vannamei* haemocytes (Destoumieux et al., 1997, 2000b).

Penaeidins are composed of an N-terminal domain rich in proline residues and a C-terminal domain containing six cysteines that form three disulphide bridges. These features are usually associated with two distinct groups of antimicrobial peptides found in insects, that is proline rich peptides, active against gram negative bacteria, and insect defensins anti-gram positive peptides. Besides this chimera-like overall structure, penaeidins undergo post-translational modifications, such as C-terminal amidation (Destoumieux et al., 1997) which has also been observed in other marine invertebrate antimicrobial peptides such as the tachyplesins from *Tachypleus tridentatus* (Nakamura et al., 1988) and in insect cecropins (Li et al., 1988) where it is shown to be functionally important by increasing antimicrobial activity compared with the same peptides, which have a free carboxyl group. Penaeidins were constitutively expressed in their mature and active form in granular haemocytes of naive shrimps and were stored

within cytoplasmic granules of granular haemocyte populations (Munoz et al., 2002, 2003).

One of the most striking characteristics of the penaeidins is the presence of a 5′ UTR and leader sequence that is nearly identical within and between species. This contrasts with the proline rich domain (PRD) and cysteine rich domain (CRD) which are only about 40% similar across penaeidin classes regardless of species (O'Leary and Gross, 2006). Adding to the structural complexity of the penaedin family, each of these classes display a significant degree of isoform diversity (Bachere et al., 2000a; Gueguen et al., 2006). Multiple isoforms, generated by substitutions and deletions within the proline and cysteine-rich domains, have been reported at the mRNA levels for all the classes of penaeidins suggesting that this is a highly diverse gene family. O'Leary and Gross (2006) examined the genomic sources for the penaedin class penaeidin-2, -3 and -4 and has proved that penaeidin class is encoded by a unique gene and that isoform diversity is generated by polymorphism within each penaeidin gene locus.

Most groups working on shrimp from different parts of the world have now isolated numerous penaeidin sequences mostly by genomic approaches, and this family appears to be ubiquitous among penaeid shrimp species. At the moment more than 200 sequences can be found in the EMB/GenBank/DDBJ databases, some of which have yet to be published. Expressed sequence tag (EST) analysis from haemocyte cDNA libraries has shown that penaeidin transcripts are very abundant. In fact, penaeidins appear to represent 10.7 and 20% of all the sequences isolated from haemocytes of *L. vannamei* and *L. setiferus*, respectively (Gross et al., 2001).

The amino acid sequences deduced from the cDNA revealed that the penaeidins isolated from haemocytes are synthesized as precursor molecules consisting of a signal peptide (19–21 amino acids) immediately preceding the bioactive molecule (Destoumieux et al., 1997). The role of post-translational modifications observed in native penaeidin, like C terminal amidation and N

terminal pyroglutamic acid have been studied with a set of penaeidin variants. The results showed that these modifications had little effect on penaeidin AMPs (Destoumieux et al., 1999), but they increased the stability of penaeidin, to proteolysis (Destoumieux et al., 1997).

### *2.1.3.2. Diversity of penaeidins*

Recent studies based on genomic approaches have revealed the presence of penaedins in different shrimp species. Penaeidin sequences have been isolated and characterized from *L. vannamei* (Pacific White shrimp) (Destoumieux et al., 1997, Bartlett et al., 2002; Cuthbertson et al., 2002); *L. setiferus* (Atlantic White shrimp) (Cuthbertson et al., 2002; Bartlett et al., 2002); *L. stylirostris* (Blue shrimp) (Munoz et al., 2004); *M. japonicus* (Kuruma prawn) (Rojtinnakorn et al., 2002); *P. monodon* (Black Tiger shrimp) (Supungul et al., 2002; Chen et al., 2004b; Chiou et al., 2005, 2006; Hu et al., 2006); *F. chinensis* (Fleshy prawn) (Kang et al., 2004); *F. paulensis* (Sao Paulo shrimp) (Barracco et al., 2005); *L. schmitti* (Southern White shrimp) (Barracco et al., 2005).

All these peptides share high sequence similarities that have allowed the establishment of a penaeidin signature. The family appears to be characterized by (i) a highly conserved signal peptide (ii) an N-terminal proline-rich domain with the following signature (Y,F)T(R,G)P(X)2(R,K)P, and (iii) a C-terminal cysteine-rich domain with the following signature C(X)2-3C(X)7RXC C(X)5CC (Fig.2.3). Penaeidins have been divided to three subgroups, penaeidin-2, -3, -4 and -5, based on amino acid sequence comparisons and the position of specific amino acids (Cuthbertson et al., 2002; Bachere et al., 2004). In most of the cases, the various penaeidin subgroups have been shown to be expressed in one single individual (Gross et al., 2001), suggesting that the various penaeidins may have different biological functions in shrimp. Expressed sequence tag (EST) analysis from haemocyte cDNA libraries has also revealed the great abundance of penaeidin transcripts found in the shrimps. Penaeidins represent 10.7 and

20%, of all the sequences isolated in the shrimp species *L. vannamei* and *L. setiferus*, respectively. Finally, as shown by biochemical approach, the penaeidin-3 subgroup is dramatically the most abundantly produced (Destoumieux et al., 1997). It represents more than 90% of all the penaeidin mRNA sequences detected in both shrimp species (Cuthbertson et al., 2002).

### *2.1.3.3. Biological properties of penaeidins*

Penaeidin anti-bacterial activity is predominantly directed against gram-positive bacteria via a strain-specific inhibition mechanism and through multiple modes of action. Penaeidins display weak anti-bacterial activity in vitro against gram-negative strains including Vibrionaceae species. The C-terminal domain of penaeidins presents some similarities and partial conservation of a primary sequence motif common to several chitin-binding proteins isolated from plants (Raikhel et al., 1993). The penaeidin C-terminal domain was shown to confer to the whole molecule an ability to attach chitin tightly (Destoumieux et al., 2000b). Chitin-binding ability is most often related to an anti-fungal activity (Cuthbertson et al., 2006).

Penaeidins have broad-spectrum fungicidal activity against filamentous fungi but are found to be inactive against yeast such as *S. cerevisiae* or *Candida albicans* (Destoumieux et al., 1999). Interestingly, penaeidins are active against the shrimp pathogen *Fusarium oxysporum*, which is responsible for infections in different *Penaeid* shrimps (Rhoobunjongde et al., 1991). Many phytopathogenic fungal strains such as *Nectria haematococca*, *Alternaria brassicola*, *Neurospora crassa* and *Botritys cinerea* were shown to be sensitive to the peptides, indicating that penaeidins could have applications in agronomy as therapeutic agents. Penaeidin fungicidal activity against the different strains was shown to be associated with inhibition of spore germination. At lower concentrations, the peptides cause growth inhibition of fungal hyphae, resulting in morphological abnormalities.

One can assume that the peptides, bound to the shrimp cuticle tissues through chitin-binding property carried by the C-terminal domain, exert antimicrobial activity and initiate opsono-phagocytosis via the free N-terminal tail. This domain can adopt a conformation upon interaction with the bacteria membrane, which displays antimicrobial activity. From all hypotheses, the two domains are likely to have complementary activities, and their presence in one single peptide would be necessary for its full activity. Studies are being undertaken to address this question. Penaeidins, whose N-terminal sequence shares some similarities with plant extensin modules, may participate in protein–protein interactions and thus display various functions. Penaeidin chitin binding ability could participate in anti-microbial activity and in wound healing and chitin assembly. The peptides may play a role in the protection of the animals during molting cycle, when the animals are particularly exposed to potential infections. This dual function of penaeidins is likely determinant for the survival of the animals. To address this question, it is now under investigation whether the penaeidins conserve their anti-microbial activity when bound to the shrimp cuticle (Bachere et al., 2004).

### *2.1.3.4. Role of penaeidin $NH_2$- and COOH-terminal regions*

Penaeidins combine two domains in their overall structure, one proline-rich and the other cysteine-rich, usually observed in distinct groups of antimicrobial peptides (Destoumieux et al., 2000a). The overall biological activity of penaeidin may be associated with distinct properties of their two characteristic regions.

#### *$NH_2$-terminal region*

The penaeidin N-terminal proline-rich region shares sequence similarities with other proline-rich antimicrobial peptides (Gennaro et al., 1989; Schnapp et al., 1996). A synthetic peptide corresponding to the N-terminal proline-rich domain of penaeidin (residues 1–20) was produced and found to be inactive against both bacterial and fungal strains. Hence, it is

assumed that the penaeidin proline rich region is similarly involved more in target cell interaction than in direct antimicrobial activity. This is also consistent with the spectrum of penaeidin antimicrobial activity, which is directed mainly against fungi and gram-positive bacteria (Destoumieux et al., 1999), in contrast to most of the known proline-rich peptides, which have essentially anti-Gram-negative properties (Bulet et al., 1999).

***COOH-terminal region***

Since the penaeidin N -terminal region failed to exhibit antimicrobial activity, it is tempting to speculate that the C-terminal region mediates penaeidin antimicrobial properties. In addition, most of the antimicrobial peptides with intramolecular disulfide bridges, such as defensins, have antimicrobial properties similar to those observed for penaeidins. The penaeidin C-terminal region was found to display partial conservation of a repeated motif common to several chitin-binding proteins of the hevein family (Allen et al., 1996). Chitin binding has also been demonstrated for intact penaeidins but was absent for their synthetic N-terminal domain (20 amino acids) (Destoumieux et al., 1999). Thus it is believed that binding of penaeidins to chitin, and consequently their antifungal activity, is mediated by their C-terminal domain.

### 2.1.3.5. Synthesis and localization of penaeidins

In shrimp, haemocytes are the main source of penaeidin production (Destoumieux et al., 2000a, 2000b; Munoz et al., 2002, 2003, 2004; Kang et al., 2004). Penaeidins are constitutively expressed in their mature and active form in granular haemocytes of naive shrimps. About 30–40% of the circulating haemocytes express penaeidins (Meister et al., 1997). The peptides are stored within cytoplasmic granules of granular haemocyte populations, namely haemocytes with large granules and to a lesser extent also in haemocytes with small granules. The population of hyaline cells is devoid of penaeidins. The penaeidins could be secreted or released from haemocytes

by degranulation into the blood upon immune response stimulation (Bachere et al., 2000a). The distribution of penaeidin transcripts and proteins is restricted to haemocytes that are present strikingly in almost all the shrimp tissues, both circulating in blood vessels irrigating the tissues or infiltrating tissues such as the brain, subcuticular epithelia, midgut cecum, or muscle.

### *2.1.3.6. Effect of post-translational modifications on bioactivity of penaeidins*

Post-translational modifications were found to have little effect on penaeidin antimicrobial properties (Destoumieux et al., 1999), but possibly they increase the stability of penaeidins, which are highly resistant to proteolysis (Destoumieux et al., 1997). N-terminal blocking has no effect on penaeidin antimicrobial properties (Destoumieux et al., 2000a). Moreover, when C-terminal amidation is replaced by an extra glycine residue, penaeidin antifungal properties are unaltered, and antibacterial activity is decreased only two-fold. The partial loss of antibacterial activity that occurs in non-amidated penaeidins may be due to the loss of a positive charge at the C-terminus, and consequently to less efficient interaction of the peptide with bacterial membranes. Until now in shrimp, only penaeidins, a family of antimicrobial peptides, have been intensively studied in terms of biological properties, antimicrobial activities, gene expression and localization in response to infection (Bachere et al., 2004). The other antimicrobial effectors recently identified such as crustins or ALFs remain to be characterized for their biological properties and immune functions.

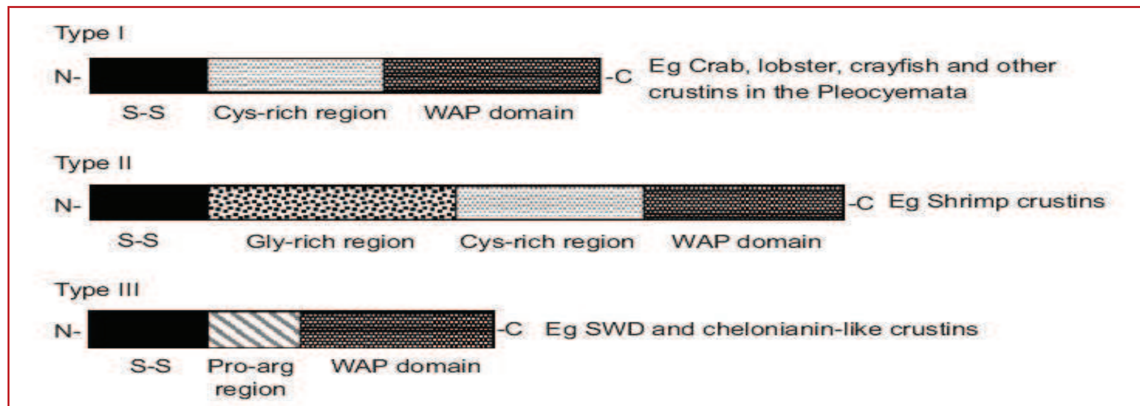



**Fig.2.1. Schematic representation (not to scale) of the domain organization of the three main crustin types from decapods. S-S = Signal sequence. (Adapted from Smith et al., 2008)**

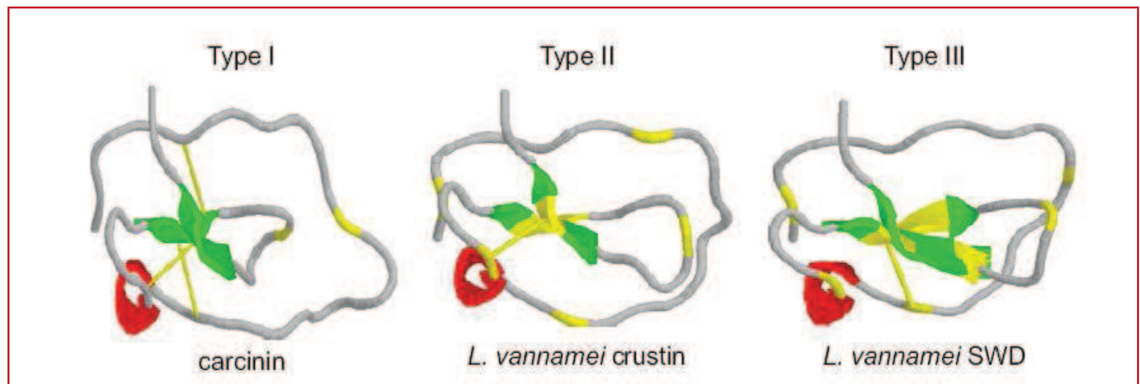



**Fig.2.2. Structural models WAP domain in various decapod crustins created with SWISS-MODEL Server. (Adapted from Smith et al., 2008)**

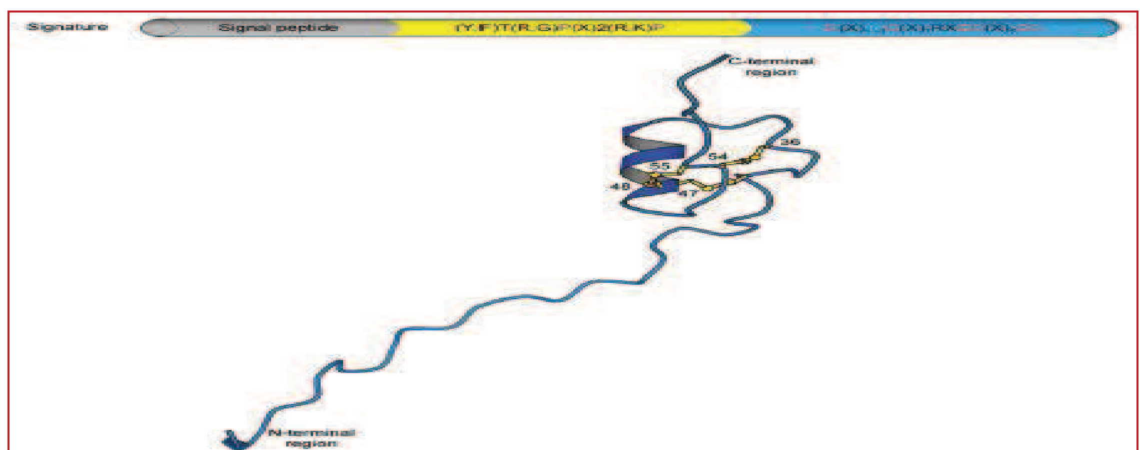



**Fig.2.3. The penaeidin signature (Adapted from Bachere et al., 2004)**

# 2.2. Materials and Methods

## *2.2.1. Experimental animals*

Healthy adult *P.monodon* (25-30 g) (Fig. 2.4) were collected from a shrimp farm at Vypeen, Kochi and were brought to the laboratory.

**Fig.2.4. Experimental animal - Adult black tiger shrimp, *Penaeus monodon***

## *2.2.2. RNase control*

### *2.2.2.1. Basic precautions*

Some basic precautions were taken when working with RNA. These include:

1. Wearing gloves throughout experiments to prevent contamination from RNases found on most human hands
2. Changing gloves after touching skin, door knobs, and common surfaces
3. Having a dedicated set of pipettors that are used solely for RNA work
4. Using tips and tubes that are tested and guaranteed to be RNase-free
5. Using RNase-free chemicals and reagents and
6. Designating a "low-traffic" area of the lab that is away or shielded from air vents or open windows as an "RNase-free zone".

These common sense precautions helped to minimize RNase contamination problems.

#### 2.2.2.2. RNase-free solutions, glassware and metalware

RNase free water and anticoagulant solutions were made RNase-free by treating with diethylpyrocarbonate (DEPC). Briefly, the solutions including anticoagulant and water, glasswares, metalwares and gloves were incubated with 0.1% DEPC at room temperature, overnight. This is followed by autoclaving the solution for 1 hr. to eliminate residual DEPC.

### 2.2.3. Haemolymph collection

Haemolymph was collected from the rostral sinus of *P. monodon* using specially designed capillary tubes (RNase-free) rinsed using pre-cooled anticoagulant solution (RNase free, 10% sodium citrate, pH 7.0). Haemolymph was suspended in TRI reagent (Sigma) for total RNA isolation.

### 2.2.4. RNA isolation

Total RNA was extracted from the haemocytes using TRI Reagent (Sigma) following manufacturer's protocol. Briefly, about 1 ml haemolymph was homogenized in 1 ml TRI Reagent. To ensure complete dissociation of the nucleoprotein complexes, samples were allowed to stand for 5 min at room temperature. Chloroform (0.2 ml per 1 ml TRI Reagent) was added to the homogenate, sample was covered tightly, vigorously shaken for 15 sec and allowed to stand for 2-15 min at room temperature. The resulting mixture was centrifuged at 12,000 x g for 15 min at 4 $^0$C. Centrifugation separates the mixture into three phases: a red organic phase (containing protein), an interphase (containing DNA) and a colourless upper aqueous phase (containing RNA).

The aqueous phase was transferred to a fresh tube and 0.5 ml isopropanol per ml TRI Reagent was added. The samples were allowed to stand for 5-10 min at room temperature and then centrifuged at 12000 x g for 10 min at 4 $^0$C. The RNA precipitated out in the side and bottom of the tube was

washed by adding 1 ml 75 % ethanol per 1 ml of TRI Reagent. The samples were vortexed and centrifuged at 7500 x g for 5 min at 4 $^{0}$C. RNA pellets were dried for 5-10 min and dissolved in RNase free water by repeated pipetting with a micropipette at 55-60 $^{0}$C for 10-15 min.

### *2.2.5. Determination of the quantity and quality of RNA*

RNA was quantified and qualified by measuring optical density (O.D) at 260 and 280 nm in a UV spectrophotometer and visualizing RNA using electrophoresis. The ratio of absorbance at 260 nm and 280 nm is an indication of RNA quality. Only RNAs with absorbance ratios ($A_{260}$: $A_{280}$) greater than 1.8 were used for cDNA synthesis (Fig. 2.5). For quantification of RNA, the O.D at 260 nm was taken and the concentration of RNA was calculated as follows:

1 O.D of RNA = 40 µg/ml

RNA concentration (µg/ml) = O.D at 260 nm x Dilution factor x 40

### *2.2.6. Reverse transcription*

First strand cDNA was generated in a 20 µl reaction volume containing 5 µg total RNA, 1x RT buffer, 2mM dNTP, 2µM oligo d($T_{20}$), 20 U of RNase inhibitor and 100 U of Mu-MLV reverse transcriptase (New England Biolabs). The reaction was conducted at 42$^{0}$C for 1 hr followed by an inactivation step at 85 $^{0}$C for 15 min.

### *2.2.7. PCR amplification*

PCR of 1µl cDNA was performed in a 25 µl reaction volume containing 1x standard Taq buffer (10mM Tris-HCl, 50mM KCl, pH 8.3), 200µM dNTPs, 0.4 µM each primer and 1U Taq DNA polymerase (New England Biolabs). Amplification was performed using ALF-1, ALF-2, crustin-1, crustin-2, crustin-3, penaeidin-3, penaeidin-5, β-actin, and elongation factor primers (Table 2.1). The thermal profile used was an initial denaturation at 94 $^{0}$C for 2 min followed by 35 cycles of denaturation at 94 $^{0}$C for 15 sec, and extension at 68 $^{0}$C for 30 sec and a final extension at 68 $^{0}$C for

10 min. for the target genes. Annealing temperature and $MgCl_2$ concentration varied for the different genes as given in Table 2.1. The PCR products were visualized on 1.5% agarose gel.

## 2.2.8. Agarose gel electrophoresis

Agarose gel (1.5 %) was prepared in 1 X TBE buffer (Tris-base -10.8 g, 0.5 M EDTA – 4 ml, Boric acid – 5.5 g, double distilled water – 100 ml, pH – 8.0). Ethidium bromide (2 µl of 1 mg / ml stock stored in dark) was added to the melted agarose. After cooling to 45 $^0$C, the agarose was poured on to gel tray and was allowed to solidify. The gel try was transferred into a buffer tank and was submerged in 1 x TBE buffer. Ten microlitre of PCR product was mixed with 2 µl of 6 x gel loading buffer (1 % Bromophenol blue – 250 µl, 1 % xylene cyanol–250 µl, glycerol–300 µl, double distilled water–200 µl) and loaded onto the well. Electrophoresis was done at a voltage of 3-5 volt/cm till the bromophenol blue dye front migrate to the middle of the gel. The gel was visualized on a UV transilluminator and documentation was performed (BioRad).

## 2.2.9. Cloning of the PCR product

### 2.2.9.1. Ligation

PCR products were cloned onto the pGEM-T East vector (Promega, USA). The ligation mix (10 µl) consisted of 5 µl ligation buffer (2x), 0.5 µl of the vector (50ng/ µl), 3.5 µl PCR product (600 ng/ µl) and 1 µl of T4 DNA ligase (3U/ µl). The ligation mix was incubated at room temperature for 1 hr.

### 2.2.9.2. Transformation

JM 109 high efficiency competent cells of *E.coli* (transformation efficiency ≥ 1 x $10^8$ cells/µg DNA), provided with the pGEM®-T Easy Vector Systems were used for transformation. Ten microlitre of the ligated product was mixed with 50 µl of competent cells in a polypropylene tube and was incubated on ice for 20 min. The cells were given a heat-shock for 45–50 seconds in a water bath at exactly 42°C without shaking, to facilitate the

entry of recombinant DNA to the host cells. Then the tubes were immediately returned to ice for 2 minutes. To this, 600 µl SOC medium was added at room-temperature and incubated for 1.5 hours at 37°C with shaking (~250 rpm). The transformation mixture (200 µl) was spread onto Lauria-Bertani (LB) agar plates supplemented with ampicillin (100 µg/ml), IPTG (100 mM) and X-gal (80 µg/ml). The plates were then incubated at 37 $^0$C overight. The transformants were selected based on blue/white colony screening. The white colonies were selected and streaked on LB+Ampicillin+X-gal+IPTG plates and incubated overnight at 37 $^0$C.

### *2.2.9.3. Colony PCR*

Colony PCR was performed to confirm the presence of the insert DNA. All the individually streaked colonies were tested with colony PCR using universal vector primers T7 (5′- tgtaatacgactcactataggg-3′) and SP6 (5′-gatttaggtgacactatag- 3′) to confirm the presence of the gene of interest and electrophoresis was performed on 1% agarose gel prepared in 1X TBE buffer and stained with ethidium bromide.

White colonies (template) picked from the transformed plate were dispensed into the PCR reaction mix (25 µl) containing 1x standard Taq buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3), 200 µM dNTPs, 0.4 µM each primer (T7 and SP6) and 1U Taq DNA polymerase (New England Biolabs). The thermal profile used was an initial denaturation at 95 $^0$C for 5 min followed by 35 cycles of denaturation at 94 $^0$C for 15 sec, annealing at 57 $^0$C for 20 sec and extension at 72 $^0$C for 1 min and a final extension at 72 $^0$C for 10 min.

### *2.2.9.4. Plasmid extraction and purification*

Plasmid extraction and purification was done using GenElute HP′ plasmid miniprep kit (sigma). Cells were harvested by centrifuging 2 ml of overnight recombinant *E. coli* culture at 16000 x g. The pellets were resuspended in 200 µl resuspension solution with RNase. The resuspended cells were lysed by

adding 200 µl of the lysis buffer. The contents were mixed immediately by gentle inversion until the mixture becomes clear and viscous. The cell debris were precipitated by adding 350 µl of the neutralization buffer and were pelletted by centrifuging at 16000 x g for 10 min. Column was prepared by inserting a GenElute HP MiniPrep Binding Column into the provided microcentrifuge tube. Column preparation solution (500 µl) was added to the MiniPrep column and was centrifuged at 16000 x g for 1 min. and the flow through liquid was discarded. Wash solution I (500 µl) and wash solution II (750 µl) were added to the column in two successive steps. Centrifugation was performed at 16000 x g 1 min. and the flow through liquid was discarded. Excess ethanol was removed by centrifuging at 16000 x g for 1 min and the column was transferred to a fresh collection tube. Elution solution (100 µl of 10mM Tris-HCl) was added to the column and was again centrifuged at 16000 x g for 1 min. The DNA present in the eluate (Plasmid DNA) was stored at -20$^0$C and was sent for sequencing.

### *2.2.10. Sequencing and sequence analysis*

The plasmid DNA were sent for sequencing at Microsynth, Switzerland. Sequencing was performed using ABI PRISM 3700 Big Dye Sequencer using vector primers T7 and SP6. Sequenced data were compiled and analyzed. The nucleotide sequences obtained were assembled using BioEdit and GeneTool software. Open reading frame of the sequences were found out using GeneTool software. Gene translation and prediction of deduced protein were performed with ExPASy (http://www.au.ExPASy.org/) and GeneTool software. Molecular weight and isoelectric point of the target AMP were predicted out using ProtParam tool of ExPASy programme. Conserved domains/motifs/pattern search were also found out using Motif Scan and ScanProsite tool of ExPASy programme. The sequence homology

and the deduced amino acid sequence comparisons were carried out using BLAST (Basic Local Alignment Search Tool) algorithm (Altschul et al., 1990) at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/blast). The signal peptide was predicted by SignalP program (http://www.au.ExPASy.org/). The multiple sequence alignments were performed on amino acid sequences of known AMP/control gene sequences from shrimps using CLUSTALW and GeneDoc computer programs. Amino acid sequences of the target genes were retrieved from the NCBI GenBank and phylogenetic tree was constructed by the Neighbor-Joining (NJ) method using MEGA version 4.0 (Tamura et al., 2007). Bootstrap analysis was based on 1000 replicates. The nucleotide sequence and the deduced amino acid sequences were submitted to GenBank. The structural models of the AMPs were created using SWISS-MODEL server.

## 2.3. Results

### *2.3.1. Molecular characterization of AMP genes in P.monodon*

Expression of seven AMP genes could be observed from the haemocytes of *P. monodon.* The AMP genes were clustered into three different types viz., anti-lipopolysaccharide factor-1 (ALF-1), anti-lipopolysaccharide factor-2 (ALF-2), three crustins (crustin-1, crustin-2 and crustin-3) and two subgroups of penaeidins (penaeidin-3 and penaeidin-5).

#### *2.3.1.1. Anti-Lipopolysaccharide Factor-1 (ALF-1) (GU732817)*

##### *2.3.1.1.1. Nucleotide and deduced amino acid sequences of ALF-1*

A partial mRNA transcript of 264 bp belonging to the ALF family of AMPs was obtained from the mRNA of *P. monodon* haemocyte by RT-PCR (Fig.2.6). The nucleotide sequence encoding 87 amino acids was translated using GeneTool software (Fig.2.6). Both the nucleotide and amino acid sequences were submitted to GenBank under the accession number **GU732817.** Signal peptide could be detected at the 21$^{st}$ position (Fig. 2.7).

Peptide model of ALF-2 created using SWISS-MODEL server was found to consist of two alpha-helices crowded against a four-strand β-sheet. Two of the β-strands are in turn linked by a disulfide bond to form an amphipathic loop rich in cationic amino acid side chains (Fig.2.8).

### *2.3.1.1.2. Sequence alignment of ALF-1*

BLAST analysis of the nucleotide sequence showed that it belonged to DUF3254 superfamily, the family of ALFs, confirming the sequence to be an isoform of ALF. BLAST analysis of the nucleotide and amino acid sequence revealed the relation of ALF gene to other isoforms of ALF present in *P. monodon, F. indicus*, *F. chinensis* and *F. paulensis* (Table 2.2 and 2.3). BLASTn analysis of the nucleotide sequence revealed that the sequence shared 77% similarity to an ALF isoform ALFPm3 of *P. monodon* for a query coverage of 91% followed by 76% similarity to another isofrom ALF-9 of *P.monodon*. Similarity was also found to ALF isoforms of *F. indicus, F. chinensis* and *F. paulensis*. However, for these isoforms the query coverage was found to be less than 60% (Table 2.2). BLASTp analysis of the amino acid sequences of the ALF, also confirmed the above results (Table 2.3). Multiple alignments of nucleotide and amino acid sequences of ALFs with high degree of similarity and with other known ALFs of shrimps showed the presence of conserved sequences for these AMPs. Multiple alignments confirmed the results of the BLAST analysis, where maximum similarity was with ALF isoforms of *P. monodon* (Fig. 2.9 and 2.10).

### *2.3.1.1.3. Phylogenetic analysis of ALF-1*

The bootstrap distance tree calculated for the resulting ALF sequences confirmed that ALF possessed more similarity to that of other ALFs from *P. monodon* than to the ALF of *F. chinensis* and *F. paulensis* ALF (Fig.2.11). Phylogenetic tree drawn based on known amino acid sequences of shrimp ALF could be divided into three groups. Group I consisted of ALFs of

*Fenneropenaeus, Litopenaeus* and *Farfantepenaeus* sp. Group II and III consisted of ALFs of *P. monodon*. The present ALF was found to belong to Group II.

The phylogenetic relationships of the ALF sequence with all known ALFs of the decapods are shown in Fig.2.12. The tree topologies revealed the relationships of ALF with other invertebrate ALF-like peptides. The tree could be divided into four major groups. Group I and III consisted of shrimp ALFs, whereas, Group II and IV consisted of ALFs of lobsters and crabs respectively. Though the tree could be classified into major groups, but interestingly, it was found that in each group ALF of shrimps shared close similarity with ALF of crabs and lobsters. Molecular phylogenetic tree based on amino acid sequences suggests that all the ALF members possess a same ancestral origin, which has subsequently diverged at different phases of evolution. Another interesting fact revealed by phylogenetic analysis is that ALFs of shrimps were found to be evolutionarily closely related with ALFs of other decapods species.

### 2.3.1.2. Anti-Lipopolysaccharide factor-2 (ALF-2) (HM588914)

#### 2.3.1.2.1. Nucleotide and deduced amino acid sequences of ALF-2

A partial mRNA transcript of 301 bp, encoding 98 amino acids, belonging to the ALF family of AMPs could be obtained from the mRNA of *P. monodon* haemocyte by RT-PCR (Fig.2.13). Both the nucleotide and amino acid sequences were submitted to GenBank under the accession number **HM588914.** Peptide model of ALF-2 predicted using SWISS-MODEL was found to consist of two alpha-helices crowded against a four-strand β-sheet Two of the β-strands Are in turn linked by a disulfide bond to form an amphipathic loop rich in cationic amino acid side chains (Fig. 2.14.)

#### 2.3.1.2.2. Sequence alignment of ALF-2

BLAST analysis of the nucleotide sequence showed that it belonged to **DUF3254** superfamily, the family of ALFs, confirming the sequence to be an isoform of ALF. BLAST analysis of the nucleotide and amino acid sequence

revealed the relation of ALF gene to other isoforms of ALF present in *P. monodon*, *F. indicus*, *F. chinensis* and *F. paulensis* (Table 2.4 and 2.5). BLASTn analysis of the nucleotide sequence revealed that the sequence shared 99% similarity to the ALF isoform ALFPm3 of *P. monodon* for a query coverage of 98% which showed that both are the same isoforms. Great similarity was also found to ALF isoforms of *F. indicus*, *F. chinensis* and *F. paulensis* also (Table 2.4). BLASTp analysis of the amino acid sequences of the ALF, also confirmed the above results (Table 2.5).

Multiple alignments also revealed high degree of similarity among the amino acid sequences of ALFs. Multiple alignments performed for the amino acid sequences of ALFs that are more similar and with other known ALFs of shrimps showed the presence of conserved sequences for these peptide molecules. Multiple alignments confirmed the results of the BLAST analysis, where maximum similarity was shown to ALF isoforms of *P. monodon* (Fig.2.15 and 2.16).

### *2.3.1.2.3. Phylogenetic analysis of ALF-2*

The bootstrap distance tree calculated for the resulting ALF sequences confirmed that ALF possessed more similarity to that of other ALFs from *P. monodon* than to the ALF of *Fenneropenaeus* and *Farfantepenaeus* sp. (Fig.2.17). Phylogenetic tree drawn based on known amino acid sequences of shrimp ALF could be divided into three groups. Group I consisted of ALFs of *P. monodon*. Group II and III consisted of ALFs of *Fenneropenaeus* and *Farfantepenaeus* sp. respectively.

Phylogenetic tree drawn based on all known ALF sequences of decapods could be divided into five major groups Fig.2.18. Group I consisted of shrimp ALFs, Group II and IV consisted of ALFs of lobsters, Group III consisted of ALs of shrimsp and crabs, Group V consisted of ALFs of crabs.

## 2.3.1.3. *Crustin-1 (GQ334395)*

### *2.3.1.3.1. Nucleotide and deduced amino acid sequences of Crustin-1*

The full length cDNA consists of 456 base pairs encoding 141 amino acids with an ORF of 124 amino acids (Fig. 19). The sequence was submitted to GenBank under the accession number **GQ334395**. The crustin-1 cDNA encoded a polypeptide of 141 amino acids residues. Analysis of the putative signal peptidase cleavage site (Signal P software) indicated a cleavage site located after position 17 which implied that a crustin-1 was a secreted peptide (Fig. 2.20). The calculated molecular mass of the mature protein (124 amino acids) was 12.56 kDa and the isoelectric point (p*I*) was estimated to be 8.03 (predicted by the ProtParam software). The predicted secondary structure of crustin-1, created using SWISS-MODEL, indicated a random coiled structure that is with two possible β-sheets but no helices (Fig. 2.21). WAP domain structure with 4DSC could also be obtained using ScanProsite tool (Fig.2.22 A). Glycine rich region and cysteine rich regions could be detected in the crustin sequence using ScanProsite (Fig.2.22 B) and Motif Scan tools (Fig.2.23).

### *2.3.1.3.2. Amino acid composition of Crustin-1*

The deduced amino acid sequence of crustin-1 was found to be rich in amino acid residues Glycine (24.2%) following proline, valine and cysteine (9.7%). At the N-terminal of the mature peptide, crustin-1 contained a number of glycine-rich repeats. Following the repeat region is a cysteine-rich region. The C-terminal segment included a high proportion of cysteine-rich region (9.7%), which contained 12 Cysteine residues that participate in the formation of disulphide bonds.

### *2.3.1.3.3. Analysis of WAP domain structure of Crustin-1*

As predicted by the ScanProsite program, a whey-acidic protein (WAP) domain signature exists in the C-terminal (Fig.2.22). According to the

previous reports on the crustin-like proteins, the four-disulfide core domain was proved to play important roles in the biological function of crustins (Zhang et al., 2007). The position of the conserved cysteines for such category of 'four-disulfide core' (4DSC) domain and the location of the signature pattern is $Cys^{92}$-$Cys^{122}$, $Cys^{99}$-$Cys^{126}$, $Cys^{109}$-$Cys^{121}$, and $Cys^{115}$-$Cys^{132}$. In addition, searching against the Prosite database, analysis of Crustin-1 revealed the existence of WAP-type 4DSC domain signature, C1-(Xn)-C2-(Xn)-C3-(X5)-C4-(X5)-C5-C6-(X3-5)-C7-(X3-4)-C8 (Bartlett et al., 2002) and crustin-1 followed the same pattern with 3 residues found between $C_6$ & $C_7$ ($C_6$ $X_3$ $C_{6}$) and $C_7$ & $C_8$ ($C_7$ $X_3$ $C_8$). Several other consensus sequences were found in the 4-DSC domain: (1) the consensus KXGXCP containing C1; (2) a conserved aspartate (D) residue between C3 and C4; (3) KCC with C5 and C6; (4) CXXP with C8 (Bartlett et al., 2002). Crustin-1 follows this pattern as 'CXP with $C_8$' instead of 'CXXP with $C_8$'.

### *2.3.1.3.4. Sequence alignment of Crustin-1*

BLAST analysis confirmed the sequence to be an isoform of crustin. BLAST analysis of the nucleotide and amino acid sequence revealed the relation of crustin-1 to that of crustins from *P. monodon* and *F. chinensis* (Table 2.6 and 2.7). Maximum similarity was obtained for another crustin isoform of *P. monodon* that gave 97% similarity to the sequence (Table2.6). BLASTp analysis of the amino acid sequences confirmed the above results (Table2.7).

Multiple alignments also revealed high degree of similarity among the amino acid sequences of shrimp crustins. Conserved domains could be detected in the signal peptide region. Also the WAP domain signature was also found to be highly conserved among shrimps. Multiple alignments confirmed the results of the BLAST analysis, where maximum similarity was shown to crustins isoforms of *P. monodon* (Fig. 2.24 and 2.25).

### *2.3.1.3.5. Phylogenetic analysis of Crustin-1*

The bootstrap distance tree calculated for the resulting crustin sequences of shrimps, could be grouped into four major groups (Fig.2.26). Group I consisted of crustins of *Litopenaeus*, *Fenneropenaeus* and *Farfantepenaeus* sp. Group II consisted of crustin isoforms of *M. japonicus*, Group III of *P. monodon*, and Group IV of *F. chinensis*. Phylogenetic analysis of crustins of all decapods could be broadly divided into two Groups. Group I consisting of shrimp crustins and Group II consisting of crustins of crabs, crayfishes and lobsters (Fig.2.27).

## *2.3.1.4. Crustin-2 (FJ535568)*

### *2.3.1.4.1. Nucleotide and deduced amino acid sequences of Crustin-2*

A partial mRNA transcript of 299 bp, encoding 91 amino acids, belonging to the crustin family of AMPs was obtained from the mRNA of *P. monodon* haemocyte by RT-PCR (Fig. 2.28). The sequence was submitted to GenBank under the accession number **FJ535568**. Analysis of the putative signal peptidase cleavage site (Signal P software) indicated a cleavage site located after position 20 (Fig. 2.29). WAP domain structure could also be obtained using ScanProsite and Motif Scan tool (Fig.2.30 and 2.31).

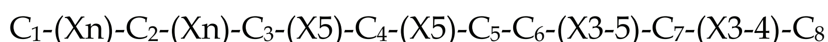
### *2.3.1.4.2. Amino acid composition of Crustin-2*

The transcript had 91 amino acid residues with conserved cysteine residues characteristic of the WAP domain (Fig 2.32). The C-terminal segment included a high proportion of cysteine-rich region that participate in the formation of disulphide bonds. The partial cDNA fragment contained the five conserved cysteine residues ($C_4$-$C_8$ residues). Multiple polyadenylation consensus sequences (AATAAA) were also present at the C-terminus. The partial cDNA fragment lacked the signal peptide region but possessed the cysteine rich region characteristic to WAP domain. The putative polyadenylation consensus signal (AATAAA) also confirms the 3′

end of the crustin-like AMP. Multiple polyadenylation sites were observed in the present fragment.

### *2.3.1.4.3. Analysis of WAP domain structure of Crustin-2*

As predicted by the ScanProsite program a partial WAP domain signature exists in the C-terminal region and one of the four disulphide core (DSC) domain was found to be located at $Cys^6$-$Cys^{23}$. Since only partial cDNA fragment and only five of the conserved cysteine regions could be retrieved from the sequence, other locations of the DSC could not be performed. Searching against the Prosite database, analysis of the crustin-like AMP revealed the existence of WAP type DSC domain signature. The expected WAP type 4DSC core domain signature is:



$C_1$-(Xn)-$C_2$-(Xn)-$C_3$-(X5)-$C_4$-(X5)-$C_5$-$C_6$-(X3-5)-$C_7$-(X3-4)-$C_8$

where X is any amino acid residue and Xn is a stretch of n residues (Bartlett et al., 2002).

The partial cDNA sequence of the crustin-like AMP showed the presence of $C_4$-$C_8$ and it followed the same pattern as expected in the WAP domain except for one extra residue between $C_7$ and $C_8$ ($C_7$-(X5)-$C_8$). Similar case was reported for the crustins isolated from *F. chinensis*, GenBank Accession No. **DQ097703**, **DQ097704** (Zhang et al., 2007). The WAP domain signature of the present crustin-like AMP could be expressed as in Fig. 2.32. Several other consensus sequences also appear in the 4DSC domain. (1) A conserved aspartate (D) residue between $C_3$ and $C_4$ (2) KCC with $C_5$ and $C_6$. (3) CXXP with $C_8$ (Bartlett et al., 2002).

### *2.3.1.4.4. Sequence alignment of Crustin-2*

BLAST analysis was performed at the nucleotide and amino acid level with other crustins in the GenBank (Table 2.8 and 2.9). BLAST analysis showed that the crustin-like AMP shared maximum similarity with other crustins of *F. paulensis* (**ABM633611**), *L. vannamei* (**AY488493**), *F. subtilis* (**ABO93323**), *F. brasiliensis* (**ABQ96197**) and *L. schmitti* (**ABM63362**). The

sequence also showed high similarities to the crustin isolated from *P. monodon* by Chen and co-workers (2004a) that have not been submitted to the GenBank. Multiple alignments showed the presence of highly conserved domains in both the nucleotide and amino acid sequences (Fig.2.33 and 2.34).

#### *2.3.1.4.5. Phylogenetic analysis of Crustin-2*

All known crustins were retrieved from the GenBank and phylogenetic analysis of both the nucleotide and amino acid sequences of the crustin-like AMP were performed. Crustins of shrimps (Fig. 2.35) and that of all groups of crustaceans (Fig. 2.36) were subjected to phylogenetic analysis. NJ trees were constructed both at nucleotide and amino acid level using MEGA 3.1 software. The phylogenetic relationship of the crustin-like AMP and other AMPs with WAP domain that showed maximum similarity with that of crustin-like AMP (**FJ535568**) and also with that of all the known crustins deposited in GenBank are shown in Fig. 2.35 and 2.36 respectively. The tree topologies revealed the relationships of crustin-like AMP with other invertebrate crustin / crustin-like peptides.

### *2.3.1.5. Crustin-3 (GQ334396)*

#### *2.3.1.5.1. Nucleotide and deduced amino acid sequences of Crustin-3*

A partial mRNA transcript of 239 bp, encoding 73 amino acids, belonging to the crustin family of AMPs was obtained from the mRNA of *P. monodon* haemocyte by RT-PCR (Fig. 2.37). The sequence was submitted to GenBank under the accession number **GQ334396**. Cysteine rich region and the WAP domain signature with the characteristic 4DSC could be detected using ScanProsite tool (Fig.2.38). Motif Scan also showed the presence of cysteine-rich regions and WAP domain structure in the crustin-2 sequence (Fig. 2.39). The predicted secondary structure of crustin-3, created using SWISS-MODEL, consisted of a coiled structure with a helical segment (Fig. 2.40).

### 2.3.1.5.2. *Amino acid composition of Crustin-3*

The transcript had 73 amino acid residues with conserved cysteine residues characteristic of the WAP domain. The C-terminal segment included a high proportion of cysteine-rich region that participate in the formation of disulphide bonds. The partial cDNA fragment contained the 12 conserved cysteine residues. The partial cDNA fragment lacked the signal peptide region but possessed the cysteine rich region characteristic to WAP domain. The other characteristic features of the WAP domain also confirm the 3′ end of the crustin-like AMP.

### 2.3.1.5.3. *Analysis of WAP domain structure of Crustin-3*

As predicted by the ScanProsite program, a whey-acidic protein (WAP) domain signature exists in the C-terminal. According to the previous reports on the crustin-like proteins, the four-disulfide core domain has proved to play important roles in the biological function of crustins (Zhang et al., 2007). The position of the conserved cysteines for such category of 'four-disulfide core' domain and the location of the signature pattern is $Cys^{24}$-Cys54, Cys31-Cys58, $Cys^{41}$-$Cys^{53}$, and $Cys^{47}$-$Cys^{64}$. In addition, searching against the Prosite database, analysis of Crustin-1 revealed the existence of WAP-type 'four-disulfide core' domain signature, $C_1$-(Xn)-$C_2$-(Xn)-$C_3$-(X5)-$C_4$-(X5)-$C_5$-$C_6$-(X3-5)-$C_7$-(X3-4)-$C_8$ (Bartlett et al., 2002) except for the presence of 5 residues between $C_7$ & $C_8$ ($C_7$ $X_5$ $C_8$). 3 residues were found between $C_6$ & $C_7$ ($C_6$ $X_3$ $C_6$). Several other consensus sequences also appears in the 4DSC domain: (1) the consensus KXGXCP containing $C_1$; (2) a conserved aspartate (D) residue between $C_3$ and $C_4$; (3) KCC with $C_5$ and $C_6$; (4) CXP with $C_8$ (Bartlett et al., 2002).

### 2.3.1.5.4. *Sequence alignment of Crustin-3*

BLAST analysis was performed at the nucleotide and amino acid level with other crustins in the GenBank. BLAST analysis showed that the crustin-like AMP shared maximum similarity with other crustins of *P. monodon*, *F.*

*chinensis* and *F. indicus* (Table 2.10 and 2.11). Sequence alignments at the nucleotide and amino acid levels showed the presence of highly conserved sequences within the crustin molecule (Fig 2.41 and 2.42)

#### ***2.3.1.5.5. Phylogenetic analysis of Crustin-3***

The bootstrap distance tree calculated for the resulting crustin sequences of shrimps could be grouped into four major groups (Fig.2.43). Group I consisted of crustins of *Litopenaeus*, *Fenneropenaeus* and *Farfantepenaeus* sp. Group II consisted of crustin isoforms of *M. japonicus*, Group III of *P. monodon*, and Group IV of *F. chinensis.* Phylogenetic analysis of crustins of all decapods could be broadly divided into two groups. Group I consisting of shrimp crustins and Group II consisting of crustins of crabs, crayfishes and lobsters (Fig.2.44).

### ***2.3.1.6. Penaeidin-3 (GU732819)***

#### ***2.3.1.6.1. Nucleotide and deduced amino acid sequences of Penaeidin-3***

A partial mRNA transcript of 138 bp, encoding 45 amino acids, belonging to the penaeidin family of AMPs was obtained from the mRNA of *P. monodon* haemocyte by RT-PCR (Fig. 2.45). The sequence was submitted to GenBank under the accession number **GU732819.** Four cysteine residues could be found in the partial sequence that form the part of the six cysteine residues organized in two doublets. Penaeidin signature could be detected within the sequence using Motif Scan tool (Fig.2.46).

#### ***2.3.1.6.2. Sequence alignment of Penaeidin-3***

BLAST analysis was performed at the nucleotide and amino acid level with other penaeidin-3 in the GenBank. BLAST analysis showed that the penaeidin-like AMP shared maximum similarity with penaeidin-3 of *P. monodon* (Table 2.12 and 2.13). Multiple alignment performed for the sequence showed the presence of highly conserved sequences both at the nucleotide and amino acid level (Fig.2.47 and 2.48).

### *2.3.1.6.3. Phylogenetic analysis of the Penaeidin-3*

Phylogenetic tree resulted could be broadly divided into two major groups. Group I consisting of penaeidins of *Litopenaeus*, F*enneropenaeus* and *Farafantepenaeus* sp. and Group II consisting of penaeidins of *P. monodon* and *F. chineneis* (Fig.2.49). The present peptide was found to belong to Group II of the phylogenetic tree obtained.

## *2.3.1.7. Penaeidin-5 (GQ334397)*

### *2.3.1.7.1. Nucleotide and deduced amino acid sequences of Penaeidin-5*

A partial mRNA transcript of 139 bp, encoding 36 amino acids, belonging to the penaeidin family of AMPs was obtained from the mRNA of *P. monodon* haemocyte by RT-PCR (Fig. 2.50). The sequence was submitted to GenBank under the accession number **GQ334397**. The proline rich domain of the N-terminal region could be seen. Analysis of the putative signal peptidase cleavage site (SignalP software) indicated a cleavage site located after position 19 which implied that a penaeidin-5 was a secreted peptide (Fig. 2.51). Penaeidin signature could be obtained using Motif Scan tool (Fig.2.52).

### *2.3.1.7.2. Sequence alignment of Penaeidin-5*

BLAST analysis was performed at the nucleotide and amino acid level with other penaeidin-5 in the GenBank. BLAST analysis showed that the penaeidin-like AMP shared maximum similarity with penaeidin-5 of *P. monodon,* followed by penaeidins of *F. chinensis, F. subtilis, F. brasiliensis* (Table 2.14 and 2.15). Multiple alignments showed the presence of highly conserved sequences at both the nucleotide and amino acid level (Fig.2.53 and 2.54).

### *2.3.1.7.3. Phylogenetic analysis of the Penaeidin-5*

Phylogenetic tree resulted could be broadly divided into three major groups. Group I consisting of penaeidins of *Litopenaeus*, *Fenneropenaeus*, *Penaeus* and *Farafantepenaeus* sp. Group II consisting of penaeidins of *P.*

*monodon* and Group III of *Litopenaeus* sp. (Fig.2.55). The present peptide was found to belong to Group II of the phylogenetic tree obtained.

### *2.3.2. Control / Reference genes*

Expression of three control genes was confirmed by RT-PCR analysis of the *P. monodon* haemocytes. Transcripts of β-actin and elongation factor (ELF) could be observed from the haemocytes of *P. monodon.* A partial mRNA transcript of 583 bp, encoding 194 amino acids, belonging to the actin family was obtained from the mRNA of *P. monodon* haemocyte by RT-PCR (Fig. 2.56). The sequence was submitted to GenBank under the accession number **GQ334394**. A partial mRNA transcript of 444 bp, encoding 147 amino acids, belonging to the ELF family was obtained from the mRNA of *P. monodon* haemocyte by RT-PCR (Fig. 2.57). The sequence was submitted to GenBank under the accession number **GU732818.**

## 2.4. Discussion

Antimicrobial peptides are important molecules of the immune defense reactions of living organisms to fight infection by microorganisms. They form a major component of the innate immune defense system in arthropods, providing an immediate and usually a rapid response to invading microorganisms. Of those AMPs characterized in arthropods, most have been isolated from insects and many share common features, which imply wide distribution across species. To date, few AMPs have been isolated and characterized from crustaceans. Given the wide variety of AMPs isolated and characterized from insects, it seems likely that crustaceans also possess a wide variety of AMPs, most of which are yet to be discovered.

Crustaceans, belonging to the phylum Arthropoda, are of enormous commercial importance in many parts of the world. Being aquatic they live in a medium rich with microorganisms and have evolved very efficient defense mechanisms to evade pathogens. Like other invertebrates, crustaceans possess cellular and humoral immune responses mainly of non-

specific nature like phagocytosis, encapsulation, nodule formation, phenol-oxidase system, reactive oxygen species and AMPs. AMPs are a major component of the innate immune defense system in crustaceans, providing an immediate and broad-spectrum microbicidal activity against pathogenic microbes. They are considered as endogenous antibiotics, encoded in the host genome directly. Most AMPs display cationic properties, have a molecular mass less than 10 kDa, with less than 50 amino acid residues and adopt an amphipathic structure that is believed to be essential for their antimicrobial action. In most cases, AMPs were shown to disrupt microbial membrane by pore formation or detergent effect and cause immediate cell death. So far more than 800 AMPs have been isolated and more than half of them have been isolated from Arthropods.

Among crustaceans AMPs have been isolated from artemia, crayfishes, lobsters, crabs and prawns. AMPs including penaeidins, crustins, astacidins and anti-lipopolysaccharide factors (ALFs) have been isolated and characterized from many crustaceans. Among crustaceans, the first described AMPs were the carcinins, a crustin isolated from *C. maenas*, and penaeidins from *L. vannamei*. Among crustaceans, penaeidins are the most studied AMPs, being isolated from more than ten penaeid prawn species. Several ALFs and crustins have also been recently isolated and characterized in many crustacean species including crabs, lobsters, prawns and crayfishes. During the past several years, a variety of AMP families were characterized from shrimps.

In penaeid shrimps, to date, three kinds of antimicrobial peptides have been fully characterized, namely the crustins (Bartlett et al., 2002), penaeidins and the anti-lipopolysaccharide factor (ALF) from haemocytes (Destoumieux et al., 2001; Somboonwiwat, et al., 2005). Penaeidins constitute an original peptide family, whose molecular structure is unique, composed of an N-terminal proline–rich region and a C-terminal domain containing six cysteines forming three intramolecular disulphide bridges (Yang et al., 2003).

Tiger shrimp (*P. monodon*) is one of the most important cultivated penaeid shrimps in the world. However, the shrimp aquaculture industry is suffering from diseases linked to infection by viral pathogens, which cause a drastic decrease in shrimp production, resulting in huge economic losses. To overcome this problem, studies should be focused on developing strategies of controlling viral diseases to ensure long-term survival of shrimp aquaculture. The increased prevalence of disease outbreak is contributing to the rising costs of aquaculture of shrimp and marine fishes. Furthermore, bacterial resistance arising from over usage of conventional antibiotics against infection by pathogens in aquaculture, agriculture, and human medicine is becoming widespread, and has led to a corresponding increase in the number of attempts to find effective replacements. AMPs are a largely unexplored group of antimicrobial compounds that are gaining prominence as potential therapeutic agents (Hancock and Patrzykat, 2002).

In the present study, seven AMP genes viz., ALF-1 ALF-2, crustin-1, crustin-2, crustin-3, penaeidin-3 and penaeidin-5 were successfully isolated from the haemocytes of *P. monodon*.

ALFs are small basic proteins of approximately 100 amino acids that were initially characterized from the haemolymph of distantly related arthropods, the horseshoe crabs *T. tridentatus* (Aketagawa et al., 1986) and *L. polyphemus* (Muta et al., 1987). Thereafter, several ALF homologs have been identified from *P. monodon* (Supungul et al., 2004) and many other shrimp species including *L. setiferus* (Gross et al., 2001), *F. chinensis* (ALFFc) (Liu et al., 2005), *M. japonicas* (M-ALF) (Nagoshi et al., 2006), *P. leniusculus* (Liu et al., 2006), *L. vannamei, L. styrirostris* lobster and mud crab (Imjongjirak et al., 2007). The growing number of relatively conserved ALF genes identified with apparently conserved functions being characterized across taxa seems to indicate the likely importance of ALF in shrimps besides the AMPs in the penaeidin family (Gueguen et al., 2006).

ALFs bind to cell walls of gram-negative bacteria and inhibit the lipopolysaccharide-mediated coagulation cascade (Chaby, 2004). A growing number of studies on crustacean ALF are available in recent years, describing primarily the molecular cloning, sequencing and expression analysis of ALF in various shrimp species (Supungul et al., 2004; Liu et al., 2005; Nagoshi et al., 2006; Somboonwiwat et al., 2006). Recombinant *P. monodon* ALF showed antimicrobial activity against fungi, gram-positive and gram-negative bacteria (Somboonwiwat et al., 2005), and in the crayfish *P. leniusculus* ALF has been implicated in the reduction of WSSV replication. Thus, it is possible to predict that ALF could function in shrimp as a broad spectrum AMP.

Two partial mRNA sequences predicted to encode ALFs could be identified from the haemocytes of *P. monodon* in the present study. Herein, the nucleotide and amino acid sequences of these two isoforms were analyzed. ALF-1 that displayed a lower degree of similarity to already reported ALFs could be denoted as a novel isoform of ALF in shrimps/ however ALF-2 was found to be already reported in *P. monodon*. Phylogenetic tree analysis clearly indicated that the ALF sequences were clustered according to species. The crab and lobster ALF peptides were clustered separately from those of the shrimp ALFs. However, the analysis also shows that the present ALF molecule is more closely related to crab ALFs rather than to the lobster ALFs. However, within the crab cluster, the black tiger shrimp ALFs grouped together suggested that corresponding genes are the closest to crab ALFs. Peptide model of the partial ALF-1 predicted using SWISS-MODEL tool, was found to consist of both alpha-helical structure and beta-sheets (Fig.2.8). Four β-sheets and two α-helix could be detected in the ALF-1 of *F. indicus*. As per Hoess et al. (1993) and Beale et al. (2008), the ALF molecule is supposed to consist of a single domain with three α-helices crowded against a four-strand β-sheet. Since

ALF-1 was a partial sequence, the two of the α-helix that is required for the complete structure could not be obtained.

The ALF-2 molecule was found to consist of a single domain with two α-helices crowded against a four-strand β-sheet (Fig. 2.14). Two of the β-strands are linked by a disulfide bond to form an amphipathic loop rich in cationic amino acid side chains as reported by Hoess et al. (1993) and Beale et al. (2008). The structure predicted from both ALF-1 and -2 of *P.monodon* was also found to agree with the above mentioned findings. Synthetic cyclic versions of this loop are found to be functionally active against gram-negative bacteria including *Vibrio harveyi* (Imjongjirak et al., 2007) and *Pseudomonas aeruginosa* (Pan et al., 2007) and against gram-positive bacteria such as *Micrococcus luteus* (Imjongjirak et al., 2007).

A variety of ALF isoforms have been described in shrimp, including five (ALFPm1–5) in the giant tiger prawn *P. monodon* discovered during the course of expressed sequence tag analysis (Supungul et al., 2004; Tassanakajon et al., 2006). The antimicrobial activities of *P. monodon* ALFs are interesting as the ALFs can be applied in the aquaculture as an alternative to antibiotics (Tharntada, et al., 2008).

Among the innate immune responses in crustaceans, WAP domain containing proteins collectively termed the crustins deserve much attention (Christie et al., 2007). Crustins that have been described to date are classified by diverse amino acid sequences with conservation in the C-terminus of 12 cysteine residues including a single whey acidic protein (WAP) domain. The WAP domain generally consists of 50 amino acid residues with eight cysteine residues at defined positions. They form four intracellular disulfide bonds creating a tightly packed structure. The WAP domain-containing proteins are widespread throughout the Metazoan and have diverse biological functions such as the secretory leukocyte protease inhibitor (SLPI) and elafin which have both antibacterial activity and antiprotease activity (Sallenave, 2002) and mouse single WAP motif protein1 (SWAM1) and

SWAM2, as antibacterial proteins (Hagiwara et al., 2003). According to the previous reports on the crustin-like proteins, the 4DSC domain played important roles in the biological function of crustins (Zhang et al., 2007).

Herein, the nucleotide and amino acid sequences of the three crustins viz. crustin-1, crustin-2 and crustin-3 were analyzed. The sequence diversity between the signal peptide and the WAP domain in all of these peptides complicates comparisons of the relationship of these genes. As a result, phylogenetic analysis was carried out using only the WAP domain sequences which are the key to the tertiary structure and functioning of these peptides, as described in Hauton et al. (2006). Based on the crustin signature described (Bartlett et al., 2002) the crustins were found to belong to two different types. Crustin-1 and crustin-2 was found to belong to crustin type-2 and crustin-3 was found to belong to crustin type-3, i.e. the family of SWD proteins. The WAP domain with the characteristic 4DSC could be observed in all the three crustin isoforms. The C-terminus exhibited a unique cysteine array that is predicted to form six disulphide bonds in the tertiary structure as reported by Brockton et al. (2007). This 12 cysteine array arrangement is conserved in expressed sequence tags (ESTs) from related genera and seems to represent a novel tertiary structure amongst AMPs, unique to the Crustacea.

Glycine rich regions and the cysteine rich regions, characteristics of the crustin molecules could also be detected in the sequences. This molecular motif, also found in many other mammal-secreted proteins, seems to exert a protease inhibitor activity, as well as other biological functions (Ranganathan et al., 1999). It is well established that protease inhibitors exert several important immunological functions, such as the inhibition of microbial proteases and the regulation of the prophenoloxidase activating system in crustaceans (Cerenius and Soderhall, 2004). Therefore, it would be interesting to examine the activity of crustins in other immune contexts, and not only as AMPs.

As mentioned before, the presence of a hydrophobic region containing an over representation of glycine residues at the N-terminal portion is a marked characteristic of penaeid shrimp crustins that is not encountered in the carcinins and crustin-like peptides of several nonpenaeid decapoda (crabs and lobsters). The glycines, being small amino acids, might render the glycine-rich region flexible and/or allow tight bends in the structure, but, as yet, it is unclear why this glycine-rich region occurs so frequently in shrimp crustins and so rarely in the Pleocyemata or what functional properties it confers on the mature proteins (Smith et al., 2008). Interestingly, a recent report on the crayfish *P. leniusculus* (Jiravanichpaisal et al., 2007) referred to the occurrence of both peptide forms in the same crustacean species. Apart from this recent report, crustins with an over representation of glycine residues have been reported only in penaeid shrimps. However, only crustin-2 was found to be a novel isoforms of crustin, the other two being already reported from shrimp species. The analysis of the results of BLAST, multiple alignment and phylogenetic analysis, all suggests that the crustin-2 reported here is a new crustin-like antimicrobial peptide in crustacean. The predicted molecular weight and isoelectric point all fall into the characteristic features described for the crustin family.

The predicted secondary structure of crustin-1 indicated a random coiled structure that is with two possible β-sheets. But no helices are predicted in the secondary structure. Hence, based on these factors and as per Powers and Hancock (2003) and Brockton et al. (2007), crustin is probably proposed to be a β-sheet, or possibly a loop protein, that may have structural similarity to the horseshoe crab defensins. However, peptide structure could not be created for crustin-2 as no suitable templates were available. The predicted secondary structure of crustin-3 was found to be different from crustin-1. A coiled structure with a helical segment could only be detected. The sequence being partial, complete structural elucidation of the peptide molecule was not possible.

As an important antimicrobial protein in the plasma and haemocyte granules of crustaceans, crustin-like protein has received the attention of many researchers. Until recently, many sequence characterization about crustin had been described in several penaeid species (Gross et al., 2001; Bartlett et al., 2002; Rattanachai et al., 2004; Vargas-Albores et al., 2004; Zhang et al., 2006).

Because crustins have antibacterial properties, they must contribute to a greater or lesser extent in defense against bacterial infections, at least in decapods. However, the variable pattern in their expression makes these molecules highly enigmatic. Their haemocytic location points to an association with host defense and whilst immunity is a major preoccupation for these cells, it is not the only one. Certainly, those, such as carcinin, that are expressed at very high levels must play an important role for the host but whether the function of these proteins is solely for bacteriostatis or for some other aspect of homoeostasis remains to be determined. The expression of two Type I crustins in regenerating tissues (Stoss et al., 2003; Durica et al., 2006) is suggestive of a possible role for crustins in recovery from trauma or response to physiological stress or as negative regulators of other host-defense factors. Since infection, injury and stress are common bedfellows and recovery involves numerous whole-body compensatory processes, often mediated through the haemocytes, crustins or crustin-like molecules may well be up-regulated by a variety of triggers. Clearly, to resolve the enigmas about crustins further studies are required to elucidate the variety of functions of the crustin-like peptides in decapods. The wide distribution of crustins in crustaceans indicates the importance of the proteins in the innate immunity and further studies are required for investigation of their biological functions.

Penaeidins are antimicrobial peptides isolated and characterized from penaeid shrimps where they appear to be ubiquitous (Destoumieux, et al., 1997, Gross et al., 2001; Rojtinnakorn, et al., 2002; Supungul, et al., 2002;

Munoz et al., 2004). The functions of the penaeidins in defense reactions were fully characterized in terms of biological activity invitro and gene expression and shrimp tissue distribution in response to microbial challenges in vivo (Destoumieux, et al., 1999; 2000; Munoz, et al., 2002, 2003). More recently, penaeidins were also detected in other penaeid species (Gross et al., 2001; Rojtinnakorn, et al., 2002; Supungul, et al., 2002; Munoz et al., 2004). Penaeidins have the potential to be used by the immune system to target-specific types of microbes that cause infection, infestation and the spread of disease. Furthermore, the variability in this peptide family provides a reservoir of diversity generated in nature that may hold promise for the treatment of human disease (Cuthbertson et al., 2008).

In the present study two partial mRNA sequences of penaeidins belonging to subgroup 3 and 5 was characterized at the molecular level. All these peptides share amino acid sequence similarities and according to the international penaeidin classification (PenBase, http://www.penbase.immunaqua.com), three distinct subgroups of penaeidins are now recognized, i.e. Penaeidin-2, -3, -4 and -5. After sequencing, the deduced amino acid sequences showed evident homology with the penaeidin antimicrobial peptide family. The amino acid structure of these peptides was similar to that described for penaeidins already reported from *P. monodon* and was in agreement with the proposed signature for the penaeidin family. Phylogenetic analyses of penaeidins of penaeid shrimps revealed that regardless of species, the penaeidins clustered together with strong nodal support. Thus, suggesting that these peptides of penaeid shrimps are paralogous and evolved by gene duplication events. Previous studies on the molecular evolution of AMPs in many vertebrates and invertebrates also showed strong evidence of gene duplication in AMPs of respective taxonomic groups.

Penaeidins represent a reservoir of diversity for the development of potential peptide antibiotics that can be exploited through translational science. The penaeidins which are present in different tissues and locations

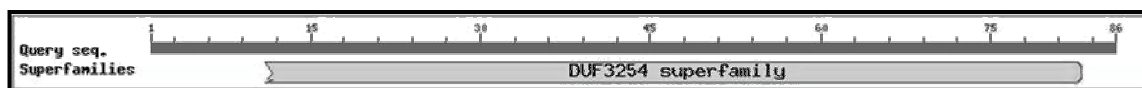
in the shrimp body, by the presence of haemocytes, could combine antimicrobial and chitin-binding properties that may be important in interactions between immune function and developmental function through the synthesis of exoskeleton in shrimp. These possible multifunctional properties of antimicrobial peptides represent an important new area to be investigated. The study of such AMP functions and expression in shrimp is particularly important for fundamental understanding of shrimp immunity and for further establishment of disease control in shrimp aquaculture (Bachere, 2003). Any progress in this field would contribute to a better understanding of the penaeid shrimp physiology and of their capacity to respond to pathological injuries (Bachere et al., 2000b). Moreover, from their unusual features both at the structural level and in terms of biological activity, penaeidins could represent, along with other AMPs, a new generation of therapeutic agents and find potential applications in aquaculture.

The recent discovery of AMPs in crustaceans provides new clues for fundamental understanding of crustacean immunity. The study of such AMP functions and expression in shrimp is particularly important for fundamental understanding of shrimp immunity and for further establishment of disease control in shrimp aquaculture (Bachere, 2003). Moreover, from their unusual features both at the structural level and in terms of biological activity, AMPs represent a new generation of therapeutic agents and find potential applications in aquaculture. Moreover, AMP represents a new generation of therapeutic agents with potential applications in aquaculture.

**Table 2.1. Primers used for the study**

| Target gene | Sequence (5′-3′) | Product Size (bp) | Annealing Temp. ($^0C$) | $MgCl_2$ Conc. (mM) |
|---|---|---|---|---|
| ALF-1 | F- gcacgagggagcttcatatt<br>R- gagcaaagggcctatgagtta | 264 bp | 62 | 1.5 |
| ALF-2 | F- caagggtgggaggctgtgg<br>R- tgagctgagccactggttgg | 300 bp | 62 | 1.5 |
| Crustin-1 | F- cgcacagccgagagaaacactatcaagat<br>R- ggcctatccctcagaacccagcacg | 456 bp | 55 | 3.5 |
| Crustin-2 | F- tgttcccacgacttcaagtgtgc<br>R- caaagattcaactaaataaacag | 299 bp | 60 | 3.5 |
| Crustin-3 | F- tccctggaggtcaattgagtg<br>R- agtcgaacatgcaggcctatcc | 233 bp | 60 | 1.5 |
| Penaeidin-3 | F- aggatatcatccagttcctg<br>R- acctacatcctttccacaag | 240 bp | 60 | 1.5 |
| Penaeidin-5 | F- acctgaccctcacctgcagaggcc<br>R- ttcgttgtcttctccatcaacc | 300 bp | 60 | 1.5 |
| β-actin | F- cttgtggttgacaatggctccg<br>R- tggtgaaggagtagccacgctc | 520 bp | 60 | 1.5 |
| ELF | F- atggttgtcaactttgcccc<br>R- ttgacctccttgatcacacc | 440 bp | 60 | 1.5 |

**Table.2.2. BLASTn analysis of ALF-1 (GU732817) in *P. monodon***

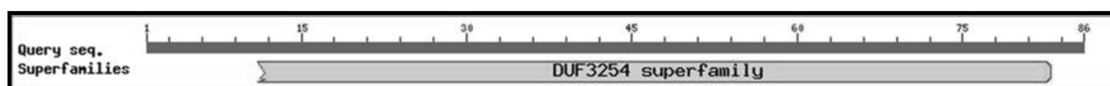



| GENBANK ACCESSION NUMBER | DESCRIPTION | QUERY COVERAGE | E-VALUE | % IDENTITY |
|---|---|---|---|---|
| EF523559 | *P. monodon* ALF isoform 3 mRNA, complete cds | 91% | 1e-38 | 77% |
| GU299806 | *P. monodon* clone ALF-9 mRNA, complete cds | 91% | 2e-34 | 76% |
| GU727863 | *F. indicus* ALF mRNA, complete cds | 60% | 2e-28 | 79% |
| AY859500 | *F. chinensis* ALF mRNA, complete cds | 60% | 2e-28 | 79% |
| EF601051 | *F. paulensis* ALF isoform 1 mRNA, complete cds | 54% | 2e-04 | 69% |

**Table .2.3. BLASTp analysis of ALF-1 (GU732817) in *P. monodon***

| GENBANK ACCESSION NUMBER | DESCRIPTION | MAX SCORE | TOTAL SCORE | QUERY COVERAGE | E-VALUE |
|---|---|---|---|---|---|
| ACC86067 | *P. monodon* ALF | 81.3 | 81.3 | 91% | 3e-14 |
| ADC32520 | *P. monodon* ALF | 79.7 | 79.7 | 91% | 9e-14 |
| AAX63831 | *F. chinensis* ALF | 79.0 | 79.0 | 92% | 2e-13 |
| ADE27980 | *F. indicus* ALF | 79.0 | 79.0 | 91% | 2e-13 |
| ABJ90465 | *L. schmitti* ALF | 68.2 | 68.2 | 92% | 3e-10 |

**Table 2.4. BLASTn analysis of ALF-2 (HM588914) in *P. monodon***




| GENBANK ACCESSION NUMBER | DESCRIPTION | QUERY COVERAGE | E-VALUE | % IDENTITY |
|---|---|---|---|---|
| **EF523559** | *P. monodon* ALF isoform 3 mRNA, complete cds | 98% | 5e-151 | 99% |
| **GU299806** | *P. monodon* clone ALF-9 mRNA, complete cds | 98% | 3e-144 | 98% |
| **GU727863** | *F. indicus* ALF mRNA, complete cds | 98% | 4e-122 | 93% |
| **AY859500** | *F. chinensis* ALF mRNA, complete cds | 98% | 2e-115 | 92% |
| **EF601051** | *F. paulensis* ALF isoform 1 mRNA, complete cds | 96% | 1e-68 | 83% |

**Table 2.5. BLASTp analysis of ALF-2 (HM588914) in *P. monodon***

| GENBANK ACCESSION NUMBER | DESCRIPTION | MAX SCORE | TOTAL SCORE | QUERY COVERAGE | % IDENTITY |
|---|---|---|---|---|---|
| **ABP73289** | *P. monodon* ALF isoform 3 | 204 | 204 | 100% | 2e-51 |
| **ACC86067** | *P. monodon* ALF | 203 | 203 | 100% | 4e-51 |
| **ADE27980** | *F.indicus* ALF | 196 | 196 | 100% | 8e-49 |
| **AAX63831** | *F. chinensis* ALF | 177 | 177 | 100% | 3e-43 |
| **ABQ96193** | *F. paulensis* ALF isoform 1 | 173 | 173 | 100% | 5e-42 |

**Table.2.6. BLASTn analysis of Crustin-1 (GQ334395) in *P. monodon***

| GENBANK ACCESSION NUMBER | DESCRIPTION | QUERY COVERAGE | E-VALUE | % IDENTITY |
|---|---|---|---|---|
| **EF654658** | *P. monodon* crustin-like antimicrobial peptide mRNA, complete cds | 100% | 0.0 | 97% |
| **EF654659** | *P. monodon* crustin-like antimicrobial peptide gene, complete cds | 94% | 0.0 | 98% |
| **FJ539178** | *P. monodon* clone PMC-36 crustin-like antimicrobial peptide mRNA, complete cds | 93% | 0.0 | 94% |
| **GU299808** | *P. monodon* clone pcm21 crustin-like peptide mRNA, complete cds | 93% | 1e-173 | 93% |
| **DQ097703** | *F. chinensis* crustin-like protein fc-1 mRNA, complete cds | 100% | 1e-123 | 85% |

**Table. 2.7. BLASTp analysis of Crustin-1 (GQ334395) in *P. monodon***

| GENBANK ACCESSION NUMBER | DESCRIPTION | MAX SCORE | TOTAL SCORE | QUERY COVERAGE | % IDENTITY |
|---|---|---|---|---|---|
| **ACL97376** | *P.monodon* crustin-like AMP | 161 | 161 | 100% | 3e-38 |
| **AAZ76017** | *F. chinensis* crustin-like protein fc-1 | 133 | 133 | 48% | 6e-30 |
| **AAZ76018** | *F. chinensis* crustin-like protein fc-2 | 67.0 | 67.0 | 48% | 8e-10 |
| **BAD15064** | *M. japonicus* crustin-like peptide type 3 | 59.3 | 59.3 | 46% | 1e-07 |
| **ABQ96197** | *F. brasiliensis* crustin | 53.1 | 53.1 | 33% | 9e-06 |

**Table 2.8. BLASTn analysis of Crustin-2 (FJ535568) in *P. monodon***

| GENBANK ACCESSION NUMBER | DESCRIPTION | QUERY COVERAGE | E-VALUE | % IDEN TITY |
|---|---|---|---|---|
| **FJ853146** | *F. chinensis* crustin 1 mRNA, partial cds | 93% | 2e-90 | 88% |
| **FJ686015** | *P. monodon* crustin Pm4 antimicrobial peptide mRNA, complete cds | 36% | 3e-49 | 100% |
| **AF430076** | *L. vannamei* clone ZAP 18 putative antimicrobial peptide mRNA, complete cds | 40% | 5e-32 | 88% |
| **EF182748** | *L. schmitti* crustin mRNA, complete cds | 42% | 2e-31 | 87% |
| **EF182747** | *F. paulensis* crustin mRNA, complete cds | 31% | 2e-31 | 94% |

**Table 2.9. BLASTp analysis of Crustin-2 (FJ535568) in *P. monodon***

| GENBANK ACCESSION NUMBER | DESCRIPTION | MAX SCORE | TOTAL SCORE | QUERY COVERAGE | % IDENTITY |
|---|---|---|---|---|---|
| **ACQ66005** | *P. monodon* crustin Pm4 antimicrobial peptide | 80.5 | 80.5 | 38% | 6e-14 |
| **ACZ43781** | F. chinensis crustin 1 | 76.6 | 76.6 | 38% | 9e-13 |
| **ABO93323** | *F.subtilis* crustin | 67.0 | 67.0 | 38% | 7e-10 |
| **ABM63361** | *F.paulensis* crustin | 67.0 | 67.0 | 38% | 7e-10 |
| **AAS59735** | *L. vannamei* crustin I | 66.6 | 66.6 | 38% | 1e-09 |

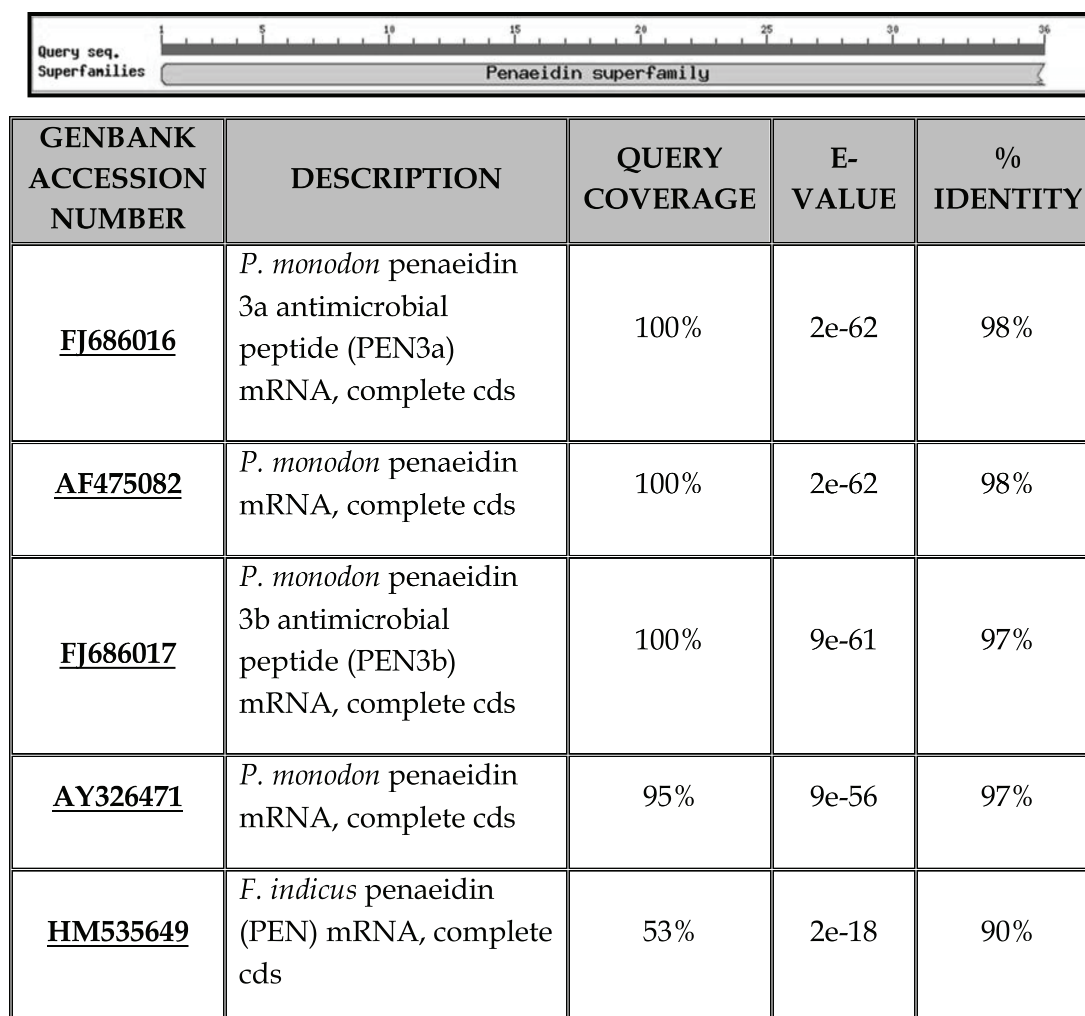
**Table 2.10. BLASTn analysis of Crustin-3 (GQ334396) in *P. monodon***

| GENBANK ACCESSION NUMBER | DESCRIPTION | QUERY COVERAGE | E-VALUE | % IDENTITY |
|---|---|---|---|---|
| **EF654659** | *P. monodon* crustin-like AMP gene, complete cds | 100% | 9e-118 | 99% |
| **FJ539178** | *P. monodon* clone PMC-36 crustin-like antimicrobial peptide mRNA, complete cds | 93% | 2e-105 | 98% |
| **GU299808** | *P. monodon* clone pcm21 crustin-like peptide mRNA, complete cds | 93% | 4e-92 | 94% |
| **DQ097703** | *F. chinensis* crustin-like protein fc-1 mRNA, complete cds | 97% | 3e-88 | 92% |
| **DQ097704** | *F. chinensis* crustin-like protein fc-2 mRNA, complete cds | 61% | 2e-25 | 94% |

**Table 2.11. BLASTp analysis of Crustin-3 (GQ334396) in *P. monodon***

| GENBANK ACCESSION NUMBER | DESCRIPTION | MAX SCORE | TOTAL SCORE | QUERY COVERAGE | % IDENTITY |
|---|---|---|---|---|---|
| **ABV25094** | *P. monodon* crustin-like AMP | 138 | 138 | 95% | 2e-31 |
| **AAZ76017** | *F.chinensis* crustin-like protein fc-1 | 125 | 125 | 94% | 1e-27 |
| **AAZ76018** | *F.chinensis* crustin-like protein fc-2 | 58.9 | 58.9 | 94% | 2e-07 |
| **BAD15063** | *M.japonicus* crustin-like peptide type 2 | 56.6 | 56.6 | 80% | 1e-06 |
| **ABQ96197** | *F. brasiliensis* crustin | 50.4 | 50.4 | 68% | 6e-05 |

**Table 2.12. BLASTn analysis of Penaeidin-3 (GU732819) in *P. monodon***

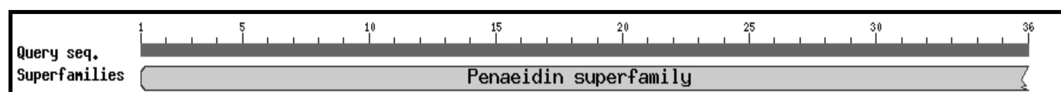



| GENBANK ACCESSION NUMBER | DESCRIPTION | QUERY COVERAGE | E-VALUE | % IDENTITY |
|---|---|---|---|---|
| FJ686016 | *P. monodon* penaeidin 3a antimicrobial peptide (PEN3a) mRNA, complete cds | 100% | 2e-62 | 98% |
| AF475082 | *P. monodon* penaeidin mRNA, complete cds | 100% | 2e-62 | 98% |
| FJ686017 | *P. monodon* penaeidin 3b antimicrobial peptide (PEN3b) mRNA, complete cds | 100% | 9e-61 | 97% |
| AY326471 | *P. monodon* penaeidin mRNA, complete cds | 95% | 9e-56 | 97% |
| HM535649 | *F. indicus* penaeidin (PEN) mRNA, complete cds | 53% | 2e-18 | 90% |

**Table 2.13. BLASTp analysis of Penaeidin-3 (GU732819) in *P. monodon***

| GENBANK ACCESSION NUMBER | DESCRIPTION | MAX SCORE | TOTAL SCORE | QUERY COVERAGE | % IDENTITY |
|---|---|---|---|---|---|
| AAQ05769 | *P. monodon* penaeidin | 44.3 | 44.3 | 48% | 0.005 |
| ACQ66007 | *P. monodon* penaeidin 3b antimicrobial peptide | 40.4 | 40.4 | 48% | 0.062 |
| ADN43390 | *F.indicus* penaeidin | 35.0 | 35.0 | 39% | 3.0 |

**Table 2.14. BLASTn analysis of Penaeidin-5 (GQ334397) in *P. monodon***




| GENBANK ACCESSION NUMBER | DESCRIPTION | QUERY COVERAGE | E-VALUE | % IDENTITY |
|---|---|---|---|---|
| **AF475082** | *P. monodon* penaeidin mRNA, complete cds | 99% | 2e-78 | 98% |
| **DQ154152** | *F. chinensis* penaeidin 5-2 (PEN5-2) gene, complete cds | 94% | 2e-53 | 91% |
| **HM535649** | *F. indicus* penaeidin (PEN) mRNA, complete cds | 64% | 1e-40 | 95% |
| **AY956417** | *F. paulensis* antimicrobial peptide PEN2-2 (PEN2-2) mRNA, complete cds | 78% | 2e-39 | 90% |
| **EF450742** | *F. subtilis* penaeidin mRNA, complete cds | 78% | 3e-36 | 88% |

**Table 2.15. BLASTp analysis of Penaeidin-5 (GQ334397) in *P. monodon***

| GENBANK ACCESSION NUMBER | DESCRIPTION | MAX SCORE | TOTAL SCORE | QUERY COVERAGE | % IDENTITY |
|---|---|---|---|---|---|
| **AAQ05769** | *P. monodon* penaeidin | 74.3 | 74.3 | 100% | 4e-12 |
| **ADN43390** | *F. indicus* penaeidin | 70.5 | 70.5 | 100% | 7e-11 |
| **AAX58695** | *F.paulensis* antimicrobial peptide PEN2-1 | 67.0 | 67.0 | 100% | 6e-10 |
| **ABO93321** | *F. subtilis* penaeidin | 65.5 | 65.5 | 100% | 2e-09 |
| **ABO93324** | *F. brasiliensis* penaeidin | 63.9 | 63.9 | 100% | 7e-09 |

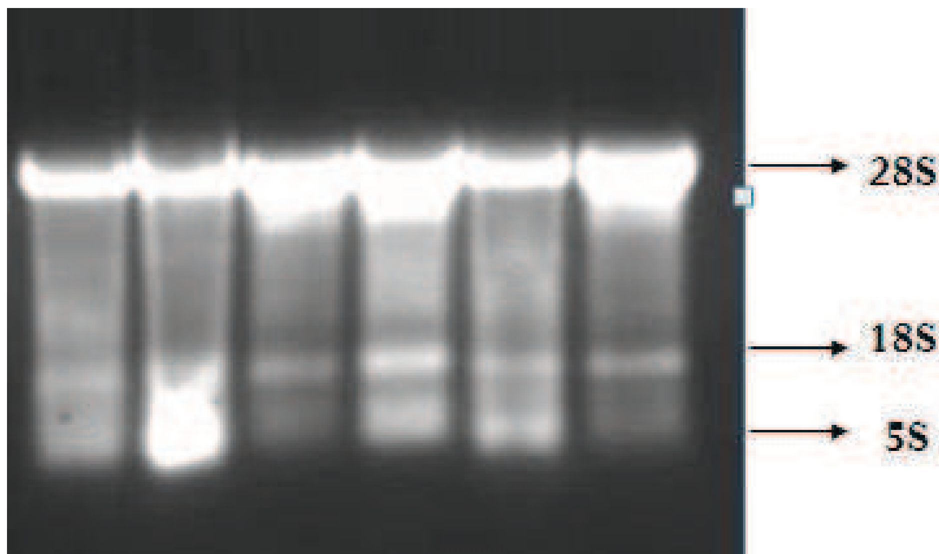
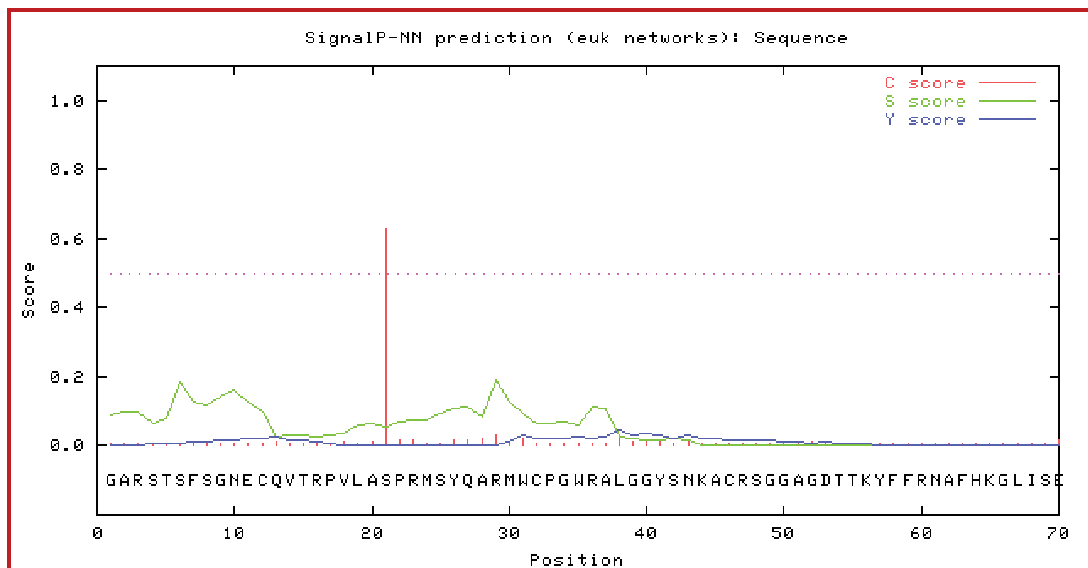



**Fig.2.5. Agarose gel electrophoretogram of RNA**

**Fig. 2.6. Nucleotide and amino acid sequences of ALF-1 from the haemocyte of the giant tiger shrimp, *P. monodon* (GU732817).**




**SIGNAL PEPTIDE (21$^{ST}$ POSITION)**

GARSTSFSGNECQVTRPVLASPRMSYQARMWCPGWRALGGYSNKACR
SGGAGDTTKYFFRNAFHKGLISEQEANQCLPPI

**Fig.2.7. Signal peptide analysis of ALF-1 (GU732817) in *P. monodon* as predicted by the SignalP 3.0 server. The underlined amino acid residues indicate a putative signal sequence.**

**Fig.2.8. Structural model of ALF-1 (GU732817) in *P. monodon* created using SWISS-MODEL Server**

**Fig.2.9. Multiple alignment of amino acid sequences of the *P. monodon* ALF-1 (GU732817) with other ALFs (*F. paulensis* EF601054, *P. monodon* EU617325, *F. paulensis* EF601052, *F. paulensis* EF601051, *P. monodon* EF523562, *F. chinensis* AY859500, *F. indicus* GU727863, *P. monodon* GU299806, *P. monodon* EF523559) obtained using GeneDoc programme Version 2.7.0. Black and grey indicates conserved sequences.**

**Fig.2.10. Multiple alignment of nucleotide sequence of the *P. monodon* ALF-1 (GU732817) with other ALFs (*F. paulensis* EF601054, *P. monodon* EU617325, *L. schmitti* DQ991357, *P. monodon* EF523559, *L. vannamei* DQ208702, *L. vannamei* DQ208703, *L. vannamei*DQ208704, *L. vannamei* DQ208705, *L. vannamei* DQ208706, *L. vannamei* GQ227486, *F. chinensis* AY859500) obtained using GeneDoc programme Version 2.7.0. Black and grey indicates conserved sequences.**

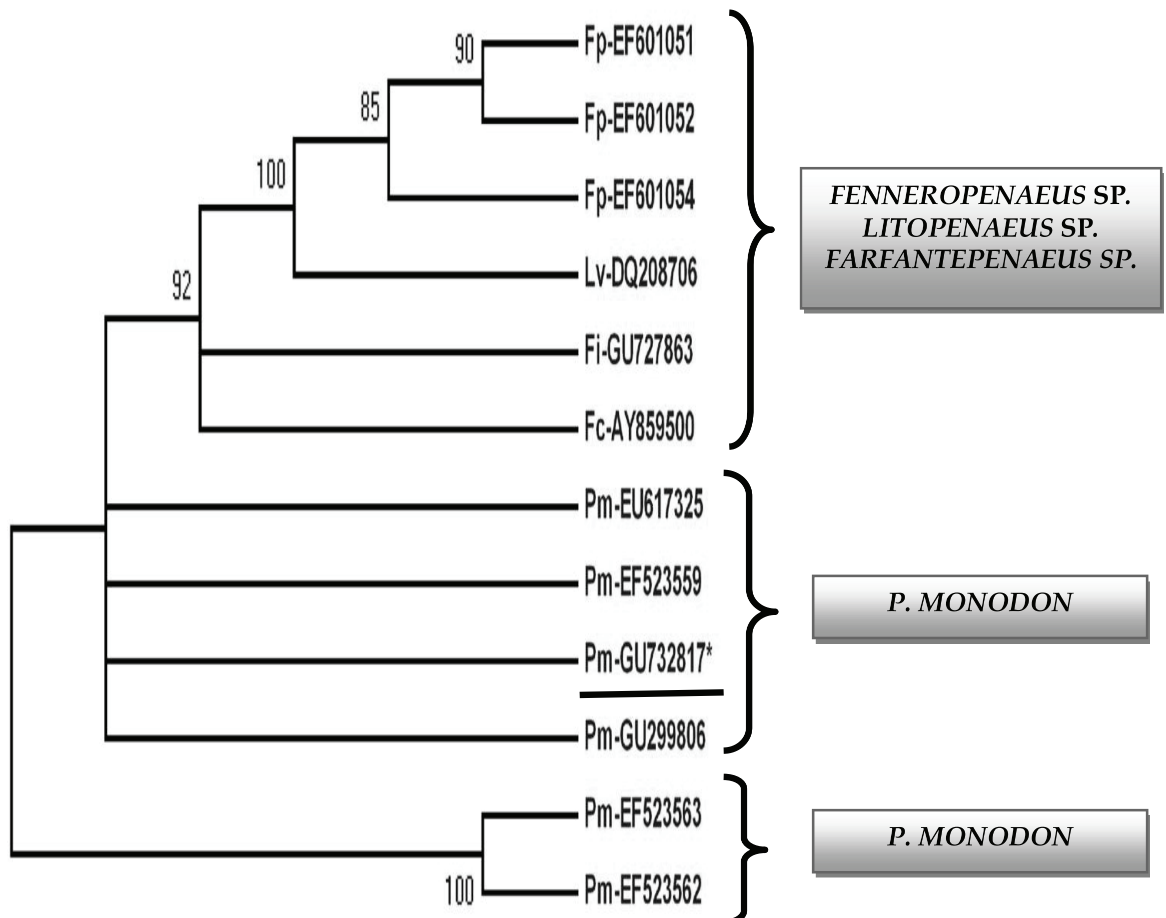



**Fig.2.11 A bootstrapped neighbour-joining tree obtained using MEGA version 4.0 illustrating relationships between the deduced amino acid sequence of the *P. monodon* ALF-1, (GU732817) with other ALFs of shrimps (*F. paulensis* EF601051, *F. paulensis* EF601052, *F. paulensis* EF601054, *L. vannamei* DQ208706, *F. indicus* GU727863, *F. chinensis* AY859500 *P. monodon* EU617325, P. *monodon* EF523559, *P. monodon* GU299806, *P. monodon* EF523562, *P. monodon* EF523563). Values at the node indicate the percentage of times that the particular node occurred in 1000 trees generated by bootstrapping the original deduced protein sequences.**

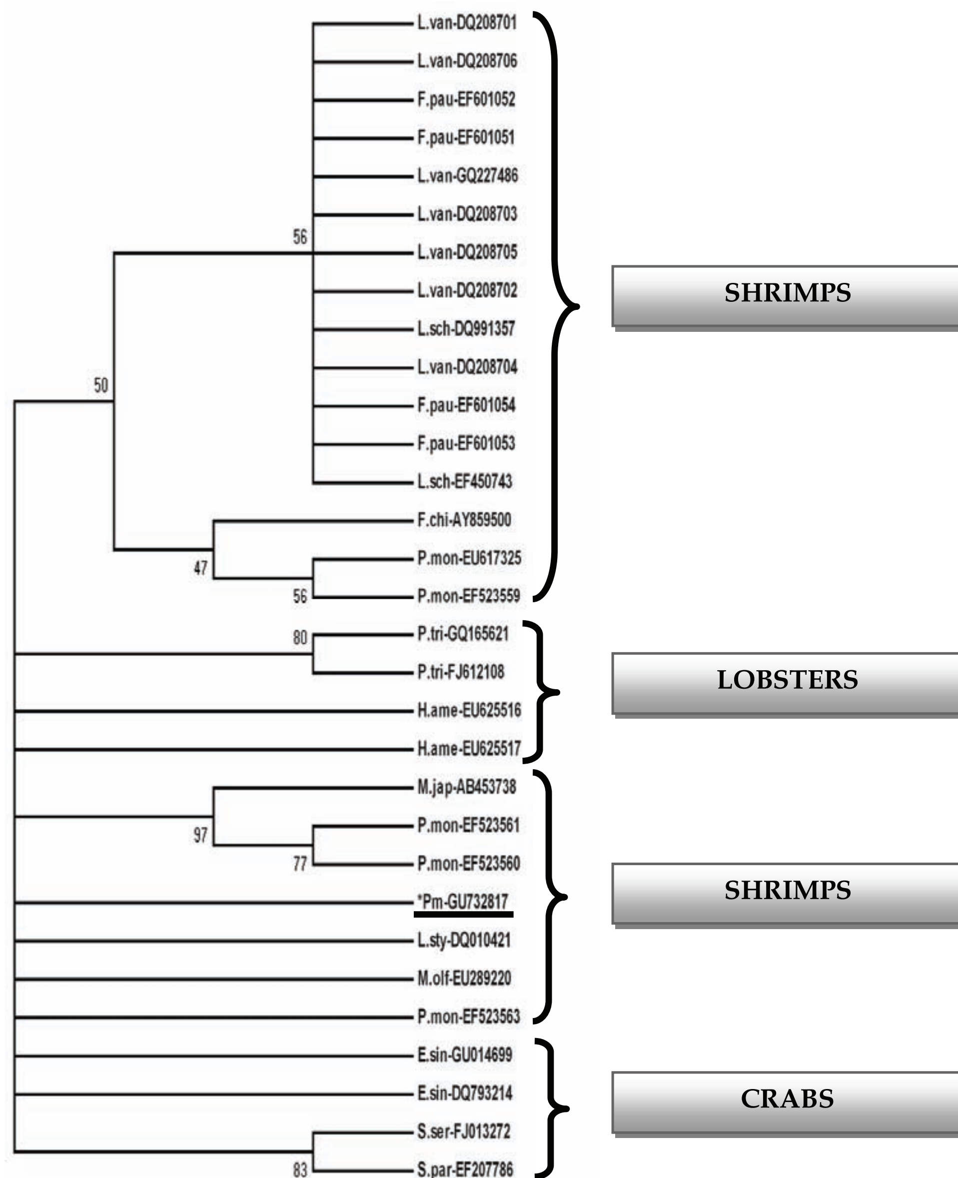



**Fig. 2.12 A bootstrapped neighbour-joining tree obtained using MEGA version 4.0 illustrating relationships between the deduced amino acid sequence of the *P. monodon* ALF-1 (GU732817) with other ALFs of decapod crustaceans (*F. chinensis* AY859500, *L. vannamei* DQ208701, *L. vannamei* DQ208702, *L. vannamei* DQ208703, *L. vannamei* DQ208704, *L. vannamei* DQ208705, *L. vannamei* DQ208706, *L. stylirostris* DQ010421, *P. trituberculatus* GQ165621, *E. sinensis* DQ793214, *S. serrata* FJ013272, *P. trituberculatus* FJ612108, *L. vannamei* GQ227486, *M. japonicus* AB453738, *H.americanus* EU625516, *H. americanus* EU625517, *P. monodon* EU617325, *E. sinensis* GU014699, *P. monodon* EF523563, *P. monodon* EF523559, *P. monodon* EF523561, *P. monodon* EF523560, *M. olfersii* EU289220, *F. paulensis* EF601054, *F .paulensis* EF601053, *F. paulensis* EF601052, *F. paulensis* EF601051, *L. schmitti* DQ991357, *L. schmitti* EF450743, *S. paramamosain* EF207786). Values at the node indicate the percentage of times that the particular node occurred in 1000 trees generated by bootstrapping the original deduced protein sequences.**

➢ NUCLEOTIDE SEQUENCE (301 bp)

acagtgccaggctcaagggtgggaggctgtggcagcggccgtcgccagcaa
gatcgtggggttgtggaggaacgaaaaactgaacttctcggccacgagtg
caagttcaccgtcaagccttatttgaagagattccaggtgtactacaaggg
gaggatgtggtgcccaggctggacggccatcagaggagaagccagcacacg
cagtcagtccggggtagctggaaagacagccaaagacttcgttcggaaagc
tttccagaaaggtctcatctctcaacaggaggccaaccagtaaaaa

➢ DEDUCED AMINOACID SEQUENCE:FRAME-2

QCQAQGWEAVAAAVASKIVGLWRNEKTELLGHECKFTVKPYLKRFQVYY
KGRMWCPGWTAIRGEASTRSQSGVAGKTAKDFVRKAFQKGLISQQEANQ

acagtgccaggctcaagggtgggaggctgtggcagcggccgtcgccagcaagatcgtgggg
Q C Q A Q G W E A V A A A V A S K I V G
ttgtggaggaacgaaaaactgaacttctcggccacgagtgcaagttcaccgtcaagcct
L W R N E K T E L L G H E C K F T V K P
tatttgaagagattccaggtgtactacaagggggaggatgtggtgcccaggctggacggcc
Y L K R F Q V Y Y K G R M W C P G W T A
atcagaggagaagccagcacacgcagtcagtccggggtagctggaaagacagccaaagac
I R G E A S T R S Q S G V A G K T A K D
ttcgttcggaaagctttccagaaaggtctcatctctcaacaggaggccaaccagtaaaaa
F V R K A F Q K G L I S Q Q E A N Q * K

**Fig. 2.13 Nucleotide and amino acid sequences of ALF-2 (HM588914) from the haemocyte of the giant tiger shrimp, *P. monodon*.**

**Fig. 2.14. Structural model of ALF-2 (HM588914) in *P. monodon* created uisng SWISS-MODEL Server**

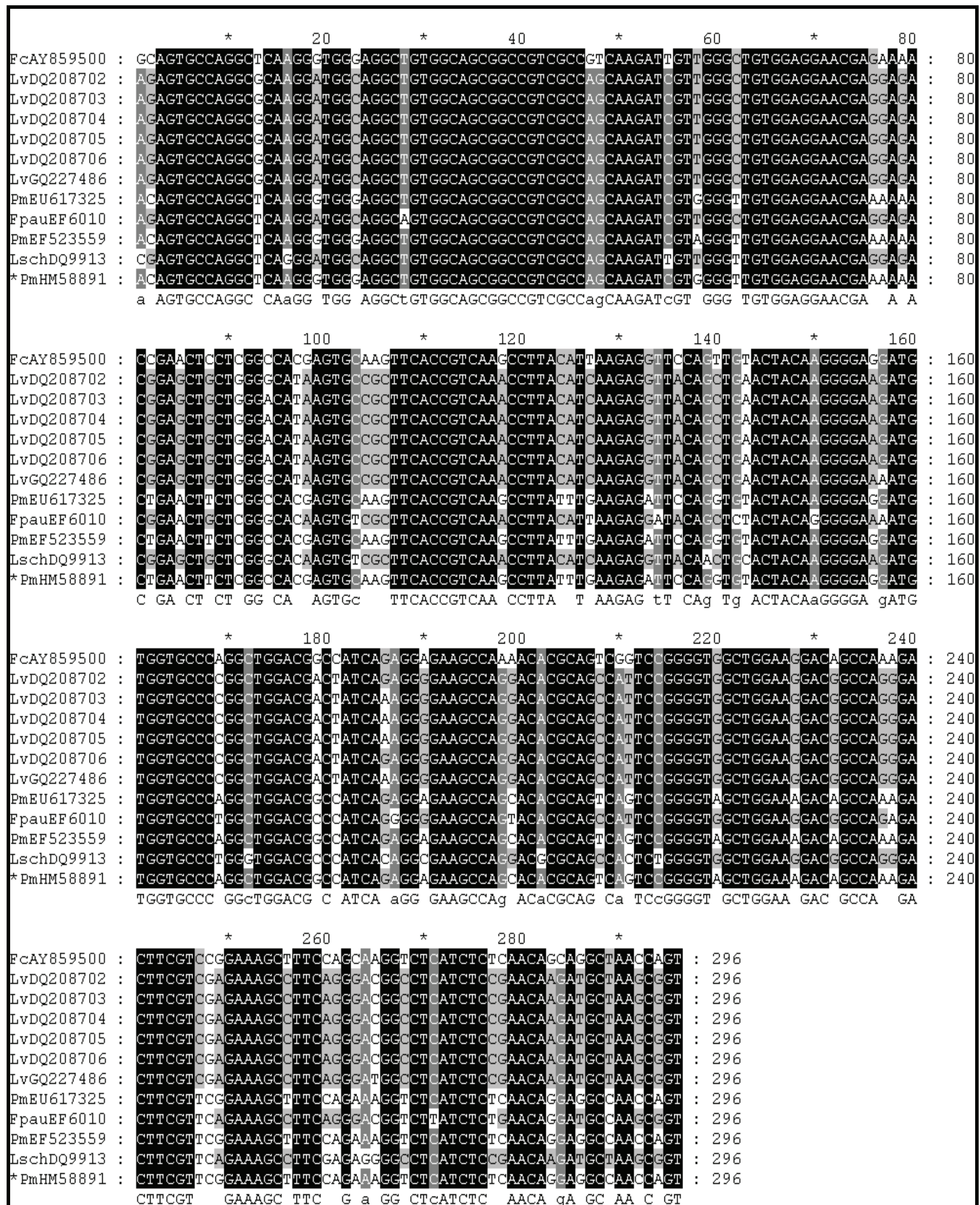



**Fig. 2.15. Multiple alignment of amino acid sequences of the *P. monodon* ALF-2 (HM588914) with other ALFs (*F. chinensis* AY859500, *L. vannamei* DQ208702, *L. vannamei* DQ208703, *L. vannamei* DQ208704, *L. vannamei* DQ208705, *L. vannamei* DQ208706, *L. vannamei* GQ227486, *P. monodon* EU617325, *F. paulensis* EF601054, *P. monodon* EF523559, *L. schmitti* DQ991357) obtained using GeneDoc programme Version 2.7.0. Black and grey indicates conserved sequences.**

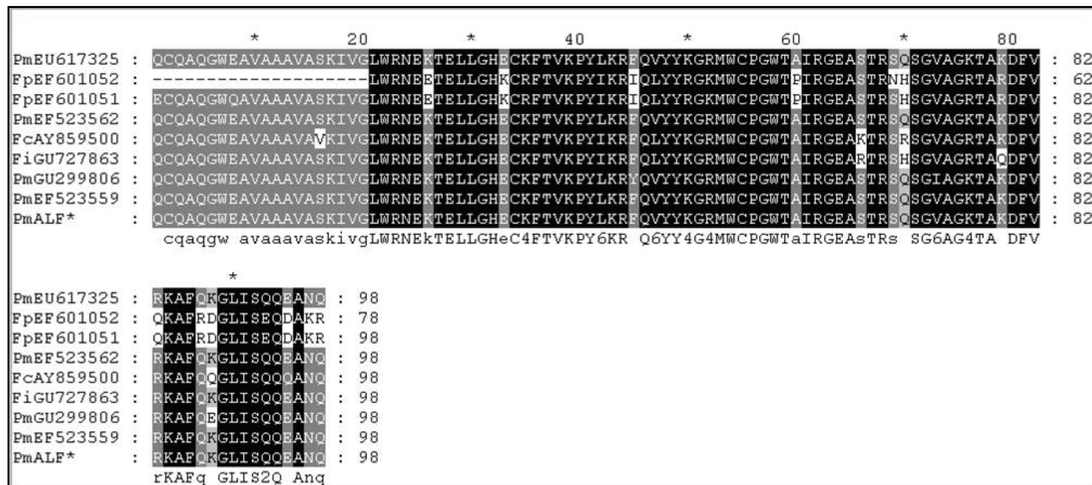



**Fig.2.16. Multiple alignment of amino acid sequence of the *P. monodon ALF-2* (HM588914) with other shrimp ALFs (*P. monodon* EU617325, *F. paulensis* EF601051, *F. paulensis* EF601052, *P. monodon* EF523562, *F. chinensis* AY859500, *F. indicus* GU727863, *P. monodon* GU299806, *P. monodon* EF523559) obtained using GeneDoc programme Version 2.7.0. Black and grey indicates conserved sequences.**

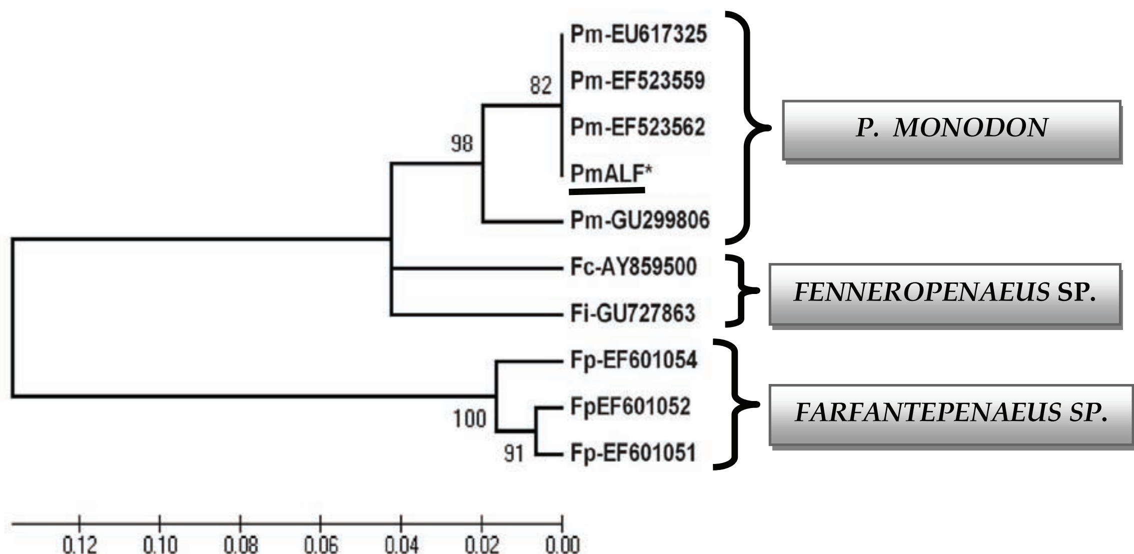



**Fig.2.17. A bootstrapped neighbour-joining tree obtained using MEGA version 4.0 illustrating relationships between the deduced amino acid sequence of the *P. monodon* ALF-2, (HM588914) with other ALFs of shrimps (*P. monodon* EU617325, *P. monodon* EF523559, *P. monodon* EF523562, *P. monodon* GU299806, *F. chinensis* AY859500, *F. indicus* GU727863, *F. paulensis* EF601054, *F. paulensis* EF601051, *F. paulensis* EF601052). Values at the node indicate the percentage of times that the particular node occurred in 1000 trees generated by bootstrapping the original deduced protein sequences.**

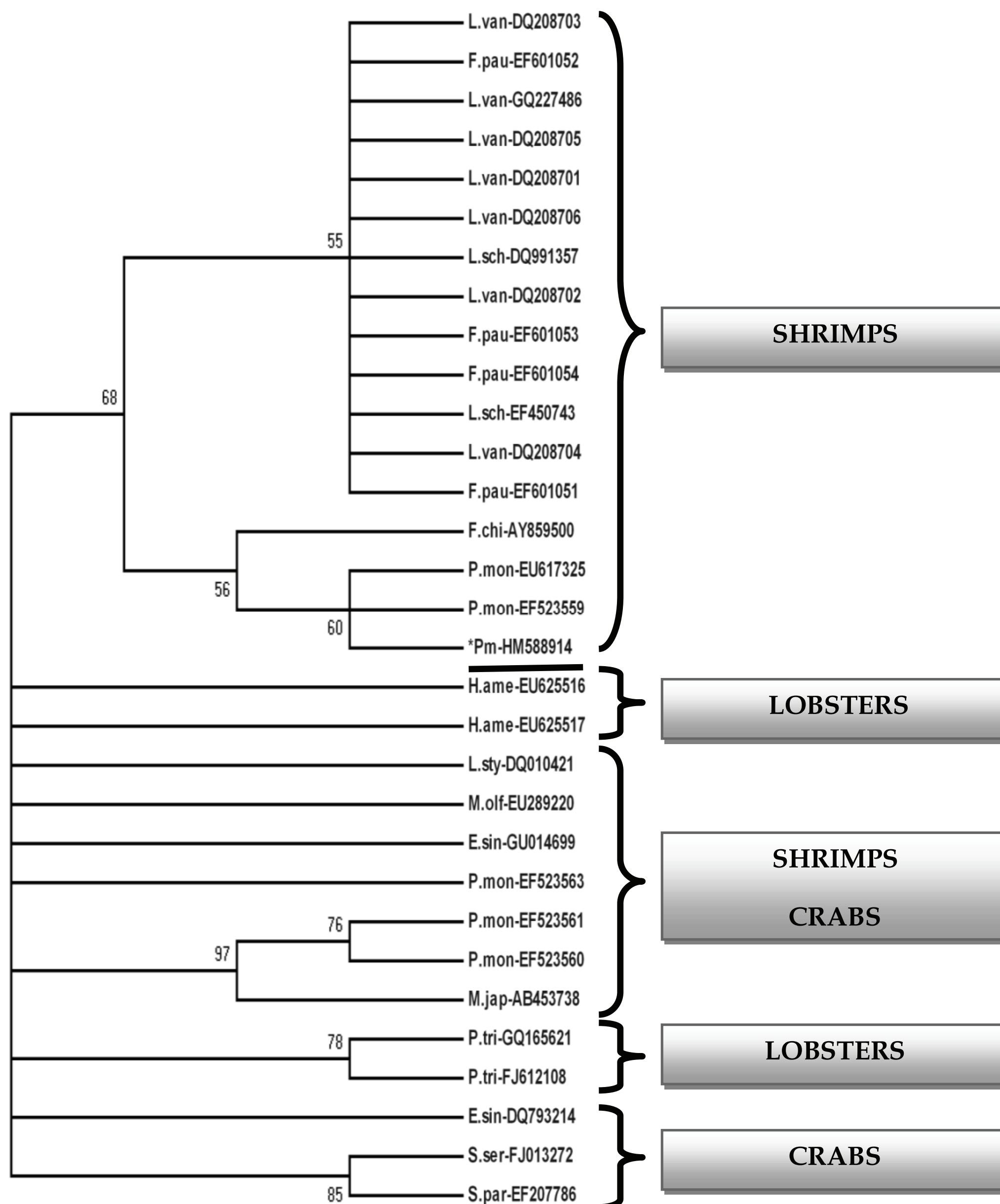



**Fig.2.18. A bootstrapped neighbour-joining tree obtained using MEGA version 4.0 illustrating relationships between the deduced amino acid sequence of the *P. monodon* ALF-2 (HM588914) with other ALFs of decapod crustaceans (*F. chinensis* AY859500, *L. vannamei* DQ208701, *L. vannamei* DQ208702, *L. vannamei* DQ208703, *L. vannamei* DQ208704, *L. vannamei* DQ208705, *L. vannamei* DQ208706, *L.stylirostris* DQ010421, *P. trituberculatus* GQ165621, *E. sinensis* DQ793214, *S. serrata* FJ013272, *P. trituberculatus* FJ612108, *L. vannamei* GQ227486, *M. japonicus* AB453738, *H. americanus* EU625516, *H. americanus* EU625517, *P. monodon* EU617325, *E. sinensis* GU014699, *P. monodon* EF523563, *P.monodon* EF523559, *P.monodon* EF523561, *P.monodon* EF523560, *M. olfersii* EU289220, *F. paulensis* EF601054, *F. paulensis* EF601053, *F. paulensis* EF601052, *F. paulensis* EF601051, *L. schmitti* DQ991357, *L. schmitti* EF450743, *S. paramamosain* EF207786). Values at the node indicate the percentage of times that the particular node occurred in 1000 trees generated by bootstrapping the original deduced protein sequences.**

➢ NUCLEOTIDE SEQUENCE (456 bp)

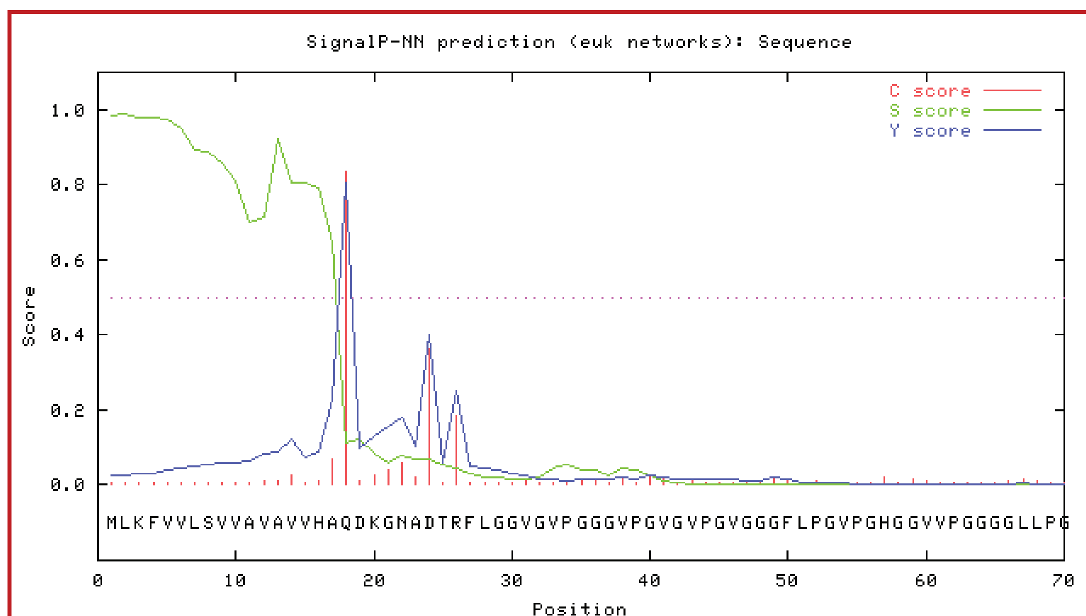
cgcacagccgagagaaacactatcaagatgctaaagtttgtagtattatccgttgtcgct
gtggctgtggtacacgcgcaggataaaggcaatgccgatactcgcttcctaggtggagtt
ggagttcctggaggtggagttcctggagttggagttcctggagttggaggtggattcctg
ccgggggttcctgggcatggtggcgttgttcctggaggcggtggccttctccctggaggt
caattcgagtgcaattactgcagaacgaggtacggatacgtatgctgcaagcccggcagg
tgtccacagattcgcgaaacctgcccaggcctcagaaagggtatcccgatctgccgtcag
gacactgactgcttcggctccgacaaatgctgcttcgacacctgcttgaacgacaccgtc
tgcaaacccatcgtgctgggttctgagggataggcc

➢ DEDUCED AMINOACID SEQUENCE:FRAME-1

MLKFVVLSVVAVAVVHAQDKGNADTRFLGGVGVPGGGVPGVGVPGVGGGFLPG
VPGHGGVVPGGGGLLPGGQFECNYCRTRYGYVCCKPGRCPQIRETCPGLRKGI
PICRQDTDCFGSDKCCFDTCLNDTVCKPIVLGSEG

cgcacagccgagagaaacactatcaagatgctaaagtttgtagtattatccgttgtcgct
R T A E R N T I K M L K F V V L S V V A
gtggctgtggtacacgcgcaggataaaggcaatgccgatactcgcttcctaggtggagtt
V A V V H A Q D K G N A D T R F L G G V
ggagttcctggaggtggagttcctggagttggagttcctggagttggaggtggattcctg
G V P G G G V P G V G V P G V G G G F L
ccgggggttcctgggcatggtggcgttgttcctggaggcggtggccttctccctggaggt
P G V P G H G G V V P G G G G L L P G G
caattcgagtgcaattactgcagaacgaggtacggatacgtatgctgcaagcccggcagg
Q F E C N Y C R T R Y G Y V C C K P G R
tgtccacagattcgcgaaacctgcccaggcctcagaaagggtatcccgatctgccgtcag
C P Q I R E T C P G L R K G I P I C R Q
gacactgactgcttcggctccgacaaatgctgcttcgacacctgcttgaacgacaccgtc
D T D C F G S D K C C F D T C L N D T V
tgcaaacccatcgtgctgggttctgagggataggcc
C K P I V L G S E G * A

**Fig. 2.19. Nucleotide and amino acid sequences of Crustin-1 (GQ334395) from the haemocyte of the the giant tiger shrimp, *P. monodon*. The underlined amino acid residues in red indicate a putative signal sequence. The underlined aminiacid residues indicate the WAP domain present in crustin-1. An asterisk is the stop codon.**




**SIGNAL PEPTIDE (1-17 = SIGNAL PEPTIDE)**

MLKFVVLSVVAVAVVHAQDKGNADTRFLGGVGVPGGGVPGVGVPGVGGGFLPG
VPGHGGVVPGGGGLLPGGQFECNYCRTRYGYVCCKPGRCPQIRETCPGLRKGI
PICRQDTDCFGSDKCCFDTCLNDTVCKPIVLGSEG

**Fig.2.20. Signal peptide analysis of Crustin-1 (GQ334395) in *P. monodon* as predicted by the SignalP 3.0 server. The underlined amino acid residues indicate a putative signal sequence.**

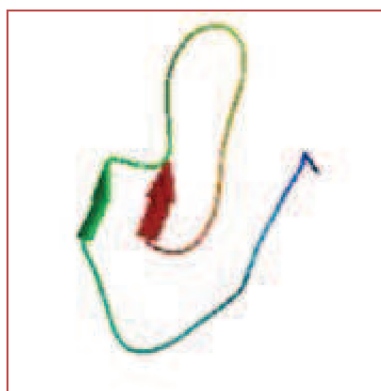


**Fig.2.21. Structural model of Crustin-1 (GQ334395) in *P. monodon* created uisng SWISS-MODEL Server**

A

B

**Fig. 2.22. ScanProsite analysis for the presence of conserved domain / pattern / motif in Crustin-1 (GQ334395) of *P. monodon.***
**(A) WAP domain signature (B) Glycine and cysteine rich regions**

**Fig.2.23. Schematic representation of matches map and list of matches obtained for Crustin-1 (GQ334395) of *P. monodon* from the Motif Scan search**

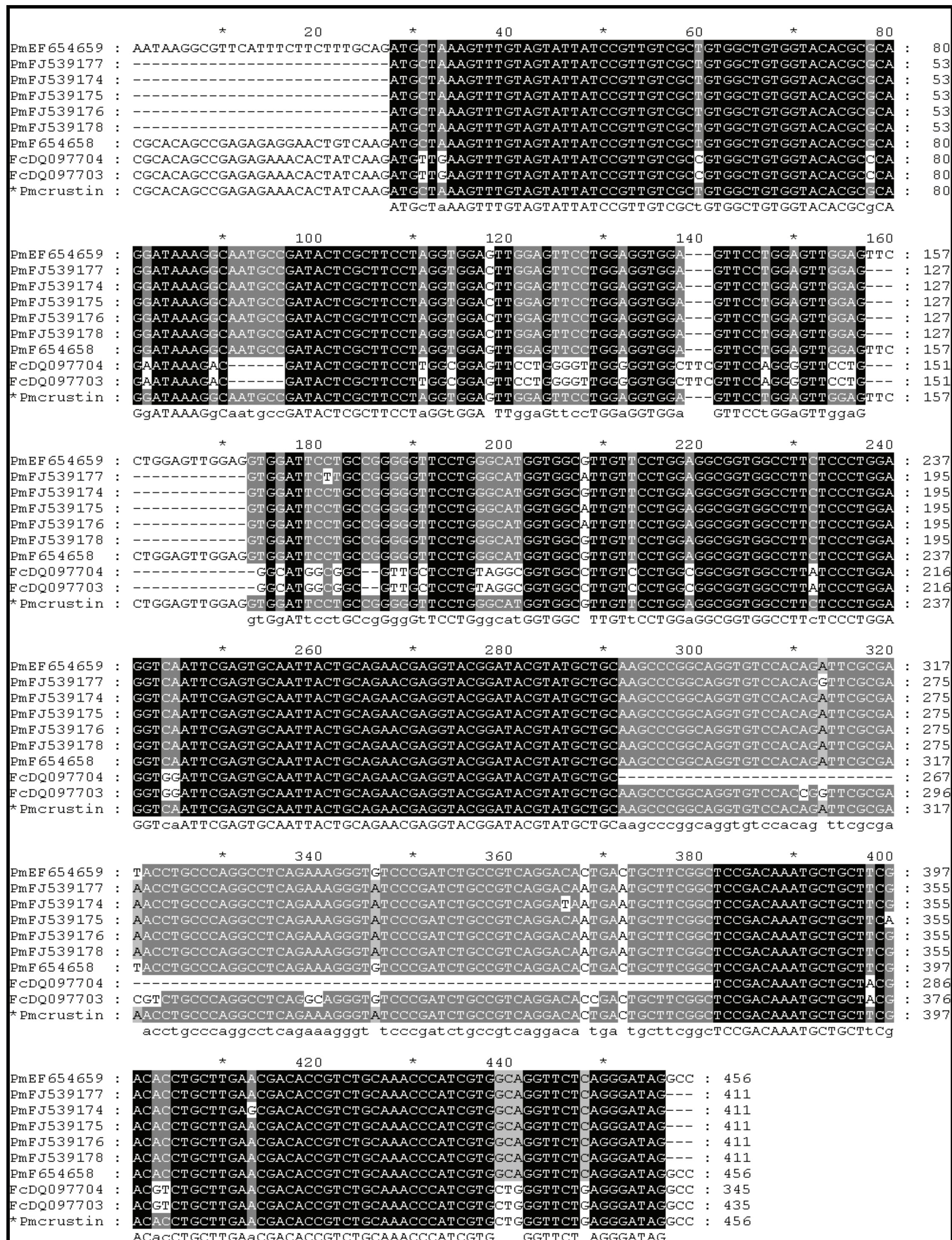


**Fig.2.24 Multiple alignment of nucleotide sequence of the *P. monodon* Crustin-1 (GQ334395) with other shrimp crustins (*P. monodon* EF654659, *P. monodon* FJ539177, *P. monodon* FJ539174 , *P. monodon* FJ539175 , *P. monodon* FJ539176 , *P. monodon* FJ539178 , *P. monodon* EF654658, *F. chinensis* DQ097704, *F. chinensis* DQ097703) obtained using GeneDoc programme Version 2.7.0. Black and grey indicates conserved sequences.**

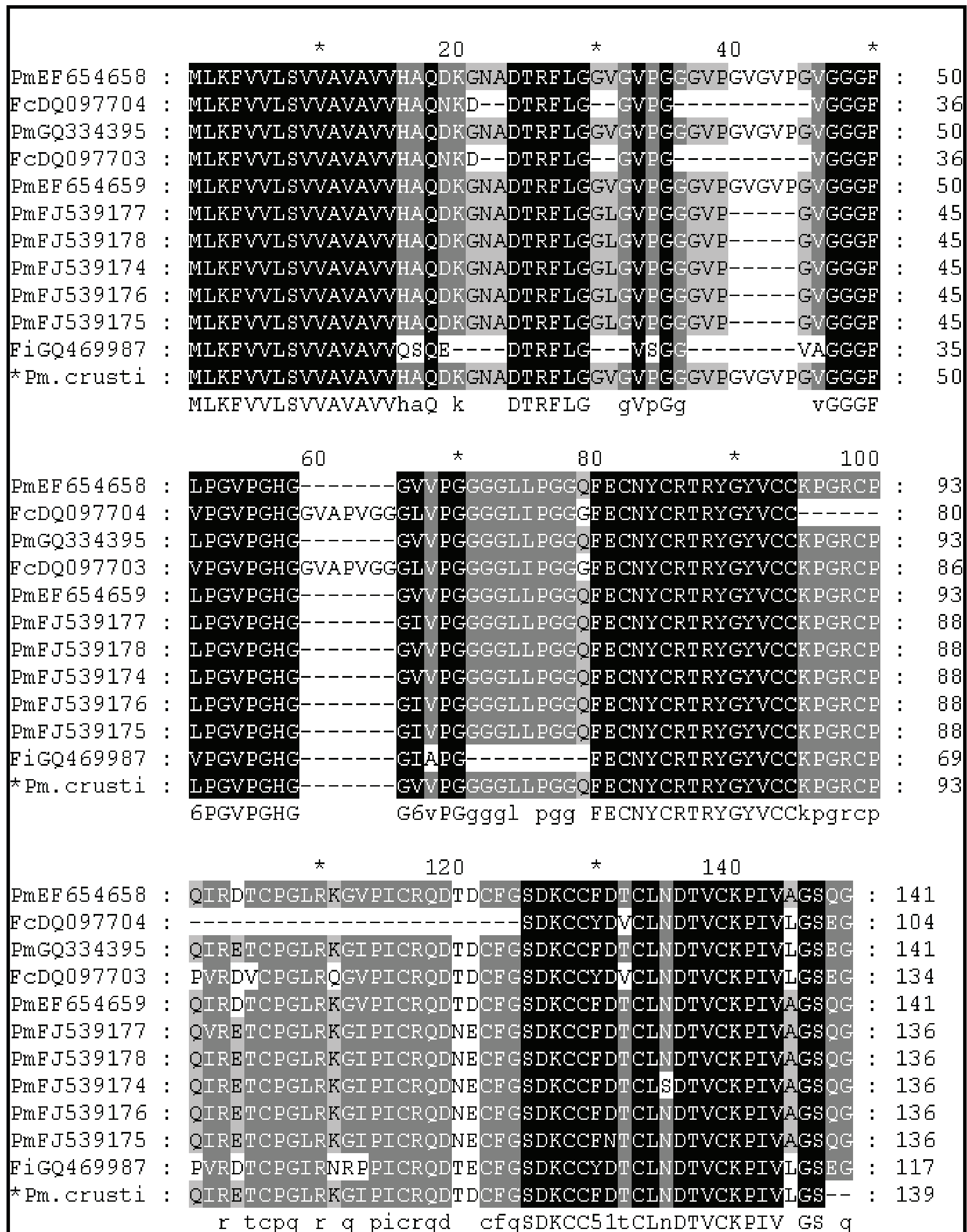



**Fig.2.25 Multiple alignment of amino acid sequence of the *Penaeus monodon* crustin-1 (GQ334395) with other shrimp crustins (*P. monodon* EF654658 , *F. chinensis* DQ097704, *P. monodon* GQ334395, *P. monodon* EF654659, *P. monodon* FJ539177 , *P. monodon* FJ539178 , *P. monodon* FJ539174 , *P. monodon* FJ539176 , *F. indicus* GQ469987)obtained using GeneDoc programme Version 2.7.0. Black and grey indicates conserved sequences.**

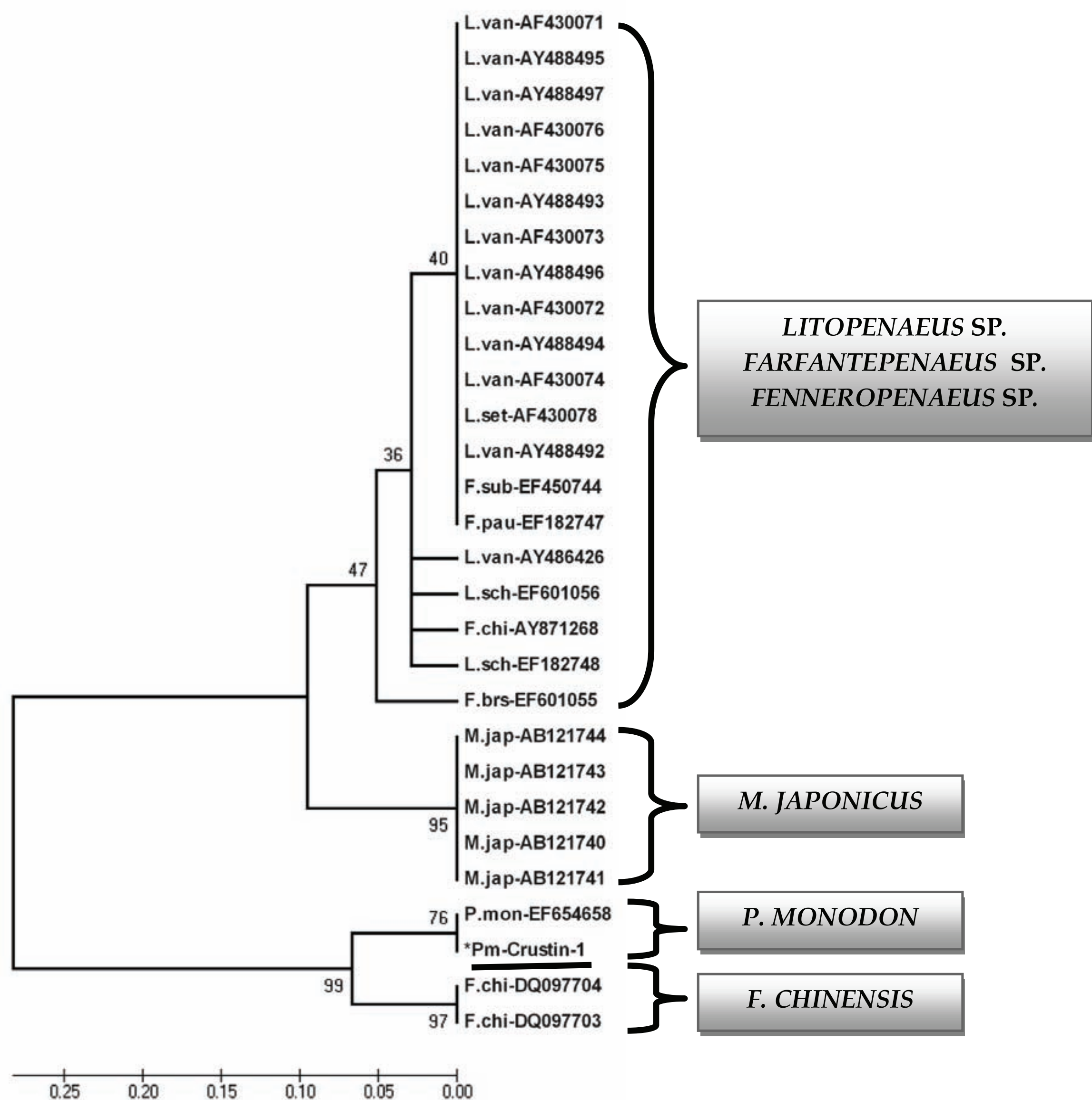



**Fig.2.26 A bootstrapped neighbour-joining tree obtained using MEGA version 4.0 illustrating relationships between the nucleotide sequence of the *P. monodon* crustin-1 (GQ334395) with other shrimp crustins *L. setiferus* AF430078, *L. vannamei* AF430075, *L. vannamei* AF430073, *L. vannamei* AF430072, *L.vannamei* AF430071, *L.vannamei* AF430076, *L. vannamei* AF430074, *L. schmitti* EF182748, *P. monodon* EF654658, *L. schmitti* EF601056 , *F. brasiliensis* EF60105, *F. subtilis* EF450744, *F. paulensis* EF182747 , *M. japonicus* AB121740, *M.japonicus* AB121744, *M.japonicus* AB121743, *M.japonicus* AB121742, *M. japonicus* AB121741, *F. chinensis* DQ097704, *F.chinensis* DQ097703, *F.chinensis* AY871268, *L. vannamei* AY486426, *L. vannamei* AY488497, *L. vannamei* AY488496, *L. vannamei* AY488495, *L. vannamei* AY488494, *L. vannamei* AY488493, *L. vannamei* AY488492). Values at the node indicate the percentage of times that the particular node occurred in 1000 trees generated by bootstrapping the original deduced protein sequences.**

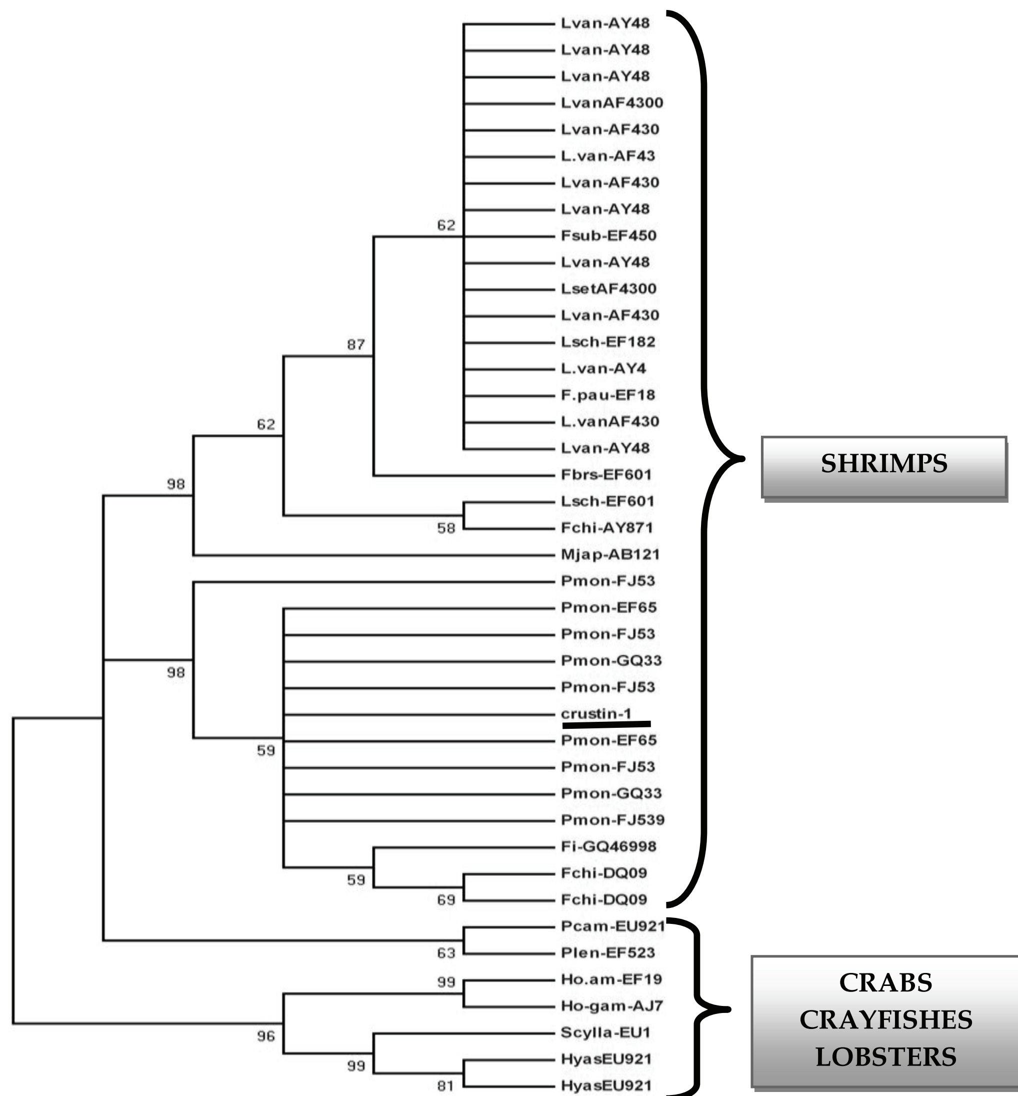



**Fig. 2.27. A bootstrapped neighbour-joining tree obtained using MEGA version 4.0 illustrating relationships between the deduced amino acid sequence of the *P. monodon* crustin-1 (GQ334395) with other crustins of decapod crustaceans (*L. vannamei* AF430071, *L.vannamei* AF430074, *L.vannamei* AY488497, *L.vannamei* AY488492, *L.setiferus* AF430078, *F.paulensis* EF182747, *L.vannamei* AY488493, *L.vannamei* AF430073, *L.vannamei* AY486426, *L.vannamei* AF430072, *F.subtilis* EF450744, *L.schmitti* EF182748, *L.vannamei* AY488496, *L. vannamei* AY488495, *L.vannamei* AF430075, *L.vannamei* AF430076, *L.vannamei* AY488494, *F.brasiliensis* EF601055, *L.schmitti* EF601056, *F.chinensis* AY871268, *M. japonicus* AB121740, *P.monodon* FJ539174, *P.monodon* EF654659, *P.monodon* FJ539178, *P.monodon* EF654658, *P.monodon* GQ334395, *P.monodon* GQ334396, *P.monodon* FJ539175, *P.monodon* FJ539177, *F. chinensis* DQ097704, *F.chinensis* DQ097703, *P.camtschaticus* EU921643, *P.leniusculus* 1 EF523614, *H. americanus* EF193003, *H. gammarus* AJ786653, *S. paramamosain* EU161287, *H. araneus* EU921642, *H. araneus* EU921641). Values at the node indicate the percentage of times that the particular node occurred in 1000 trees generated by bootstrapping the original deduced protein sequences.**

> **NUCLEOTIDE SEQUENCE (299 bp)**

gttcccacgacttcaagtgtgctggcctcgataagtgttgcttcgacaggtgtttgggag
aacacgtgtgcaagcctccttctttctacggaaggaatgttaaaggatgatgagaataaa
caaaagaccaactgacagacaaccgatgatttggaatttaggaccacgaatgttcaatct
actgttatgtcaagtaccaagcaatctgagagtactattatctgtaaaaaataaataaac
aaataataaatgtaacagggaatgaactactcctttctgtttatttagttgaatctttg

> **DEDUCED AMINOACID SEQUENCE:FRAME-3**

**SHDFKCAGLDKCCFDRCLGEHVCKPPSFYGRNVKG**

gttcccacgacttcaagtgtgctggcctcgataagtgttgcttcgacaggtgtttgggagaa
S H D F K C A G L D K C C F D R C L G E
cacgtgtgcaagcctccttctttctacggaaggaatgttaaaggatgatgagaataaaca
H V C K P P S F Y G R N V K G * * E * T
aaagaccaactgacagacaaccgatgatttggaatttaggaccacgaatgttcaatctac
K D Q L T D N R * F G I * D H E C S I Y
tgttatgtcaagtaccaagcaatctgagagtactattatctgtaaaaaataaataaacaa
C Y V K Y Q A I * E Y Y Y L * K I N K Q
ataataaatgtaacagggaatgaactactcctttctgtttatttagttgaatctttg
I I N V T G N E L L L S V Y L V E S L

**Fig. 2.28. Nucleotide and amino acid sequences of Crustin-2 (FJ535568) from the haemocyte of the the giant tiger shrimp, *P. monodon*. An asterisk is the stop codon.**

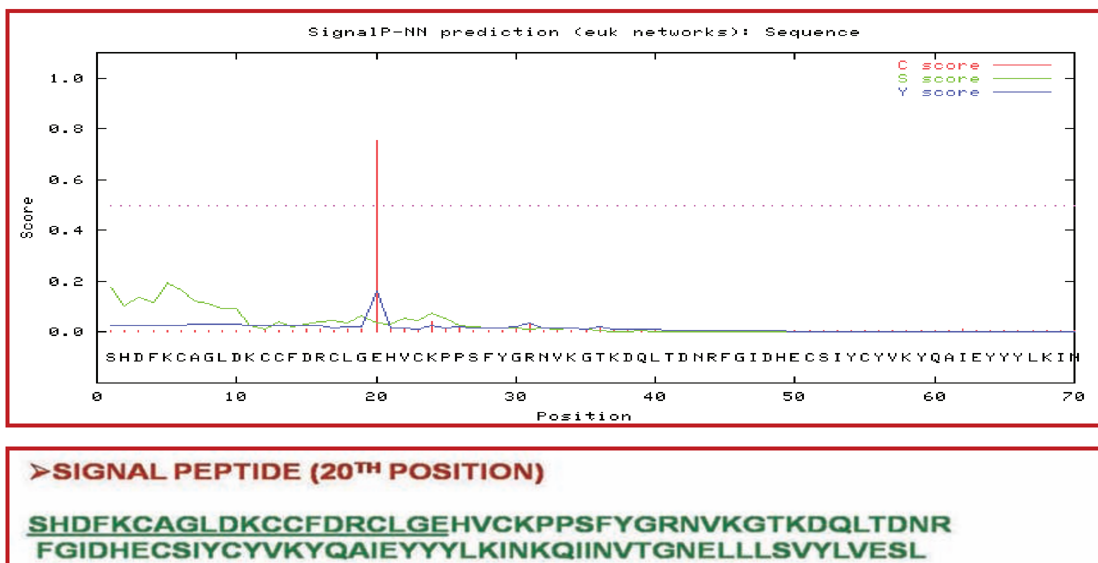



**Fig.2.29 Signal peptide analysis of Crustin-2 (FJ535568) in *P. monodon* as predicted by the SignalP 3.0 server. The underlined amino acid residues indicate a putative signal sequence.**

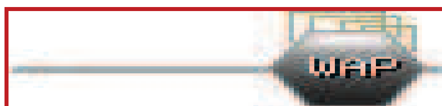



**Fig.2.30 ScanProsite analysis for the presence of conserved domain / pattern / motif in Crustin-2 (FJ535568) of *P. monodon.***

**Fig.2.31 Schematic representation of matches map and list of matches obtained for Crustin-2 (FJ535568) of *P. monodon* from the Motif Scan search**

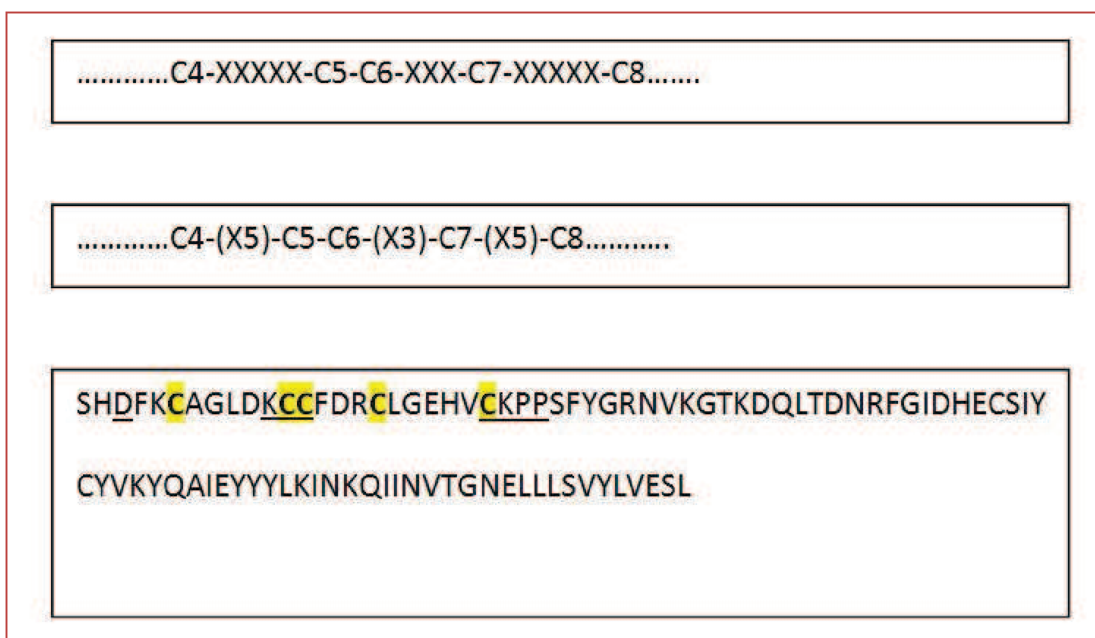



**Fig.2.32. The WAP domain signature of the Crustin-2 (FJ535568) in *P. monodon***

**(Consensus sequences that appear in the 4DSC domain are underlined and the $C_4$-$C_8$ cysteine residues are being highlighted)**

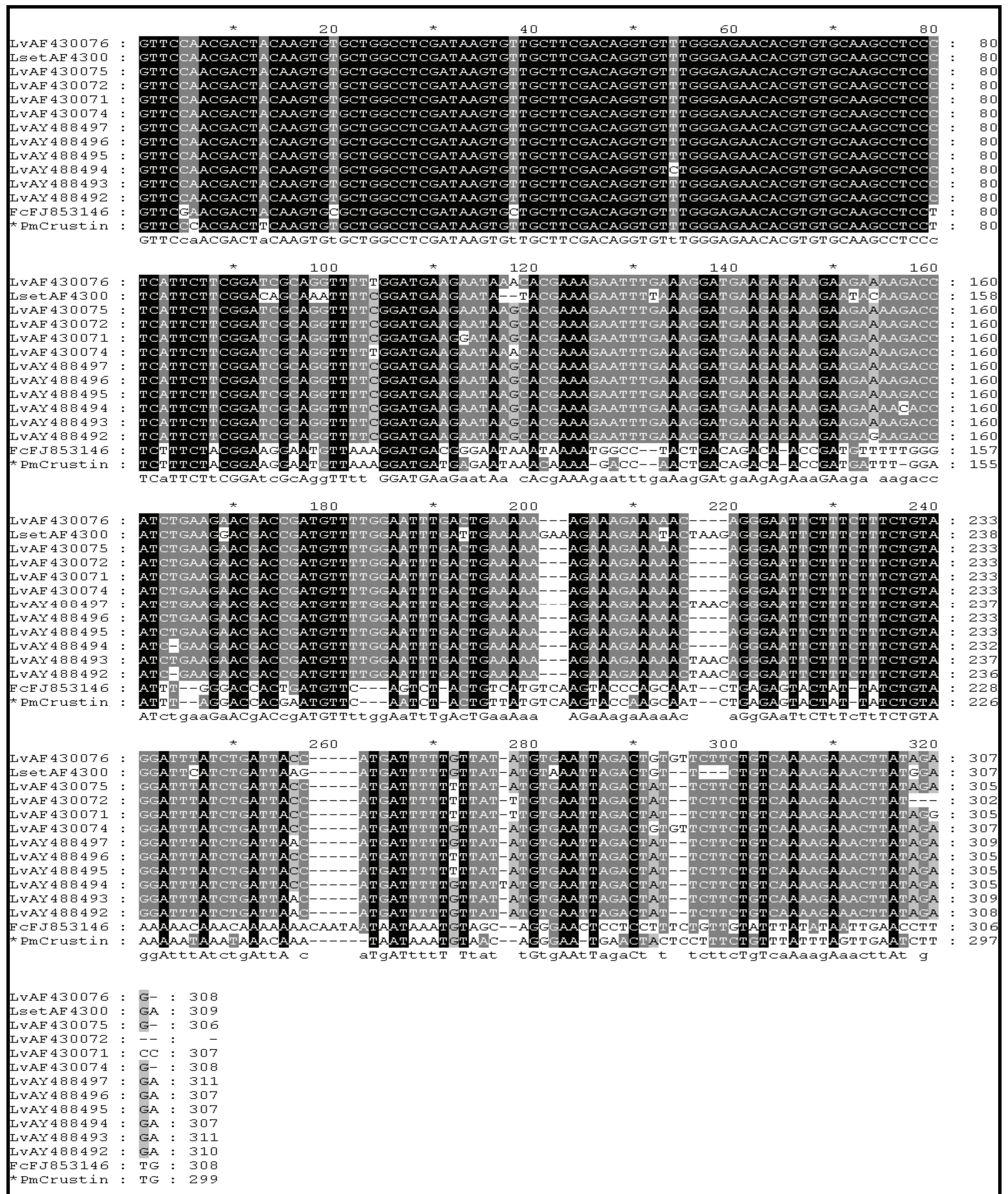



**Fig.2.33 Multiple alignment of nucleotide sequence of the *P. monodon* Crustin-2 (FJ535568) with other shrimp crustins (*L. vannamei* AF430076, *L. setiferus* AF430078, *L. vannamei* AF430075, *L. vannamei* AF430072, *L. vannamei* AF430071, *L. vannamei* AF430074, *L. vannamei* AY488497, *L. vannamei* AY488496, *L. vannamei* AY488495, *L. vannamei* AY488494, *L. vannamei* AY488493, *L. vannamei* AY488492) obtained using GeneDoc programme Version 2.7.0. Black and grey indicates conserved sequences.**

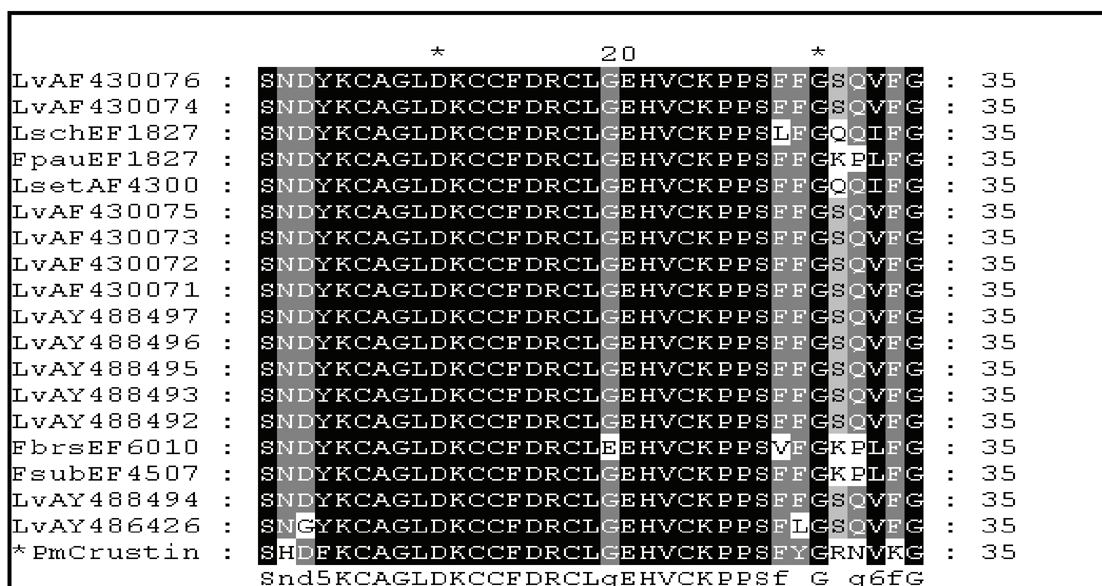



**Fig. 2.34 Multiple alignment of deduced amino acid sequence of the *P. monodon* Crustin-2 (FJ535568) with other shrimp crustins (*L. setiferus* AF430078, *L. vannamei* AF430075, *L. vannamei* AF430073, *L. vannamei* AF430072, *L. vannamei* AF430071, *L. vannamei* AF430076, *L. vannamei* AF430074, *L. vannamei* AY486426, *L. vannamei* AY488497, *L. vannamei* AY488496, *L. vannamei* AY488495, *L. vannamei* AY488494, *L. vannamei* AY488493, *L. vannamei* AY488492, *L. schmitti* EF182748, *F. brasiliensis* EF601055, *F. subtilis* EF450744, *F. paulensis* EF182747) obtained using GeneDoc programme Version 2.7.0. Black and grey indicates conserved sequences.**

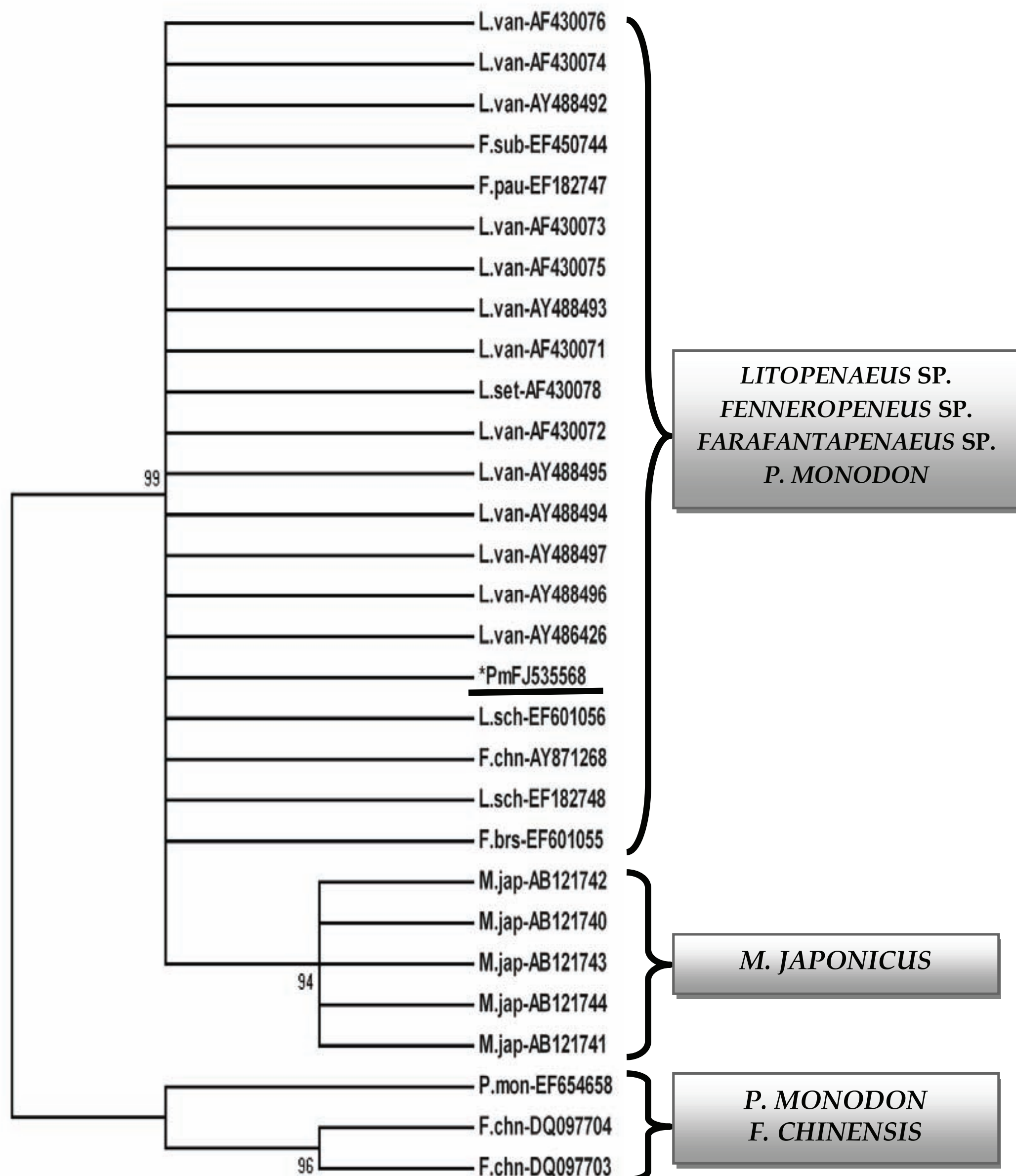



**Fig.2.35 A bootstrapped neighbour-joining tree obtained using MEGA version 4.0 illustrating relationships between the deduced amino acid sequence of the *P. monodon* Crustin-2 AMP (FJ535568) with other shrimp crustins (*L. setiferus* AF430078, *L. vannamei* AF430075, *L. vannamei* AF430073, *L. vannamei* AF430072), *L. vannamei* AF430071, *L. vannamei* AF430076, *L. vannamei* AF430074, *L. vannamei* AY486426, *L. vannamei* AY488497, *L. vannamei* AY488496, *L. vannamei* AY488495, *L. vannamei* AY488494, *L. vannamei* AY488493, *L. vannamei* AY488492, *L. schmitti* EF182748, *L. schmitti* EF601056, *P. monodon* EF654658, *F. brasiliensis* EF601055, *F. subtilis* EF450744, *F. paulensis* EF182747, *M. japonicus* AB121740, *M. japonicus* AB121744, *M. japonicus* AB121743, *M. japonicus* AB121742, *M. japonicus* AB121741, *F. chinensis* DQ097704, *F. chinensis* DQ097703, *F. chinensis* AY871268). Values at the node indicate the percentage of times that the particular node occurred in 1000 trees generated by bootstrapping the original deduced protein sequences.**

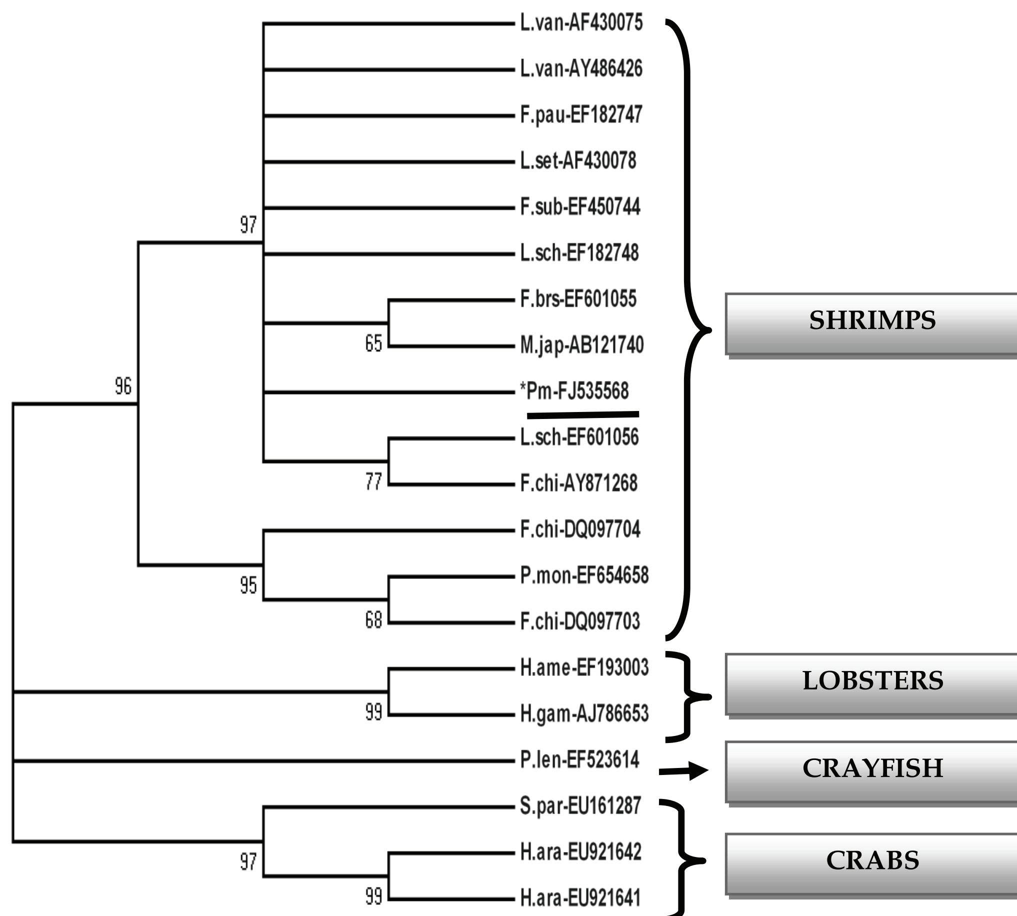



**Fig.2.36 A bootstrapped neighbour-joining tree obtained using MEGA version 4.0 illustrating relationships between the deduced amino acid sequence of the *P. monodon* Crustin-2 AMP (FJ535568) with all known crustins (*L. setiferus* AF430078, *L. vannamei* AF430075, *L. schmitti* EF182748, *L. schmitti* EF601056, *P. camtschaticus* EU921643, *H. araneus* 1 EU921642, *H. araneus* EU921641, *S. paramamosain* EU161287, *P. monodon* EF654658, *P. leniusculus* EF523614, *P. leniusculus* EF523613, *P. leniusculus* EF523612, *F. brasiliensis* EF601055, *F. subtilis* EF450744, *F. paulensis* EF182747, *M. japonicus* AB121740, *H. americanus* EF193003, *F. chinensis* DQ097704, *F.chinensis* DQ097703, *F. chinensis* AY871268, *H. gammarus* AJ786653). Values at the node indicate the percentage of times that the particular node occurred in 1000 trees generated by bootstrapping the original deduced protein sequences.**

**Fig. 2.37. Nucleotide and amino acid sequences of Crustin-3 (GQ334396) from the haemocyte of the the giant tiger shrimp, *P. monodon*. An asterisk is the stop codon.**

**Fig.2.38 ScanProsite analysis for the presence of conserved domain/pattern / motif in Crustin-3 (GQ334396) of *P. monodon.***

**(A) WAP domain signature (B) Glycine and cysteine rich regions**

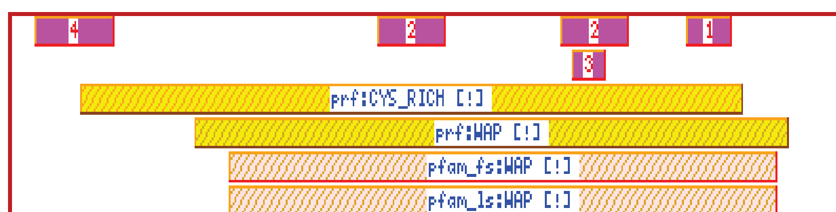



**Fig.2.39 Schematic representation of matches map and list of matches obtained for Crustin-3 (GQ334396) of *P. monodon* from the MotifScan search**

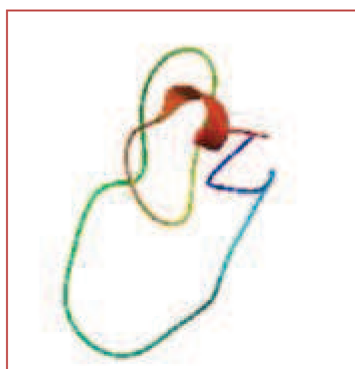


**Fig.2.40 Structural model of Crustin-3 (GQ334396) in *P. monodon* created uisng SWISS-MODEL Server**

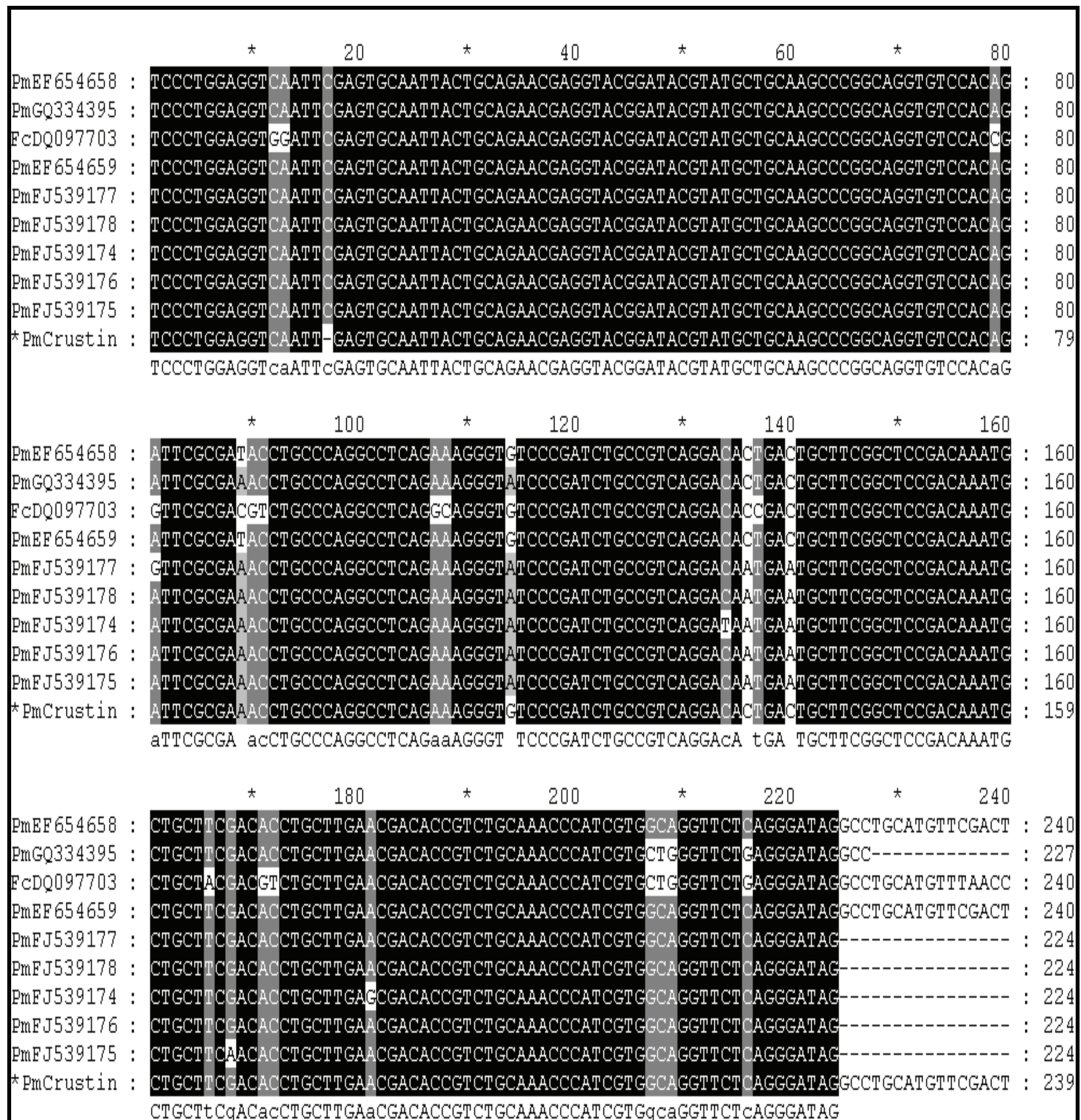



**Fig.2.41 Multiple alignment of nucleotide sequence of the *P. monodon* Crustin-3 (GQ334396) with other shrimp crustins (*P. monodon* EF654658 , *P. monodon* GQ334395, *P. monodon* FJ539175, *P. monodon* EF654659, *P. monodon* FJ539177 , *P. monodon* FJ539178 , *P. monodon* FJ539174 , *P. monodon* FJ539176 , *F. chinensis* DQ0977037) obtained using GeneDoc programme Version 2.7.0. Black and grey indicates conserved sequences.**

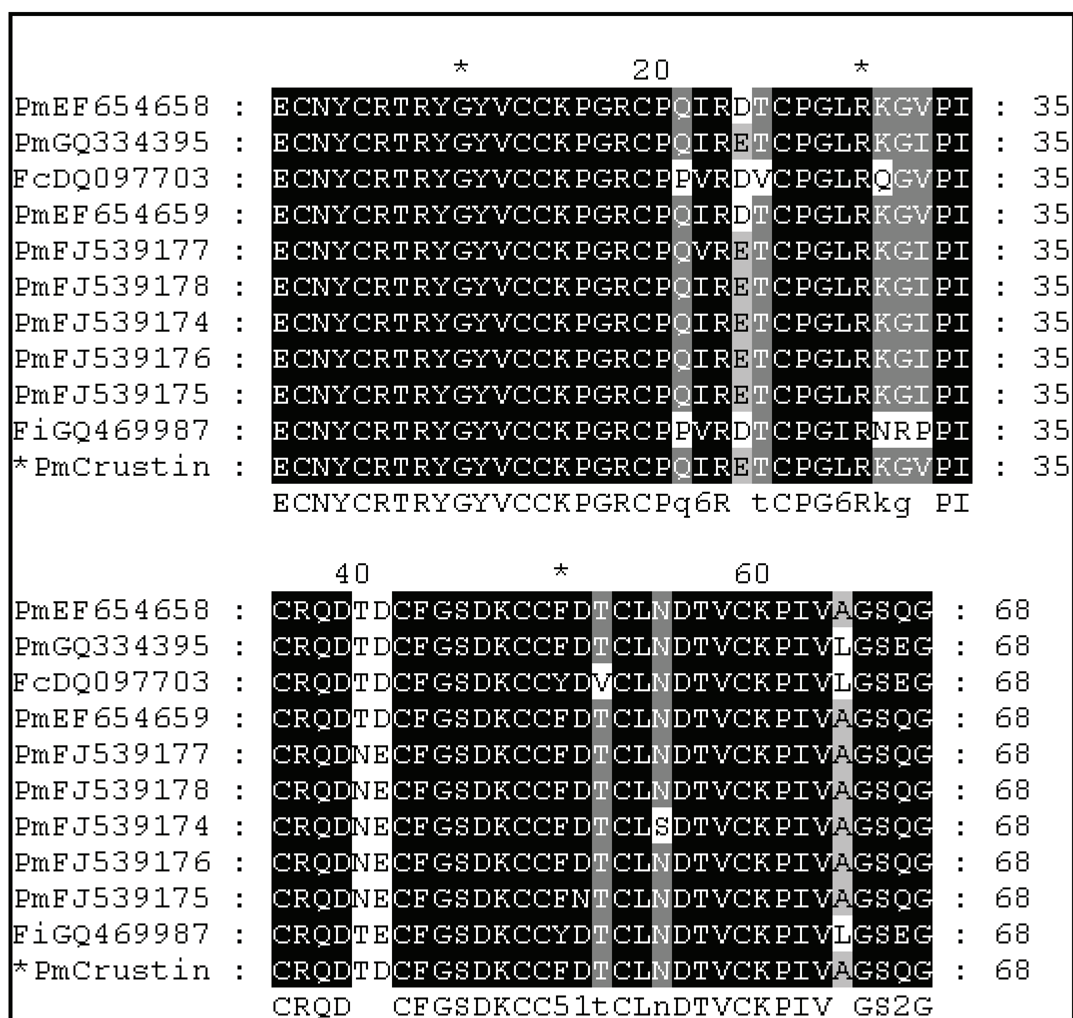



**Fig.2.42 Multiple alignment of deduced amino acid sequence of the *P. monodon* Crustin-3 (GQ334396) with other shrimp crustins (*P. monodon* EF654658, *P. monodon* GQ334395, *P. monodon* FJ539175, *P. monodon* EF654659, *P. monodon* FJ539177, *P. monodon* FJ539178 , *P. monodon* FJ539174, *P. monodon* FJ539176, *F. chinensis* DQ097703, *F. indicus* GQ469987) obtained using GeneDoc programme Version 2.7.0. Black and grey indicates conserved sequences.**

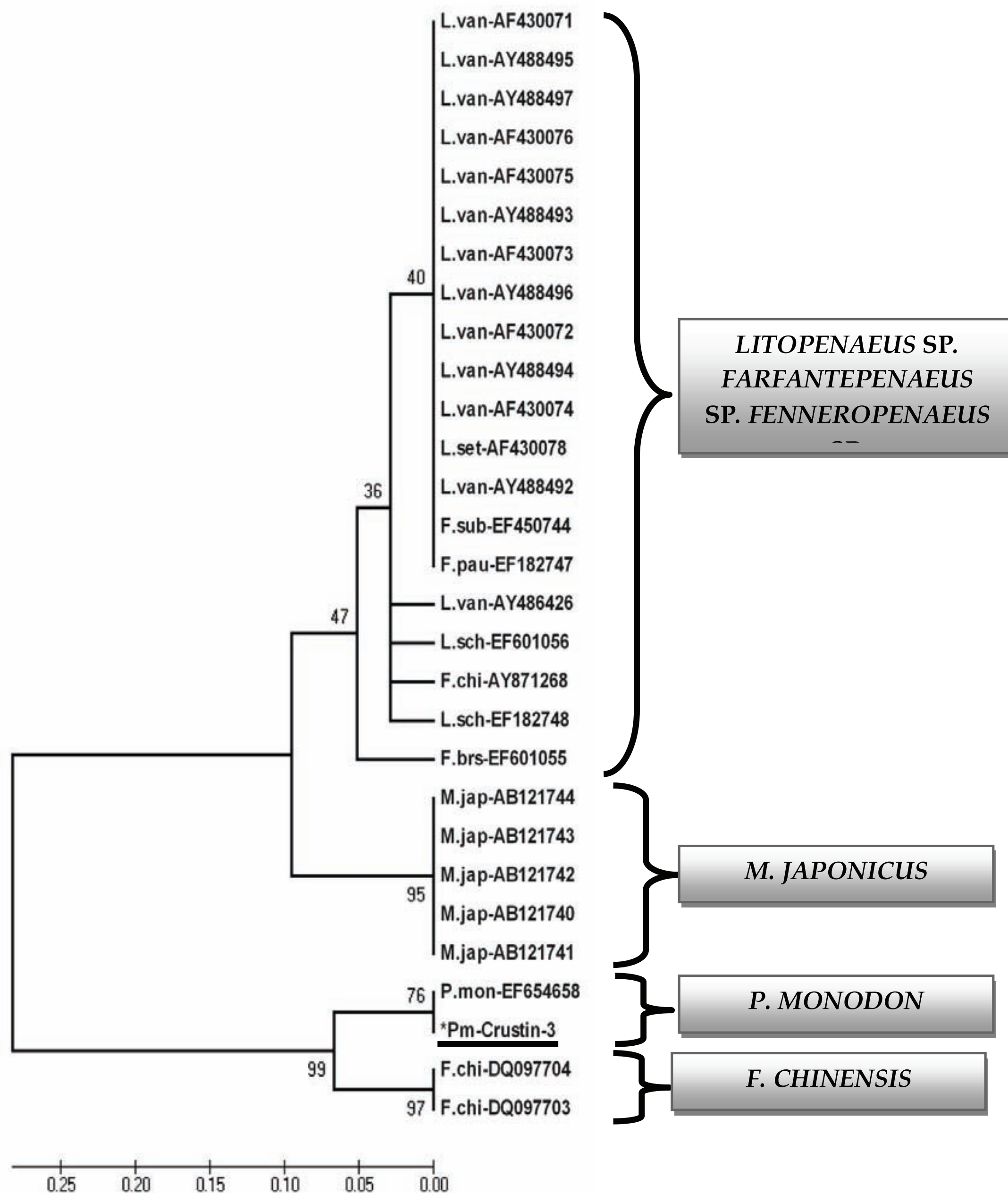



**Fig.2.43 A bootstrapped neighbour-joining tree obtained using MEGA version 4.0 illustrating relationships between the deduced amino acid sequence of the *P. monodon* Crustin-3 (GQ334396) with other shrimp crustins (*L. setiferus* AF430078, *L. vannamei* AF430075, *L.s vannamei* AF430073, *L. vannamei* AF430072, *L. vannamei* AF430071, *L.vannamei* AF430076, *L. vannamei* AF430074, *L. vannamei* AY486426, *L. vannamei* AY488497, *L. vannamei* AY488496, *L. vannamei* AY488495, *L. vannamei* AY488494, *L. vannamei* AY488493, *L. vannamei* AY488492, *L. schmitti* EF182748, *L. schmitti* EF601056, *P. monodon* EF654658, *F. brasiliensis* EF601055, *F. subtilis* EF450744, *F. paulensis* EF182747, *M. japonicus* AB121740, *M. japonicus* AB121744, *M. japonicus* AB121743, *M. japonicus* AB121742, *M. japonicus* AB121741, *F. chinensis* DQ097704, *F. chinensis* DQ097703, *F. chinensis* AY871268). Values at the node indicate the percentage of times that the particular node occurred in 1000 trees generated by bootstrapping the original deduced protein sequences.**

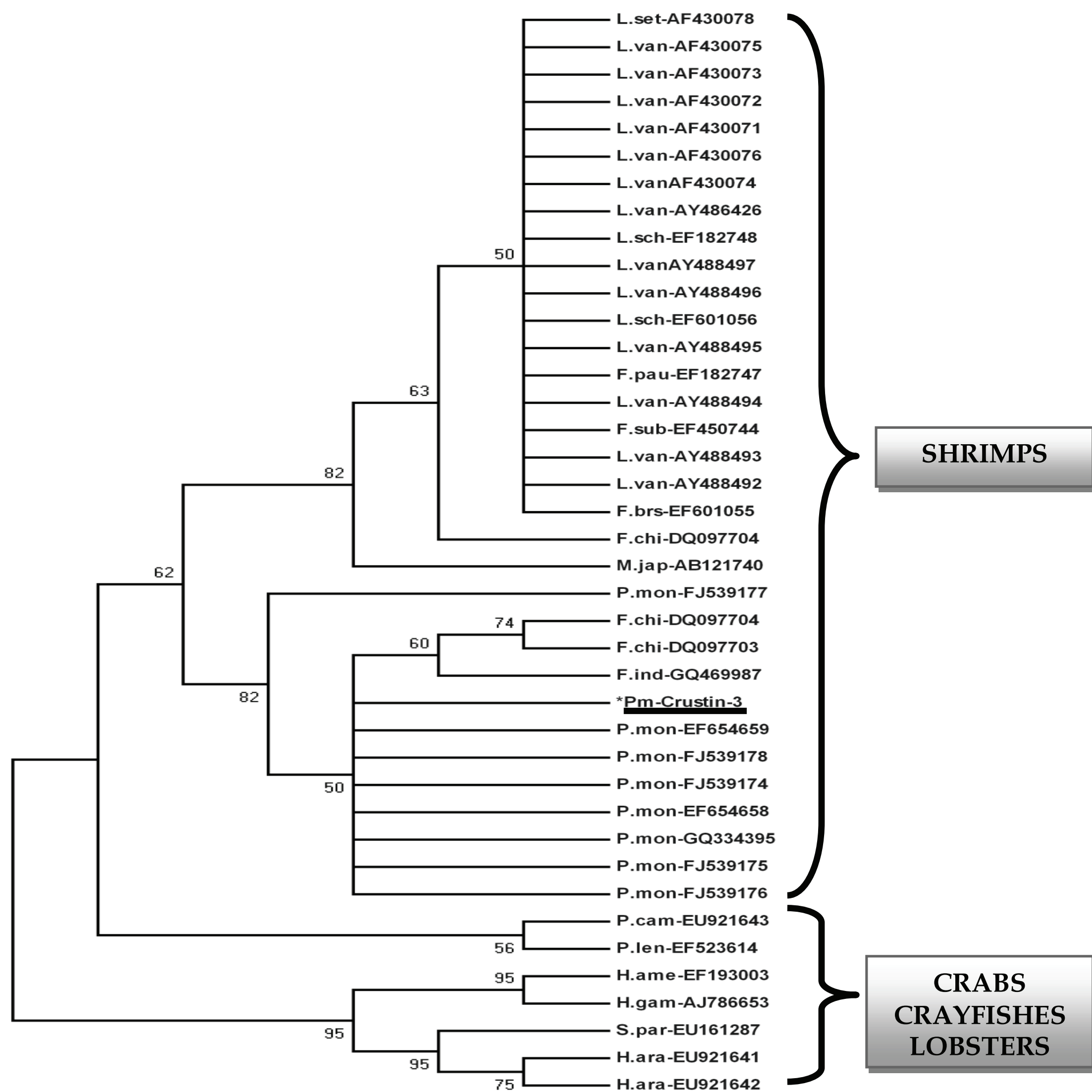



**Fig.2.44 A bootstrapped neighbour-joining tree obtained using MEGA version 4.0 illustrating relationships between the deduced amino acid sequence of the *P. monodon* crustin-3, (GQ334396) with other crustins of decapod crustaceans (*L. vannamei* AF430071, *L. vannamei* AF430074, *L. vannamei* AY488497, *L. vannamei* AY488492, *L. setiferus* AF430078, *F. paulensis* EF182747, *L. vannamei* AY488493, *L. vannamei* AF430073, *L. vannamei* AY486426, *L. vannamei* AF430072, *F. subtilis* EF450744, *L. schmitti* EF182748, *L. vannamei* AY488496, *L. vannamei* AY488495, *L. vannamei* AF430075, *L. vannamei* AF430076, *L. vannamei* AY488494, *F. brasiliensis* EF601055, *L. schmitti* EF601056, *F. chinensis* AY871268, *M. japonicus* AB121740, *P. monodon* FJ539174, *P. monodon* EF654659, *P. monodon* FJ539178, *P. monodon* EF654658, *P. monodon* GQ334395, *P. monodon* GQ334396, *P. monodon* FJ539175, *P. monodon* FJ539177, *F. chinensis* DQ097704, *F. chinensis* DQ097703, *P. camtschaticus* EU921643, *P. leniusculus* 1 EF523614, *H. americanus* EF193003, *H. gammarus* AJ786653, *S. paramamosain* EU161287, *H. araneus* EU921642, *H. araneus* EU921641). Values at the node indicate the percentage of times that the particular node occurred in 1000 trees generated by bootstrapping the original deduced protein sequences.**

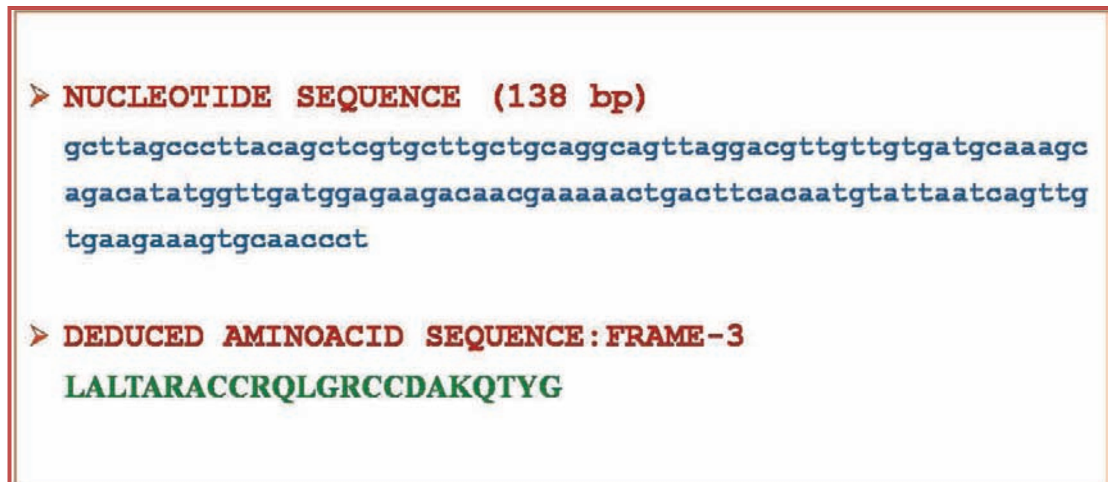


**Fig. 2.45. Nucleotide and amino acid sequences of Penaeidin-3 (GU732819) from the haemocyte of the the giant tiger shrimp, *P. monodon*. An asterisk is the stop codon.**

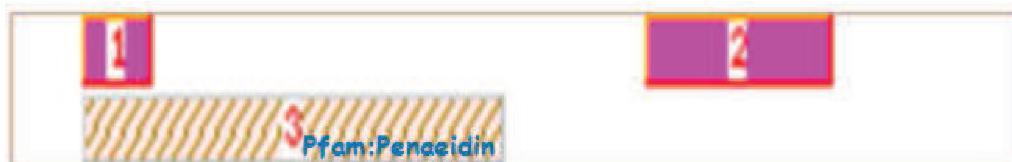



**Fig.2.46 Schematic representation of matches map and list of matches obtained for Penaeidin-3 (GU732819) of *P. monodon* from the Motif Scan search**

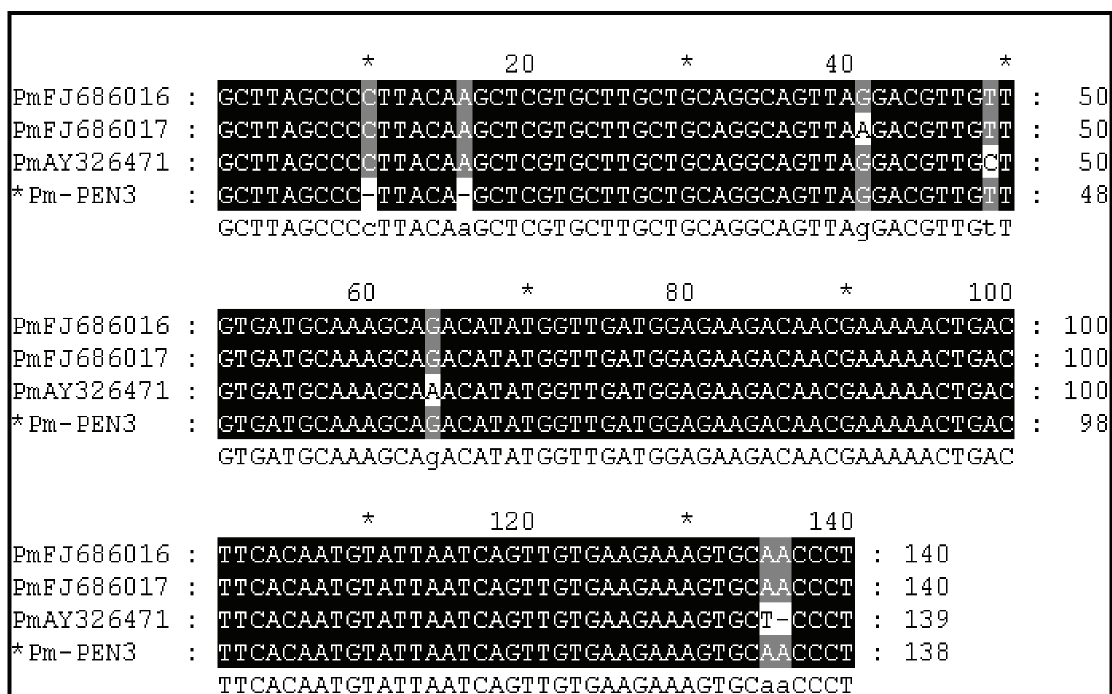



**Fig.2.47 Multiple alignment of nucleotide sequence of the *P. monodon* Penaeidin-3 (GU732819) with other shrimp penaeidins (*P. monodon* FJ686016, *P. monodon* FJ686017, *P. monodon* AY326471*)* obtained using GeneDoc programme Version 2.7.0. Black and grey indicates conserved sequences.**

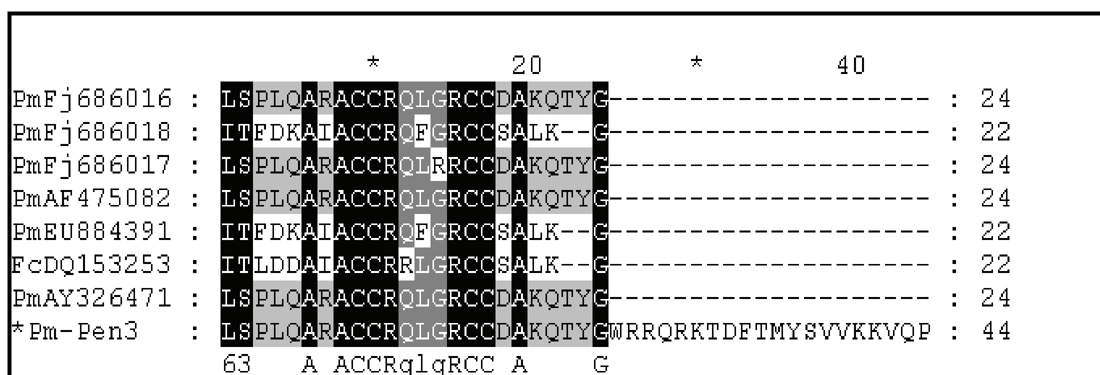



**Fig.2.48 Multiple alignment of deduced amino acid sequence of the *P. monodon* Penaeidin-3 (GU732819) with other shrimp penaeidins (*P. monodon* FJ686016, *P. monodon* FJ686018 *P. monodon* FJ686017, *P. monodon* AF475082, *P. monodon* EU884391, *P. monodon* AY326471, *F. chinensis* DQ153253*)* obtained using GeneDoc programme Version 2.7.0. Black and grey indicates conserved sequences.**

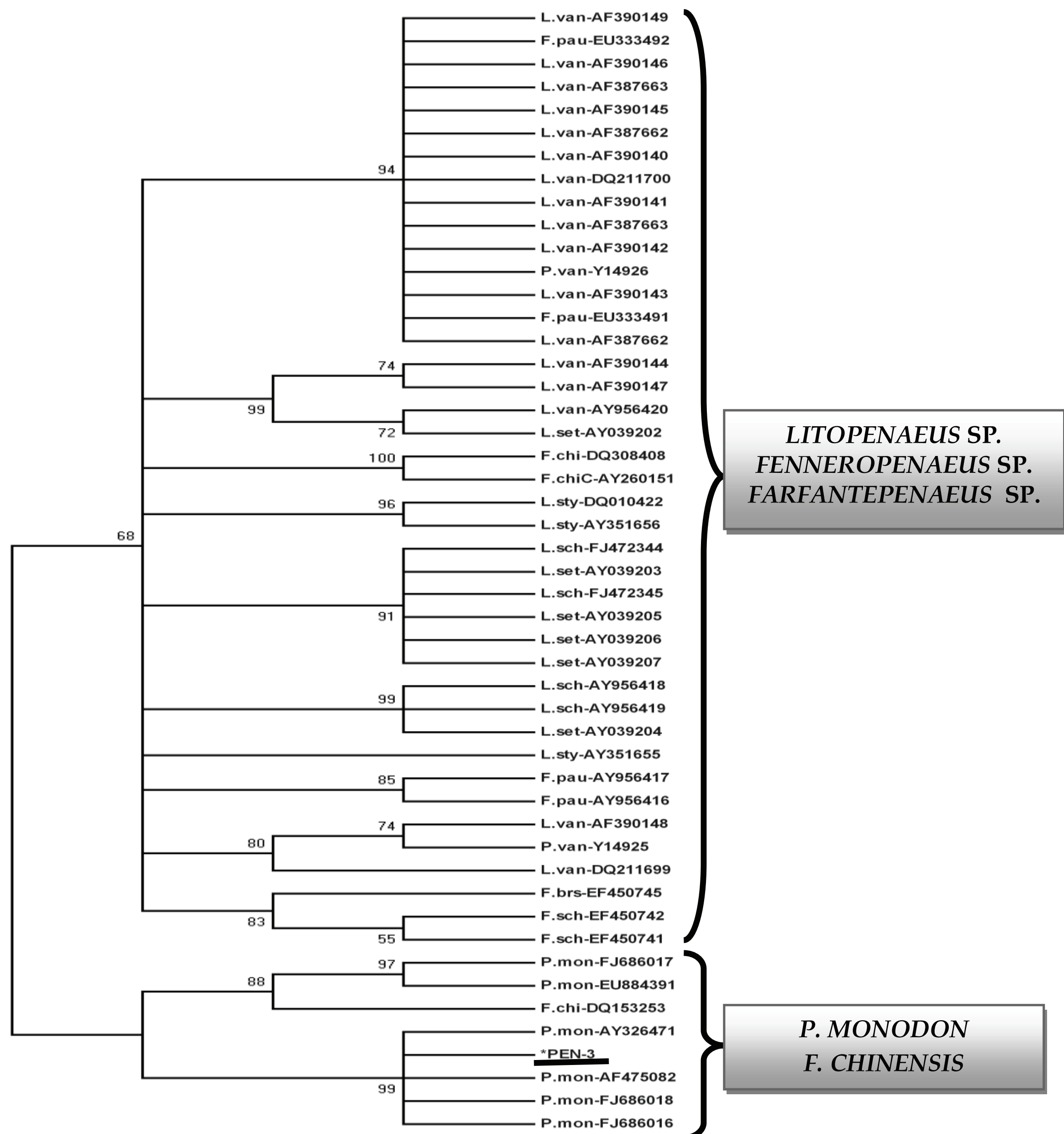



**Fig.2.49 Bootstrapped neighbor-joining tree obtained using MEGA version 4.0 illustrating relationships between the deduced amino acid sequence of the *P. monodon* Penaeidin-3 (GU732819) with all known penaeidins (*L. schmitti* AY956418, *L. schmitti* AY956419, *L. setiferus* AY039205, *P. vannamei* Y14925, *L. vannamei* AF390146, *L. stylirostris* AY351655, *L. vannamei* DQ211699, *L. stylirostris* DQ010422, *L. stylirostris* AY351656, *F. chinensis* EU884391, *P. monodon* FJ686018, *P. monodon* DQ153253, *L. schmitti* AY956420, *L. setiferus* AY039207, *L. vannamei* AF390147, *L. vannamei* AF390149, *F. brasiliensis* EF450745, *P. monodon* FJ686017, *P. monodon* GQ334397, *P. monodon* FJ686016, *P. monodon* AF475082, *P. monodon* AY326471, *L. vannamei* DQ211700, *F. penicillatus* EU333491, *L. vannamei***

**AF387660, *L. vannamei* AF390145, *L. vannamei* AF387661, *L. vannamei* AF390140, *L. vannamei* AF390142, *F. penicillatus* EU333492, *P. vannamei* Y14926, *L. vannamei* AF387662, *L. vannamei* AF387663, *L. vannamei* AF390139, *L. vannamei* AF390143, *L. vannamei* AF390144, *L. vannamei* AF390141, *F. paulensis* AY956417, *F. paulensis* AY956416, *F. subtilis* EF450742, *F. chinensis* DQ308408, *F. chinensis* AY260151, *L. setiferus* AY039204, *L. setiferus* AY039203, *L. schmitti* FJ472344, *L. setiferus* AY039206, *L. setiferus* AY039202, *L. schmitti* FJ472345). Values at the node indicate the percentage of times that the particular node occurred in 1000 trees generated by bootstrapping the original deduced protein sequences.**

➤ NUCLEOTIDE SEQUENCE (169 bp)

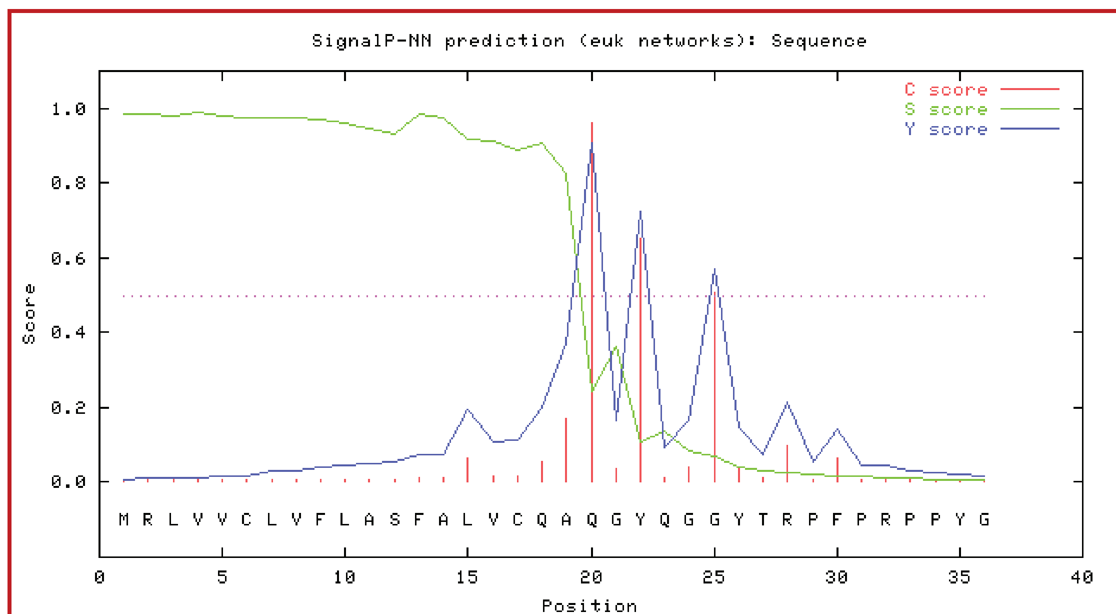
```
tacctgaccctcacctgcagaggccgagactccttgcccgggttccttcc
tgtgtccgccatgcgtctcgtggtctgcctggtcttcctggcctccttcg
ccctggtctgccaagcccaagggtaccagggtggttacacacgcccgttc
cccagaccaccctatgggg
```

➤ DEDUCED AMINOACID SEQUENCE:FRAME-1(36 aminoacid)

```
MRLVVCLVFLASFALVCQAQGYQGGYTRPFPRPPYG
```

```
tacctgaccctcacctgcagaggccgagactccttgcccgggttccttcctgtgtccgcc
 Y  L  T  L  T  C  R  G  R  D  S  L  P  G  F  L  P  V  S  A
atgcgtctcgtggtctgcctggtcttcctggcctccttcgccctggtctgccaagcccaa
 M  R  L  V  V  C  L  V  F  L  A  S  F  A  L  V  C  Q  A  Q
gggtaccagggtggttacacacgcccgttccccagaccaccctatgggg
 G  Y  Q  G  G  Y  T  R  P  F  P  R  P  P  Y  G
```

**Fig. 2.50 Nucleotide and amino acid sequences of Penaeidin-5 (GQ334397) from the haemocyte of the the giant tiger shrimp, *P. monodon*. An asterisk is the stop codon.**




➢SIGNAL PEPTIDE (1-19 = SIGNAL PEPTIDE)

➢MRLVVCLVFLASFALVCQAQGYQGGYTRPFPRPPYG

**Fig.2.51 Signal peptide analysis of Penaeidin-5 (GQ334397) in *P. monodon* as predicted by the SignalP 3.0 server. The underlined amino acid residues indicate a putative signal sequence.**

**Fig.2.52 Schematic representation of matches map and list of matches obtained for Penaeidin-5 (GQ334397) of *P. monodon* from the Motif Scan search.**

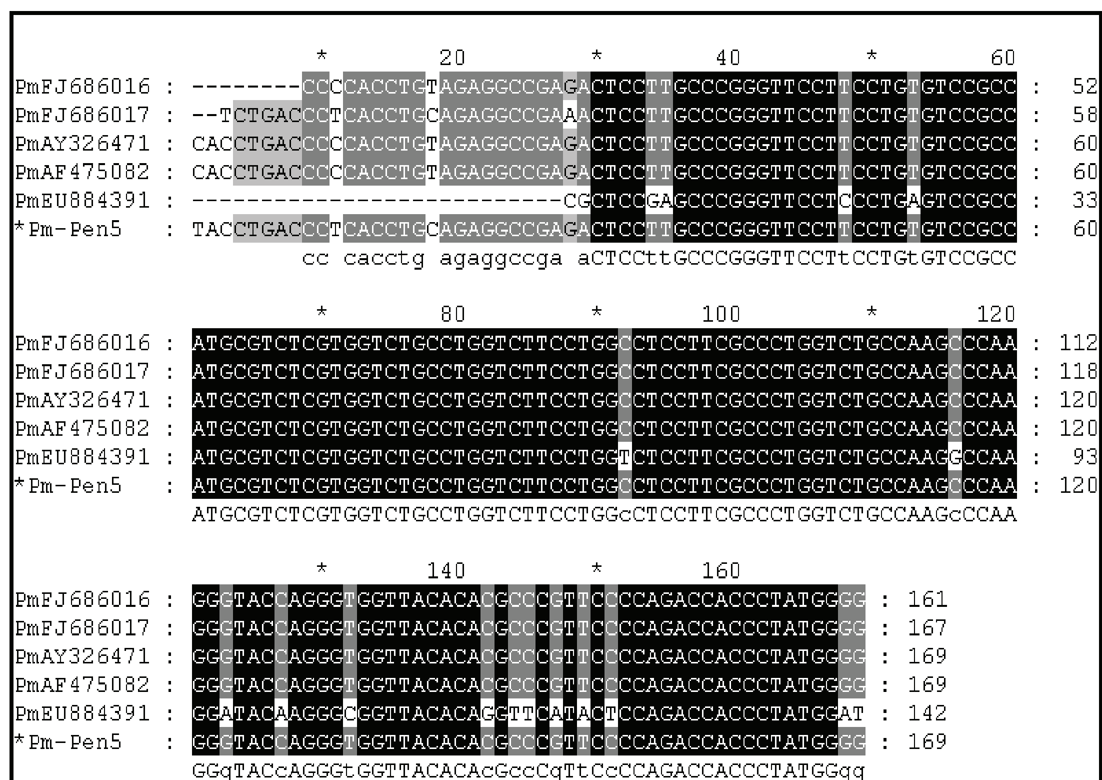



**Fig.2.53 Multiple alignment of nucleotide sequence of the *P. monodon* Penaeidin-5 (GQ334397) with other shrimp penaeidins (*P. monodon* FJ686016, *P. monodon* FJ686017, *P. monodon* AF475082, *P. monodon* EU884391, *P. monodon* AY326471*)* obtained using GeneDoc programme Version 2.7.0. Black and grey indicates conserved sequences.**

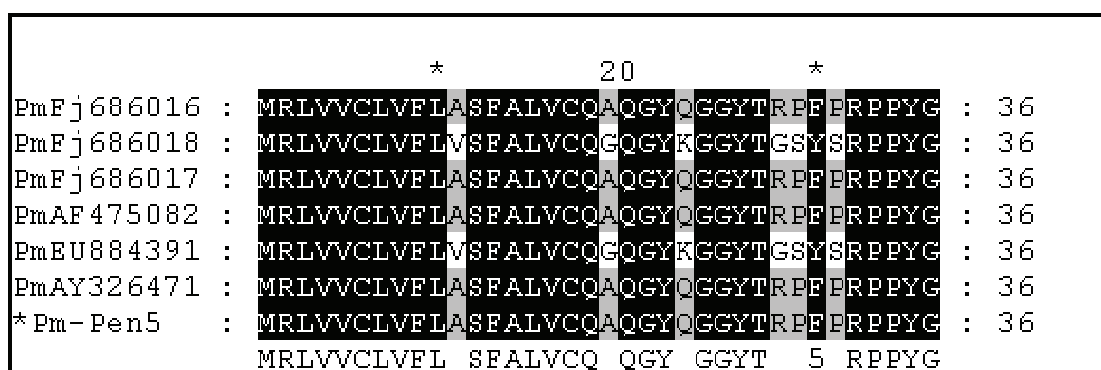



**Fig.2.54 Multiple alignment of deduced amino acid sequence of the *P. monodon* Penaeidin-5 (GQ334397) with other shrimp penaeidins (*P. monodon* FJ686016, *P. monodon* FJ686018, *P. monodon* FJ686017, *P. monodon* AF475082, *P. monodon* EU884391, *P. monodon* AY326471*)* obtained using GeneDoc programme Version 2.7.0. Black and grey indicates conserved sequences.**

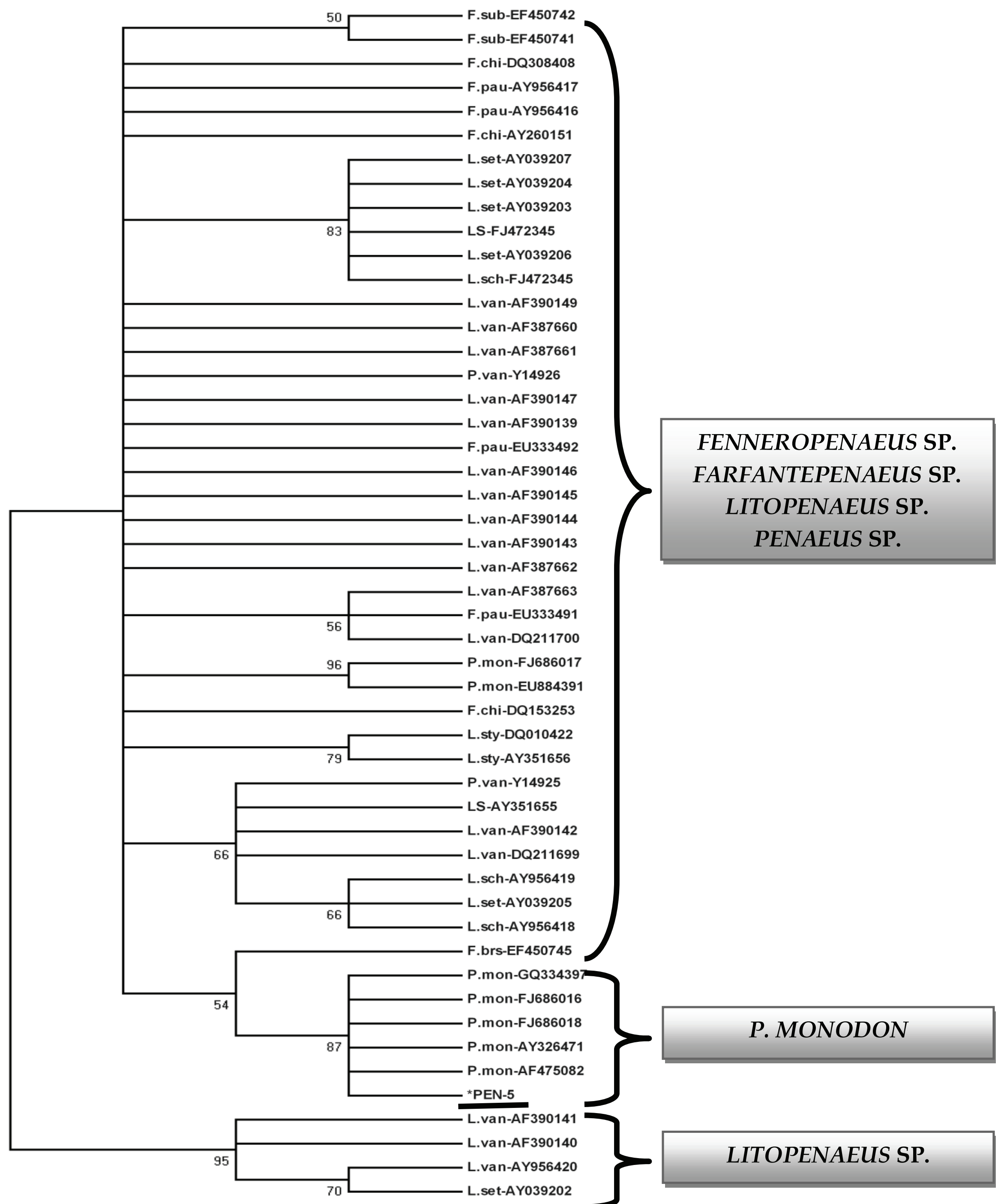



**Fig.2.55 Bootstrapped neighbor-joining tree obtained using MEGA version 4.0 illustrating relationships between the deduced amino acid sequence of the *Penaeus monodon* Penaeidin-5 (GQ334397) with all known penaeidins (*L. schmitti* AY956418, *L. schmitti* AY956419, *L. setiferus* AY039205, *P. vannamei* Y14925, *L. vannamei* AF390146, *L. stylirostris* AY351655, *L. vannamei* DQ211699, *L. stylirostris* DQ010422, *L. stylirostris* AY351656, *F. chinensis* EU884391, *P. monodon* FJ686018, *P. monodon* DQ153253, *L. schmitti* AY956420, *L. setiferus* AY039207, *L. vannamei* AF390147, *L. vannamei* AF390149, *F. brasiliensis* EF450745, *P. monodon* FJ686017, *P. monodon***

GQ334397, *P. monodon* FJ686016, *P. monodon* AF475082, *P. monodon* AY326471, *L. vannamei* DQ211700, *F. penicillatus* EU333491, *L. vannamei* AF387660, *L. vannamei* AF390145, *L. vannamei* AF387661, *L. vannamei* AF390140, *L. vannamei* AF390142, *F. penicillatus* EU333492, *P. vannamei* Y14926, *L. vannamei* AF387662, *L. vannamei* AF387663, *L. vannamei* AF390139, *L. vannamei* AF390143, *L. vannamei* AF390144, *L. vannamei* AF390141, *F. paulensis* AY956417, *F. paulensis* AY956416, *F. subtilis* EF450742, *F. chinensis* DQ308408, *F. chinensis* AY260151, *L. setiferus* AY039204, *L. setiferus* AY039203, *L. schmitti* FJ472344, *L. setiferus* AY039206, *L. setiferus* AY039202, *L. schmitti* FJ472345). **Values at the node indicate the percentage of times that the particular node occurred in 1000 trees generated by bootstrapping the original deduced protein sequences.**

➢ **NUCLEOTIDE SEQUENCE (583 bp)**

cttgtggttgacaatggctccggcatggtcaaggccggtttcgccggagacgacgcccctcgcg
ccgtcttcccctccatcgtcggccgtgcccgtcaccagggtgtgatggtcggtatgggtcagaa
ggacgcctacgtcggtgatgaggcccagagcaagcgtggtatcctcaccctcaagtaccccatt
gagcacggtatcatcaccaactgggatgacatggagaagatctggtaccacaccttctacaatg
agctccgtgttgcccctgaggagtgccccactctcctcactgaggctcccctcaaccccaaggc
caaccgtgagaagatgactcagatcatgttcgagtccttcagcgtccctgccacttacatcacc
atccaggccgtgctctccctctacgcctccggtcgtactaccggtgaggtttgcgactctggtg
atggtgtgactcactttgtccccgtctatgaaggtttcgctcttccccatgctattctccgtct
cgacttggctggtcgtgaccttacccactacctcatgaagatcatgactgagcgtggctactcc
ttcacca

➢ **DEDUCED AMINOACID SEQUENCE:FRAME-1**

LVVDNGSGMVKAGFAGDDAPRAVFPSIVGRARHQGVMVGMGQKDAYVGDEA
QSKRGILTLKYPIEHGIITNWDDMEKIWYHTFYNELRVAPEECPTLLTEAP
LNPKANREKMTQIMFESFSVPATYITIQAVLSLYASGRTTGEVCDSGDGVT
HFVPVYEGFALPHAILRLDLAGRDLTHYLMKIMTERGYSFT

**Fig. 2.56. Nucleotide and amino acid sequences of β-actin (GQ334394) from the haemocyte of the giant tiger shrimp, *P. monodon*.**

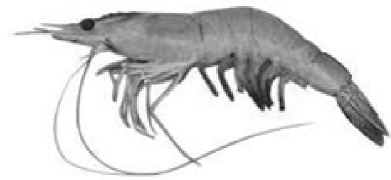
➢ **NUCLEOTIDE SEQUENCE (234 bp)**

cactgaggtaagtctgtggagatgcaccacgaagctcttaccgaggctgt
ccctggtgacaacgttggcttcaacgtgaagaacgtgtccgtgaaggacc
tgaagcgtggcttcgtcgcttccgactcgaagaacgacccagccaaggaa
gctgctgacttcaccgcccaggtgatcgtcctcaaccaccctggccagat
ccaggctggctactcacctgtgcttgattgccac

➢ **DEDUCED AMINOACID SEQUENCE:FRAME-1**

MHHEALTEAVPGDNVGFNVKNVSVKDLKRGFVASDSKNDPAKEA
ADFTAQVIVLNHPGQIQAGYSPVLDCH

**Fig. 2.57. Nucleotide and amino acid sequences of Elongation factor (GU732818) from the haemocyte of the giant tiger shrimp, *P. monodon*.**



# *CHAPTER-3*

## *Molecular Characterization and Phylogenetic Analysis of Antimicrobial Peptides in Fenneropenaeus indicus*

# 3.1. Introduction

The Indian white shrimp, *F. indicus* is the most dominant shrimp species in the shallow water shrimp fishery along the west and east coast of India. *F. indicus* is an important shrimp species which is cultivated in Pokkali paddy fields (paddy-cum-shrimp farming) practiced in the wetlands of Kerala. Antimicrobial peptides (AMPs) widely distributed in the whole living kingdom, play an important role in the immunological defense especially in those organisms which lack adaptive immunity (Dimarq et al., 1998; Bulet et al., 1999., Destoumieux et al., 2000a; Zasloff, 2002; Boman, 2003; Tincu and Taylor, 2004; Vargas-Albores et al., 2004; Cuthbertson et al., 2008). In penaeid shrimps, four main families of AMPs have been currently described and characterized from the haemocytes: penaeidins, crustins, anti-lipopolysaccharide factors (ALFs) and lysozymes. Penaeidins are mainly active against gram-positive bacteria, filamentous fungi (Destoumieux et al., 1999), viruses and protozoans (Bachere, 2003). Whereas ALFs have a broader antimicrobial spectrum, including gram-negative bacteria (Somboonwiwat et al., 2005; de la Vega et al., 2008). Conversely, crustins are reported to have a more-restricted activity spectrum, affecting mainly marine gram-positive bacteria (Relf et al., 1999; Bartlett et al., 2002; Zhang et al., 2007). A detailed literature survey on the AMPs in penaeid shrimps is given in chapter 2.

Though studies have been carried out for unraveling the biochemical aspects of defense mechanisms in *F. indicus*, no works have been carried out on AMPs in this species. So far there are no reports on AMP molecules from *F. indicus*. Discovery of new AMPs in *F. indicus* will

certainly help us to unravel the host defense mechanism of this shrimp species.

# 3.2. Materials and Methods

## *3.2.1. Experimental animals*

Healthy adult *F. indicus* (8-10 g) (Fig. 3.1) were purchased from a local shrimp farm in Vypeen, Kochi. They were transferred to aquarium tanks of 500 litres capacity and acclimatized for one week under laboratory conditions. Shrimps were fed with a standard feed (Higashi, India).

**Fig. 3.1. Experimental animal, The Indian White Shrimp, *Fenneropenaeus indicus***

## *3.2.2. Haemolymph collection*

See section 2.2.3.

### *3.2.3. Total RNA isolation and Reverse transcription*

See section 2.2.4. to 2.2.6.

### *3.2.4. PCR amplification*

PCR amplification of 1µl cDNA was performed in a 25 µl reaction volume containing 1x standard Taq buffer (10mM Tris-HCl, 50mM KCl, pH 8.3), 200µM dNTPs, 0.4 µM each primer and 1U Taq DNA polymerase (New England Biolabs). Amplification was performed using anti-lipopolysaccharide factor, crustin-1, penaeidin-2, penaeidin-5, β-actin, 18S rRNA and elongation factor primers (Table 3.1.). The thermal profile used was an initial denaturation at 94 $^0$C for 2 min followed by 35 cycles of denaturation at 94 $^0$C for 15 sec, and extension at 68 $^0$C for 30 sec and a final extension at 68 $^0$C for 10 min. for the target genes. Annealing temperature and $MgCl_2$ concentration varied for the different genes as given in Table 3.1. The PCR products were visualized on 1.5% agarose gel.

### *3.2.5. Agarose gel electrophoresis*

See section 2.2.8.

### *3.2.6. Cloning of the PCR product*

See section 2.2.9.

### *3.2.7. Sequencing and sequence analysis*

See section 2.2.10.

## 3.3. Results

### *3.3.1. Molecular characterization of AMP genes in F. indicus*

Expression of four AMP genes could be observed from the haemocytes of *F. indicus.* The AMP genes were found to belong to the three major families of AMPs viz., anti-lipopolysaccharide factor (ALF), crustins and two subgroups of penaeidins (penaeidin-2 and penaeidin-5).

### *3.3.1.1. Anti-lipopolysaccharide factor (ALF-1) (GU732814)*

#### *3.3.1.1.1. Nucleotide and deduced amino acid sequences of ALF-1*

A partial mRNA transcript of 255 bp belonging to the ALF family of AMPs was obtained from the mRNA of *F. indicus* haemocyte by RT-PCR (Fig.3.2). The nucleotide sequence encoding 85 amino acids was translated using GeneTool software. Both the nucleotide and amino acid sequences were submitted to GenBank under the accession number **GU732814.** Peptide model of ALF-1 created using SWISS-MODEL server was found to consist of one alpha-helix and four strands of beta-sheets (Fig.3.3)

#### *3.3.1.1.2. Sequence alignment of ALF-1*

BLAST analysis of the nucleotide sequence showed that it belonged to DUF3254 superfamily, the family of ALFs, confirming the sequence to be an isoform of ALF. BLAST analysis of the nucleotide and amino acid sequence revealed the relation of ALF gene to other isoforms of ALF present in *P. monodon, F. indicus*, *F. chinensis* and *F. paulensis* (Table 3.2 and 3.3). BLASTn analysis of the nucleotide sequence revealed that the sequence shared 92% similarity to an ALF isoform of *F. indicus* for a query coverage of 90%. Followed by 91% similarity to another ALF isoform of *F. chinensis*. similarity was also found to ALF isoforms of *P.monodon* (Table 3.2). BLASTp analysis of the amino acid sequences of the ALF, also confirmed the above results (Table 3.3).

Multiple alignments were performed for both the nucleotide and the deduced amino acid sequences of ALFs isolated and characterized from shrimps. Analysis of the results showed the presence of conserved sequences for these AMP molecules (Fig 3.4 and 3.5.). Multiple alignments confirmed the results of the BLAST analysis.

### 3.3.1.1.3. *Phylogenetic analysis of ALF-1*

Phylogenetic analysis was performed for both shrimps ALF sequences and another including all known ALFs of decapods. The bootstrap distance tree calculated for the resulting ALF sequences confirmed that ALF possessed more similarity to that of other ALFs from *Fenneropenaeus* sp. than to the ALF of *Farafantapenaeus, Litopenaeus* and *Penaeus sp.* (Fig.3.6). Phylogenetic tree drawn based on known amino acid sequences of shrimp ALF could be divided into three groups. Group I consisted of ALFs of *Farfantepenaeus* and *Litopenaeus* sp. Group II consisted of *Fenneropenaeus* ALF isoforms and Group III consisted of ALFs of *P. monodon*. The present ALF was found to belong to Group II and shared maximum similarity to that of *F. indicus* than to *F. chinensis*.

The phylogenetic relationships of the ALF sequence with all known ALFs of the decapods is shown in Fig.3.7. The tree topologies revealed the relationships of ALF with other invertebrate ALF-like peptides. The tree could be divided into four major groups. Group I and III consisted of shrimp ALFs, whereas, group II and IV consisted of ALFs of lobsters and crabs respectively. Molecular phylogenetic tree revealed that ALFs of shrimps are found to be evolutionarily closely related with ALFs of other decapods species.

## 3.3.1.2. *Anti-lipopolysaccharide factor (ALF-2) (HM366921)*

### 3.3.1.2.1. *Nucleotide and deduced amino acid sequences of ALF-2*

A complete mRNA transcript of 360 bp belonging to the ALF family of AMPs was obtained from the mRNA of *P. monodon* haemocyte by RT-PCR (Fig.3.8). The nucleotide sequence encoding the open reading frame containing 120 amino acids was translated using GeneTool software. Both the nucleotide and amino acid sequences were submitted to GenBank under the accession number **HM366921.** Molecular weight of the mature peptide

was predicted out to be 10.65 kDa and pI 10.18. The mature peptide of ALF-2 was found to be rich in positively charged residues viz. arginine and lysine. There were 11 arginine and 6 valine residues present in the mature peptide. Peptide model of ALF-2 created using SWISS-MODEL server was found to consist of two alpha-helices crowded against a four-strand β-sheet. Two of the β-strands are in turn linked by a disulfide bond to form an amphipathic loop rich in cationic amino acid side chains (Fig.3.9). Signal peptide could be detected at the 26$^{th}$ position (Fig. 3.10).

### *3.3.1.2.2. Sequence alignment of ALF -2*

BLAST analysis of the nucleotide sequence showed that it belonged to DUF3254 superfamily, the family of ALFs, confirming the sequence to be an isoform of ALF. However the sequence shared 98% similarity to another ALF characterized from *F. indicus*. BLAST analysis of the nucleotide and amino acid sequence revealed the relation of ALF sequence to other isoforms of ALF present in *F. chinensis, P. monodon, L. vannamei and L. schmitti* (Table 3.4 and 3.5). BLASTn analysis of the nucleotide sequence revealed that the sequence also shared 94% similarity to *F. chinensis*, followed by 92% similarity to *P. monodon*, 86% to *L. vannamei* and 84% to *L. schmitti* ALFs (Table 3.4). BLASTp analysis of the amino acid sequences of the ALF, also confirmed the above results (Table 3.5).

Multiple alignments performed for the nucleotide sequences and amino acid sequences of ALFs showed the presence of highly conserved sequences within the peptide molecule (Fig 3.11 and 3.12). Multiple alignments also confirmed the results of the BLAST analysis.

### *3.3.1.2.3. Phylogenetic analysis of ALF-2*

Two phylogenetic trees were drawn for the ALF sequences. One based on the amino acid sequences of all known ALFs of shrimps and a second tree based on all known ALFs characterized from the decapods crustaceans. The

bootstrap distance tree calculated for the resulting ALF sequences confirmed that ALF possessed more similarity to that of other ALFs of *Fenneropenaeus* sp. (Fig.3.13). Phylogenetic tree drawn based on known amino acid sequences of shrimp ALF could be divided into three groups. Group I consisted of ALFs of *Fenneropenaeus*, *Litopenaeus*, *Penaeus* and *Farfantepenaeus* sp. Group II consisted of ALFs of *Litopenaeus* and *Macrobrachium* sp. and group III consisted of ALFs of *Marsupenaeus* and *Penaeus* sp.

The phylogenetic relationships of the ALF sequence with all known ALFs of the decapods is shown in Fig.3.14. The tree topologies revealed the relationships of ALF with other invertebrate ALF-like peptides. The tree could be divided into five major groups. Group I consisted of ALFs present in shrimps; group II and IV with lobster ALFs; Group III consisted of ALFs of both shrimps and crabs; and finally group V consisting of crab ALFs. Interestingly, it was found that ALF of shrimps shared close similarity with ALF of crabs. Molecular phylogenetic tree based on amino acid sequences suggests that all the ALF members possess a same ancestral origin, which has subsequently diverged at different phases of evolution. Another interesting fact revealed by phylogenetic analysis is that ALFs of shrimps are found to be evolutionarily closely related with ALFs of other decapods species.

### *3.3.1.3. Fi-Crustin (GQ469987)*

#### *3.3.1.3.1. Nucleotide and deduced amino acid sequences of Fi-crustin*

A 371 bp fragment cDNA encoding 122 amino acids and an ORF of 117 amino acids was obtained from the mRNA of *P. monodon* haemocyte by RT-PCR (Fig.3.15). The ORF encoded 117 amino acid residues with a predicted molecular weight (MW) of 10.61 kDa and theoretical isoelectric point (pI) of 7.59 as predicted by the ProtParam software. The predicted secondary structure of Fi-crustin, created using SWISS-MODEL, indicated a

random coiled structure that is with two possible β-sheets and alpha-helical structure (Fig. 3.16). The analysis with the Signal P software revealed the presence of a signal peptide with 17 amino acids at the N-terminal region of the Fi-crustin (Fig.3.17). Being the first crustin sequence to be reported from *F. indicus*, this crustin was named Fi-crustin.

### *3.3.1.3.2. Amino acid composition of Fi-crustin*

The deduced amino acid sequence of Fi-crustin was found to be rich in amino acid residues glycine (14.5%) and valine (12.8%). At the N-terminal of the mature peptide, Fi-crustin contained a number of glycine-rich repeats between positions 25 and 49. Following the repeat region is a cysteine-rich region just like that of the crustin-like peptide from *M. japonicus* and *F. chinensis*; however they have no proline-rich domain compared with those of *M. japonicus* and hence more similar to crustin of *F. chinensis*. The C-terminal segment included a high proportion of Cysteine-rich region (10.3%), which contained 12 Cysteine residues that participate in the formation of disulphide bonds. The 12 cysteines in Fi-crustin is supposed for maintaining the tertiary structure of the peptide just like as that reported in *L. setiferus*, *L. vannamei* and *F. chinensis* crustins.

### *3.3.1.3.3. Analysis of WAP domain structure of Fi-crustin*

As predicted by the ScanProsite and Motif Scan program, a whey-acidic protein (WAP) domain signature exists in the C-terminal (Fig. 3.18 and 3.19). According to the previous reports on the crustin-like proteins, the four-disulfide core domain has proved to play important roles in the biological function of crustins. The position of the conserved cysteines for such category of 'four-disulfide core' domain and the location of the signature pattern is $Cys^{68}$-$Cys^{98}$, $Cys^{75}$-$Cys^{102}$, $Cys^{85}$-$Cys^{97}$, and $Cys^{91}$-$Cys^{108}$. Fi crustin revealed the existence of WAP-type 'four-disulfide core' domain signature, $C_1$-(Xn)-$C_2$ (Xn)-$C_3$-(X5)-$C_4$-(X5)-$C_5$-$C_6$-(X3-5)-$C_7$-(X3-4)-$C_8$ (Bartlett

et al., 2002) and Fi-crustin followed the same pattern with 4 residues were found between $C_7$ and $C_8$ ($C_7$-$X_4$-$C_8$). Several other consensus sequences also appears in the 4-DSC domain: (1) the consensus KXGXCP containing C1; (2) a conserved aspartate (D) residue between $C_3$ and $C_4$; (3) KCC with $C_5$ and $C_6$; (4) CXXP with $C_8$ (Bartlett et al., 2002). Fi-crustin follows this pattern as 'CXP with $C_8$' instead of 'CXXP with $C_8$'.

### *3.3.1.3.4. Sequence alignment of Fi-crustin*

Amino acid sequence of Fi-crustin was also compared with crustins of decapod crustaceans and it revealed maximum identity to that of *F. chinensis,* and *P. monodon*. BLAST analysis of the nucleotide sequence revealed the relation of Fi-crustin to that of crustins from *F. chinensis* and *P. monodon* (Table 3.6 and 3.7). Multiple alignment of the nucleotide sequences of Fi-crustin and other shrimp crustins showed high similarity in the signal peptide region, major gaps could be observed in the ensuing region (Fig. 3.21). Multiple alignments of amino acid sequences of crustins also showed that the deduced amino acids of Fi-crustin shared relatively high identities with those of *F. chinensis* and *P. monodon* (Fig.3.20). The $58^{th}$ - $70^{th}$ nucleotide sequences was found to be absent in the Fi-crustin when compared to *P. monodon* crustin sequence. A similar gap could be observed for the *F. chinensis* crustins between the $64^{th}$ - $70^{th}$ positions. Similarly another missing sequence region could be observed for Fi-crustin between the $88^{th}$ – $98^{th}$ positions that match with a similar gap for the *F. chinensis* crustins between the $88^{th}$ - $93^{rd}$ positions, when compared to *P. monodon*. Great variation between the sequences of *P. monodon* and *Fenneropenaeus* sp. could be observed at the $122^{nd}$ - $144^{th}$ position. Other major missing sequences of the Fi-crustins were found at $182^{nd}$ - $202^{nd}$ and also between $211^{th}$ and $216^{th}$ position. *F. chinensis* showed a major gap for the nucleotide sequences at $262^{nd}$ - $352^{nd}$ position whereas *P. monodon* and Fi-crustin did not.

#### *3.3.1.3.5. Phylogenetic analysis of Fi-crustin*

The phylogenetic relationships between Fi-crustin and other crustins with WAP domain are shown in Fig. 3.22 and 3.33. The bootstrap distance tree calculated for the resulting crustin sequences of BLAST analysis confirmed that Fi-crustin possessed more similarity to that of *F. chinensis* crustin than to the *P. monodon* crustins. The tree topologies revealed the relationships of Fi-crustin with other invertebrate crustin-like peptides. Molecular phylogenetic tree based on amino acid sequences suggests that all the crustin members possess a same ancestral origin, which has subsequently diverged at different phases of evolution. Out of all the species, crustins of shrimps are found to be evolutionarily distantly related with crustins of other decapod species. The tree could be broadly classified into three major groups. Group I included the crustins of shrimps; Group II, the king crab /crayfish crustins and Group III, the lobster/crab crustins. The bootstrap distance tree calculated for the crustin sequences clearly indicated that the Fi-crustin possessed great similarity to crustins isolated from *F. chinensis* and *P. monodon*. Great variability could also be noticed in the crustin sequences of various decapods.

### *3.3.1.4. Fi-penaeidin (HM243617)*

#### *3.3.1.4.1. Nucleotide and deduced amino acid sequences of Fi-penaeidin*

A 186 bp fragment cDNA encoding 61 amino acids and an ORF of 42 amino acids was obtained from the mRNA of *F. indicus* haemocyte by RT-PCR (Fig.3.24). The ORF encoded 42 amino acid residues with a predicted molecular weight (MW) of 4.478 kDa and theoretical isoelectric point (pI) of 5.3 as predicted by the ProtParam software. The analysis with the Signal P software revealed the presence of a signal peptide with 19 amino acids at the N-terminal region of the Fi-crustin (Fig.3.25). Being the first penaeidin

sequence to be reported from *F. indicus*, this penaeidin was named Fi-penaeidin.

### 3.3.1.4.2. Amino acid composition of Fi-penaeidin

The deduced amino acid sequence of Fi-penaeidin was found to be rich in amino acid residues leucine (13.1%) and serine (13.1%) followed by cysteine (9.8%) and proline (6.6%) residues as calculated by the ProtParam tool of ExPASy program. Fi-penaeidin was characterized by 10 conserved amino acid sequence in the signal peptide; a threonine and two proline residues conserved in the N-terminal domain; and the conserved cysteine array of the C-terminal structured domain. As per Destoumieux and co-workers (2000a) this overall structure of penaeidins is quite unique among the AMP families. Motif Scan performed on the deduced amino acid sequence also confirmed that the sequence belonged to penaeidin AMPs. A glycosylation motif was also detected at the $53^{rd}$-$56^{th}$ position (Fig. 3.26).

### 3.3.1.4.3. Sequence alignment of Fi-penaeidin

BLAST analysis of the nucleotide and amino acid sequences revealed the relation of Fi-penaeidin to that of penaeidins from *F. paulensis* and *F. subtilis* (Table 3.8 and 3.9). Multiple alignments of amino acid sequences of penaeidins showed that the deduced amino acids of Fi-penaeidin shared relatively high identities with penaeidins of *F. paulensis, F. chinensis* and *F. subtilis*. It showed high similarity in the signal peptide region of all known penaeidin AMPs followed by a major gap in the ensuing region. Slight variation could be seen in the signal peptide region of *L. vannamei* and *P. monodon*. A threonine and two proline residues could be detected conserved in the N-terminal domain which is believed to be a unique feature of the penaeidin group of AMPs. Unlike majority of the penaeidins that possess six cysteine residues at the C-terminal region, only four conserved cysteine residues could be detected in the C-terminal region of Fi-penaeidin. Similar

case has been reported in the penaeidins of *L. vannamei* (**AF390145**, **AF390144**, **AF390143**) and *L. setiferus* (**AY039204**).

Multiple alignments were also performed for penaeidins that showed great similarity with the deduced amino acid sequence of Fi-penaeidin during BLAST analysis (Fig. 3.27 and 3.28). The results showed 100% similarity in the signal peptide region. Multiple alignments confirmed the BLAST analysis which showed that Fi-penaeidin is more similar to penaeidins of *L. setiferus, F. chinensis, F. paulensis, F. subtilis* and *L. schmitti.*

### *3.3.1.4.4. Phylogenetic analysis of Fi-penaeidin*

The phylogenetic relationship of Fi-penaeidin with that of other known penaeidins is shown in Fig 3.29 and 3.30. The tree topologies revealed the relationships of Fi-penaeidin with other shrimp penaeidins. The phylogenetic tree based on amino acid sequences suggests that all the penaeidin members possess a same ancestral origin, which has subsequently diverged at different phases of evolution. Out of all the species, penaeidin of *F. indicus* was found to be evolutionarily more related with penaeidins of *F. subilis, F. paulensis* and *F. chinensis*. The phylogenetic tree obtained from all known penaeidins, could be broadly classified into five major groups. Group I consisted of penaeidins belonging to subgroup 2 isolated and characterized from the *Litopenaeus* sp. viz. *L. schmitti, L. setiferus, L. vannamei* and *L. stylirostris*. Group II included penaeidins belonging to subgroup 3 and 5 isolated and characterized from *L. stylirostris*, *F. chinensis* and *P. monodon*. Interestingly penaeidins from the same species were found to form subgroups within the major group II. Group III consisted of penaeidin-4 isolated and characterized from the *Litopenaeus* sp. Group IV consisted of penaeidin-3 isolated and characterized from *Litopenaeus* sp., *Fenneropenaeus* sp., and *Penaeus* species. Group V consisted of penaeidin 2 and 5 isolated and characterized from the shrimp species *L. setiferus, L. schmitti, F. subtilis, F.*

*chinensis, Fi-penaeidin* and *F. paulensis.* The bootstrap distance tree calculated for the penaeidin sequences clearly indicated that the Fi-penaeidin possessed great similarity to penaeidins isolated from *F. chinensis, F. subtilis* and *F. paulensis* (Fig. 3.30).

A phylogenetic tree was also constructed to study the relationship of Fi-penaeidin deduced amino acid sequence with that of other penaeidins which showed great similarity during BLAST analysis (Fig.3.29). The tree topologies revealed that Fi-penaeidin is more related to *F. subtilis* and *F. paulensis*. The tree could be broadly divided into two groups. Group I included penaeidin 3 of *L. setiferus, L. schmitti* and *F. chinensis*. Whereas Group II included Fi-penaeidin, *F. subtilis* and *F. paulensis*. Group II consisted mainly of penaeidin belonging to subgroup 2 (Fig. 3.29). The phylogeneic tree revealed that Fi-penaeidin belong to group Penaeidin-2.

## *3.3.1.5. Penaeidin-5 (HM243616)*

### *3.3.1.5.1. Nucleotide and deduced amino acid sequences of Penaeidin-5*

A partial mRNA transcript of 320 bp, encoding 106 amino acids, belonging to the penaeidin family of AMPs was obtained from the mRNA of *P. monodon* haemocyte by RT-PCR (Fig. 3.31). The sequence was submitted to GenBank under the accession number **HM243616**. Penaeidin signature could be obtained using Motif Scan tool (Fig.3.32). Structure of penaeidin-5 created using SWISS-MODEL showed the presence of α-helical structure with no β-sheets (Fig. 3.33).

### *3.3.1.5.2. Sequence alignment of Penaeidin-5*

BLAST analysis was performed at the nucleotide and amino acid level with other penaeidin-5 sequence in the GenBank. BLAST analysis showed that the penaeidin-like AMP shared maximum similarity with penaeidin-5 of *F. chinensis* and *P. monodon,* followed by penaeidins of *L. vannamei* and *L. setiferus* (Table 3.10 and 3.11).

Multiple alignments were performed for the penaeidin molecule at both nucleotide and amino acid levels. Multiple alignments revealed the presence of highly conserved sequences in the signal peptide region. Conserved cysteine residues could also be detected at the C-terminal region of the peptide molecule (Fig.3.34 and 3.35).

#### *3.3.1.5.3. Phylogenetic analysis of the Penaeidin-5*

The phylogenetic tree could be broadly divided into three major groups. Group I consisting of penaeidins of *Litopenaeus*; Group II of penaeidins of *Fenneropenaeus* sp. and Group III of *Penaeus* sp. (Fig.3.36). The present peptide was found to belong to Group II consisting of penaeidins of *Fenneropenaeus*.

### *3.3.2. Control / Reference genes*

Expression of three control genes was confirmed by RT-PCR analysis of the *F. indicus* haemocytes. Transcripts of β-actin and elongation factor (ELF) could be observed from the haemocytes of *F. indicus.* A partial mRNA transcript of 547 bp, encoding 182 amino acids, belonging to the actin family was obtained from the mRNA of *F.indicus* haemocyte by RT-PCR (Fig. 3.37). The sequence was submitted to GenBank under the accession number **GU732815**. A partial mRNA transcript of 234 bp, encoding 78 amino acids, belonging to the ELF family was obtained from the mRNA of *F.indicus* haemocyte by RT-PCR (Fig. 3.38). The sequence was submitted to GenBank under the accession number **GU732816.**

## 3.4. Discussion

AMPs play a major role in innate immunity, conserved in evolution, and present in all phyla of the living kingdom (Dimarq et al., 1998; Bulet et al., 1999; Destoumieux et al., 2000a; Zasloff, 2002; Boman, 2003; Tincu and Taylor, 2004; Vargas-Albores et al., 2004; Cuthbertson et al., 2008). The past few years brought significant advances in crustacean immunity, with a focus

on AMPs. These molecules act as endogenous antibiotics and as such are considered to be a key element of innate immunity (Bulet et al., 1999). More than 1000 AMPs have now been discovered in plants, vertebrates, and invertebrates. They are mostly cationic and amphipathic molecules and show great diversity in terms of structural features as well as biological properties and functions. Due to their small size, amphipathic structure and cationic character they can rapidly diffuse to the point of infection (Brogden, 2005), a mechanism that presumably makes it easier to circumvent microbial resistance (Bax et al., 2000). AMPs can kill bacteria in micromolar range, promptly synthesized at low metabolic cost, easily stored in large amounts and readily available shortly after an infection, to rapidly kill a broad range of microbes (Hancock, 1997, 2001; Prenner et al., 1999). Many antibacterial peptides show a remarkable specificity for prokaryotes with low toxicity for eukaryotic cells; a phenomenon which has favored their investigation and exploitation as potential new antibiotics (Zasloff, 1992). The discovery of AMPs in crustaceans provides new clues for fundamental understanding of crustacean immunity. The studies on the shrimp penaeidins have largely contributed to this knowledge as they are the first AMPs fully characterized in a crustacean and for which expression studies were performed. Soon after their discovery and initial characterization they have been subjects of extensive studies at structural and functional level.

This chapter represents the first report of AMPs from Indian white shrimp, *F. indicus*. The reported AMPs belonged to the class of ALFs, crustins and penaeidins; the three major groups of AMPs characterized from penaeid shrimps. ALF is a small basic protein which was initially isolated and characterized from haemocytes of the horseshoe crab *L. polyphemus* (Aketagawa et al., 1986; Muta et al., 1987). *L. polyphemus* ALF binds LPS and has a strong antibacterial activity, particularly on the growth of Gram-

negative bacteria. In shrimp, cDNA clones homologous to the horseshoe crab ALFs were initially identified in haemocytes of *P. monodon* and *L. setiferus* by expressed sequence tag (EST) analysis (Gross et al., 2001; Supungul et al., 2004). More recently, a similar approach has been implemented to identify ALF homologues in other shrimp species including the Chinese shrimp *F. chinensis* (Liu et al., 2005) and the kuruma shrimp *M. japonicus* (Nagoshi et al., 2006). Antimicrobial assays using recombinant *P. monodon* ALF have shown strong activity against multiple gram-positive and gram-negative bacteria and filamentous fungi (Somboonwiwat et al., 2005). In the present study two isoforms of ALFs could be characterized. The complete coding sequence of the ALF-2 could also be obtained in the present study.

In the present study two putative ALFs could be characterized from the haemocytes of the Indian white shrimp, *F. indicus*. ALF-1 was found to be a new isoform of ALF from *F. indicus*. However, only a partial sequence of ALF-1 could be characterized. Complete coding sequence of the ALF-2 could be characterized from the haemocytes of *F. indicus*. The ALF-2 sequence shared 98% similarity with another ALF recently reported from the same species. Results of BLAST, multiple alignments and phylogenetic study revealed both ALF-1 and ALF-2 to be closely related isoforms of the ALF family. ALFs characterized from *F. indicus* haemocytes were found to be more related to other ALFs of *Fenneropenaeus* sp. than to *Litopenaeus*, *Farfantepenaeus* or *Penaeus* sp. The molecular weight, pI and amino acid composition predicted out for ALF-2 were in agreement with the results of Beale et al. (2008) and Imjongjirak et al. (2007). Peptide model of the partial ALF-1 created using SWISS-MODEL server, was found to consist of both alpha-helical structure and β-sheets (Fig.3.3). Four β-sheets and one α-helix could be detected in the ALF-1 of *F. indicus*. As per Hoess et al. (1993) and Beale et al. (2008), the ALF molecules should consist of a single domain with

three α-helices crowded against a four-strand β-sheet. Since ALF-1 was a partial sequence, the two of the α-helix that is required for the complete structure could not be obtained. In case of ALF-2 obtained, the cDNA nucleotide sequence possess a 5' section encoding a hydrophobic signal peptide of approximately 25 amino acid residues, producing a nascent protein prior to signal cleavage of approximately 120 amino acids. This follows the typical pattern of ALF peptides as previously reported by Supungul et al. (2002) and Beale et al. (2008).

The ALF-2 molecule was found to consist of a single domain with two α-helices crowded against a four-strand β-sheet (Fig.3.9). Two of the β-strands are linked by a disulfide bond to form an amphipathic loop rich in cationic amino acid side chains as reported by Hoess et al. (1993) and Beale et al. (2008). The structure predicted from both ALF-1 and -2 of *F. indicus* was also found to agree with the above mentioned findings. Synthetic cyclic versions of this loop are found to be functionally active against gram-negative bacteria including *Vibrio harveyi* (Imjongjirak et al., 2007) and *Pseudomonas aeruginosa* (Pan et al., 2007) and against gram-positive bacteria such as *Micrococcus luteus* (Imjongjirak et al., 2007).

The complete coding sequence of a crustin isoform could be characterized from the haemocytes of *F. indicus*. The crustin was found to belong to type II crustins based on the classification by Smith et al. (2008). Fi-crustin characterized showed the presence of WAP domain and exhibited 91% similarity to *F. chinensis* crustins. The C-terminus exhibited a unique cysteine array that is predicted to form six disulphide bonds in the tertiary structure as reported by Brockton et al. (2007). This 12 cysteine array arrangement is conserved in expressed sequence tags (ESTs) from related genera and seems to represent a novel tertiary structure amongst AMPs, unique to the Crustacea. The predicted secondary structure of Fi-crustin

indicated a random coiled structure that is tightly coiled enclosing two β-sheets and α-helical segment. Structural models of the WAP domain suggest that the tertiary structure of this part of the molecule is well conserved between decapods species (Smith et al., 2008). Fi-crustin was found to be rich in glycine (14.5%) and valine (12.8%). The N-terminal region of Fi-crustin contained a number of glycine-rich repeats between positions 25 and 49. The glycines, being small amino acids, might render the glycine-rich region flexible and/or allow tight bends in the structure, but, as yet, it is unclear why this glycine-rich region occurs so frequently in shrimp crustins and so rarely in the Pleocyemata or what functional properties it confers on the mature proteins (Smith et al., 2008).

The phylogenetic tree analysis showed that the crustins diverged from an ancestral sequence to three major groups i.e. Group 1 with shrimps, Group II with Cray fishes/King crabs and Group III with Lobsters/Crabs (Fig. 3.23). Under Group I shrimp crustins, three subgroups were noticed 1) *L.vannamei* 2) *P.monodon* and 3) *Fenneropenaeus* sp. The wide distribution of crustins in crustaceans indicates the importance of these AMPs in the innate immunity and a detailed research is binding to reveal the enigmatic character of crustins.

Two penaeidins could be characterized from *F. indicus* haemocytes belonging to penaeidin-2 referred to as Fi-penaeidin and penaeidin-5. The phylogenetic tree analysis showed that penaeidins of *F. indicus* shared maximum similarity to other penaeidins of *Fenneropenaeus* sp. belonging to subgroup 2 and is more similar to penaeidin 2 of *F. paulensis* and *F. subtilis*. The wide distribution of penaeidins in penaeid shrimps indicates the importance of these AMPs in the innate immunity and a detailed research is required to reveal the inscrutable character of penaeidins. Peptide model was created for penaeidin-5 using SWISS-MODEL server and it showed the

presence of and α-helix in the structure of penaeidin-5. This is in agreement with results of Hu et al. (2006) where an α-helix structure was found to exist in the C-terminus of penaeidin-5. The multidomain structure, obvious sequence diversity and increasingly apparent functional complexity for penaeidins implies the need for functional studies that focus on the mechanism of action of these AMPs against specific microbial target species (Cuthbertson et al., 2004). Penaeidin proteins are probably highly regulated in their expression, potentially at multiple levels that include transcription, translation, modification and delivery. There is a great potential for class-specific utility of penaeidins within the immune system of a shrimp, and even specific isoforms may be used differentially. It is possible that each class and/or specific isoforms of a class are used as a specific defense mechanism against the pathogenesis of microbes. Much has yet to be learned about the immune response of the shrimp in regulating penaeidin expression. The diversity that is observed in penaeidin function classes and between isoforms of a single class from different species shows the potential for invivo diversity of immune function and immune (antibiotic) specificity of penaeidins. Expressing specific penaeidin isoforms in different tissues, depending on the type of immunogen detected by the shrimp and the way in which it was detected, may prove to be a most efficient way of responding to immune insult and pathogenic aggressiveness of microbes (Cuthebertson et al., 2008).

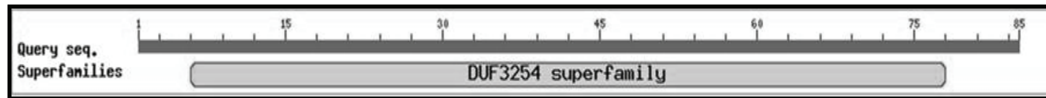
Discovery of novel AMPs and its antimicrobial spectrum might pave way to unravel the obscurity of crustacean immunity. It is interesting to notice that even if the structures are varied, some common features allow classification of these compounds into broad families. However, the recent discovery of the penaeidins suggests that this classification has to be flexible, in order to accommodate other chimeric-like molecules, such as the

horseshoe crab big defensin. The possible multifunctional properties of antimicrobial peptides represent an important new area to be investigated. Further research on the expression profile of these molecules in response to various environmental conditions and microbial infection would reveal their role in the protection of the animals from the onslaught of diseases. Any progress in this field would contribute to a better understanding of the penaeid shrimp physiology and their capacity to respond to pathological injuries.

**Table 3.1. Primers used for the study**

| Target gene | Sequence (5′-3′) | Product Size | Annealing Temp. ($^0$C) | $MgCl_2$ Conc. (mM) |
|---|---|---|---|---|
| ALF-1 | F- gcacgagggagcttcatatt<br>R- gagcaaagggcctatgagtta | 255 bp | 62 | 1.5 |
| ALF-2 | F- caagggtgggaggctgtgg<br>R- tgagctgagccactggttgg | 300 bp | 62 | 1.5 |
| Fi-Crustin | F- cgcacagccgagagaaacactatcaagat<br>R- ggcctatccctcagaacccagcacg | 456 bp | 55 | 3.5 |
| Crustin-3 | F- tccctggaggtcaattgagtg<br>R- agtcgaacatgcaggcctatcc | 233 bp | 60 | 1.5 |
| Fi-Penaeidin | F- acctgaccctcacctgcagaggcc<br>R- ttcgttgtcttctccatcaacc | 240 bp | 60 | 1.5 |
| Penaeidin-5 | F- agcctcacctgcagagaccg<br>R- tgcacttacatcccacatg | 300 bp | 60 | 1.5 |
| 18S rRNA | F-ttgtacgaggatcgagtgga<br>R-atgctttcgcagtaggtcgt | 350 bp | 52 | 2 |
| β-actin | F- cttgtggttgacaatggctccg<br>R- tggtgaaggagtagccacgctc | 520 bp | 60 | 1.5 |
| ELF | F- atggttgtcaactttgcccc<br>R- ttgacctccttgatcacacc | 440 bp | 60 | 1.5 |

**Table 3.2. BLASTn analysis of ALF-1 (GU732814) in *F. indicus***

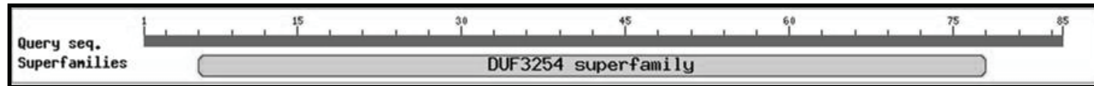



| GENBANK ACCESSION NUMBER | DESCRIPTION | QUERY COVERAGE | E-VALUE | % IDENTITY |
|---|---|---|---|---|
| **GU727863** | *F. indicus* ALF mRNA, complete cds | 97% | 4e-97 | 92% |
| **AY859500** | *F. chinensis* ALF mRNA, complete cds; plastid | 88% | 2e-80 | 91% |
| **EF523559** | *P. monodon* ALF isoform 3 mRNA, complete cds | 93% | 6e-75 | 88% |
| **GU299806** | *P. monodon* clone ALF-9 mRNA, complete cds | 93% | 7e-70 | 87% |

**Table 3.3. BLASTp analysis of ALF-1 (GU732814) in *F. indicus***

| GENBANK ACCESSION NUMBER | DESCRIPTION | MAX SCORE | TOTAL SCORE | QUERY COVERAGE | % IDENTITY |
|---|---|---|---|---|---|
| **ADE27980** | *F. indicus* ALF | 84.7 | 84.7 | 89% | 3e-15 |
| **AAX63831** | *F. chinensis* AMP | 79.3 | 79.3 | 89% | 1e-13 |
| **ADK74771** | *P. monodon* ALF | 79.0 | 79.0 | 89% | 2e-13 |
| **ABB22832** | *L. vannamei* ALF AV-R isoform | 74.3 | 74.3 | 89% | 5e-12 |
| **ABJ90465** | *L. schmitti* ALF | 73.2 | 73.2 | 89% | 9e-12 |

**Table 3.4. BLASTn analysis of ALF-2 (HM366921) in *F. indicus***




| GENBANK ACCESSION NUMBER | DESCRIPTION | QUERY COVERAGE | E-VALUE | % IDENTITY |
|---|---|---|---|---|
| **GU727863** | *F. indicus* ALF mRNA, complete cds | 99% | 3e-179 | 98% |
| **AY859500** | *F. chinensis* ALF mRNA, complete cds | 99% | 7e-156 | 94% |
| **EF523559** | *P. monodon* ALF isoform 3 mRNA, complete cds | 99% | 3e-144 | 92% |
| **DQ208702** | *L. vannamei* ALF AV-R isoform mRNA, complete cds | 93% | 2e-97 | 86% |
| **DQ991357** | *L. schmitti* ALF mRNA, complete cds | 93% | 2e-87 | 84% |

**Table 3.5. BLASTp analysis of ALF-2 (HM366921) in *F. indicus***

| GENBANK ACCESSION NUMBER | DESCRIPTION | MAX SCORE | TOTAL SCORE | QUERY COVERAGE | % IDENTITY |
|---|---|---|---|---|---|
| **ADE27980** | *F. indicus* ALF | 241 | 241 | 100% | 2e-62 |
| **ABB22832** | *L. vannamei* ALF AV-R isoform | 214 | 214 | 100% | 3e-54 |
| **ABJ90465** | *L. schmitti* ALF | 210 | 210 | 100% | 4e-53 |
| **ABQ96193** | *F. paulensis* ALF isoform 1 | 204 | 204 | 100% | 3e-51 |
| **ABP73289** | *P. monodon* ALF isoform 3 | 204 | 204 | 100% | 3e-51 |

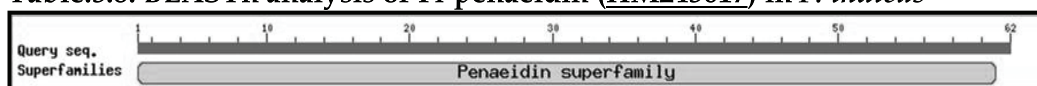
**Table 3.6. BLASTn analysis of Fi-crustin (GQ469987) in *F. indicus***

| GENBANK ACCESSION NUMBER | DESCRIPTION | QUERY COVERAGE | E-VALUE | % IDENTITY |
|---|---|---|---|---|
| **DQ097703** | *F. chinensis* crustin-like protein fc-1 mRNA, complete cds | 94% | 1e-78 | 91% |
| **EF654659** | *P. monodon* crustin-like AMP gene, complete cds | 0% | 3e-75 | 97% |
| **GQ334396** | *P. monodon* crustin-like AMP type 1 mRNA, partial cds | 57% | 2e-71 | 90% |
| **DQ097704** | *F. chinensis* crustin-like protein fc-2 mRNA, complete cds | 58% | 2e-27 | 96% |

**Table 3.7. BLASTp analysis of Fi-crustin (GQ469987) in *F. indicus***

| GENBANK ACCESSION NUMBER | DESCRIPTION | MAX SCORE | TOTAL SCORE | QUERY COVERAGE | % IDENTITY |
|---|---|---|---|---|---|
| **AAZ76017** | *F. chinensis* crustin-like protein fc-1 | 134 | 134 | 99% | 5e-30 |
| **AAZ76018** | *F. chinensis* crustin-like protein fc-2 | 70.5 | 70.5 | 99% | 6e-11 |
| **ACU25384** | *P. japonicus* crustin 3 | 67.8 | 67.8 | 93% | 4e-10 |
| **ACT82963** | *P. monodon* crustin-like AMP type 2 | 129 | 129 | 58% | 1e-28 |
| **ACL97378** | *P. monodon* crustin-like AMP | 126 | 126 | 58% | 1e-27 |

**Table.3.8. BLASTn analysis of Fi-penaeidin (HM243617) in *F. indicus***

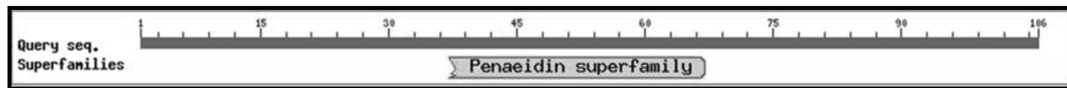



| GENBANK ACCESSION NUMBER | DESCRIPTION | QUERY COVERAGE | E-VALUE | % IDENTITY |
|---|---|---|---|---|
| **EF450745** | *F. brasiliensis* penaeidin mRNA, complete cds | 34% | 1e-20 | 96% |
| **FJ472344** | *L. schmitti* penaeidin 3-1 mRNA, complete cds | 36% | 6e-19 | 94% |
| **DQ308407** | *F. chinensis* penaeidin 5-3 gene, complete cds | 36% | 6e-19 | 94% |
| **AY039206** | *L. setiferus* penaeidin-3l mRNA, complete cds | 36% | 6e-19 | 94% |
| **EU333492** | *F. penicillatus* pen3-p precursor, mRNA, complete cds | 31% | 8e-18 | 96% |

**Table.3.9. BLASTp analysis of Fi-penaeidin (HM243617) in *F. indicus***

| GENBANK ACCESSION NUMBER | DESCRIPTION | MAX SCORE | TOTAL SCORE | QUERY COVERAGE | % IDENTITY |
|---|---|---|---|---|---|
| **AAX58696** | *F. paulensis* AMP PEN2-2 | 50.1 | 50.1 | 98% | 9e-05 |
| **AAX58695** | *F. paulensis* AMP PEN2-1 | 48.9 | 48.9 | 98% | 2e-04 |
| **AAV85945** | *F.chinensis* penaeidin | 48.9 | 48.9 | 98% | 2e-04 |
| **AAX58695** | *F. paulensis* AMP PEN2-1 | 48.9 | 48.9 | 98% | 2e-04 |
| **AAZ79334** | *F. chinensis* penaeidin 5-1 | 48.5 | 48.5 | 98% | 2e-04 |

**Table.3.10. BLASTn analysis of penaeidin-5 (HM243616) in *F. indicus***




| GENBANK ACCESSION NUMBER | DESCRIPTION | QUERY COVERAGE | E-VALUE | % IDENTITY |
|---|---|---|---|---|
| **DQ308407** | *F. chinensis* penaeidin 5-3 gene, complete cds | 65% | 1e-45 | 80% |
| **FJ686018** | *P. monodon* penaeidin 5 AMP mRNA, complete cds | 66% | 1e-44 | 79% |
| **EU884391** | *P. monodon* penaeidin 3 mRNA, complete cds | 41% | 7e-23 | 80% |
| **DQ206401** | *L. vannamei* PEN2-1 gene, complete cds | 32% | 2e-16 | 81% |
| **AY039207** | *L. setiferus* penaeidin-4d mRNA, complete cds | 35% | 1e-12 | 77% |

**Table 3.11. BLASTp analysis of penaeidin-5 (HM243616) in *F. indicus***

| GENBANK ACCESSION NUMBER | DESCRIPTION | MAX SCORE | TOTAL SCORE | QUERY COVERAGE | % IDENTITY |
|---|---|---|---|---|---|
| **ACH70378** | *P. monodon* penaeidin 3 | 42.0 | 42.0 | 31% | 0.025 |
| **AAZ79334** | *F. chinensis* penaeidin 5-1 | 41.6 | 41.6 | 46% | 0.034 |
| **AAZ80041** | *F. chinensis* penaeidin 5-2 | 40.4 | 40.4 | 46% | 0.063 |
| **AAV85945** | *F. chinensis* penaeidin | 40.4 | 40.4 | 45% | 0.077 |
| **ADN43391** | *F. indicus* penaeidin 3 | 35.0 | 35.0 | 28% | 3.0 |

➢ NUCLEOTIDE SEQUENCE (255 bp)

```
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agtgcaagttcaccgtcaagccttacattaagaggttccagttgaa
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aagccagaacacgcagtcattccggggtggctggacggacagccca
aaacttattcggaaactttccaaaaaggtttcatctctcaacaggg
ggcccccaatggctcacctcaaagt
```

➢ DEDUCED AMINOACID SEQUENCE:FRAME-1(85 aminoacid)

```
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SEEKPEHAVIPGWLDGQPKTYSETFQKGFISQQGAPNGSPQS
```

```
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 T  D  V  G  L  E  D  E  K  T  E  L  L  G  H  E  C  K  F  T
gtcaagccttacattaagaggttccagttgaactacaaggggaggatgtggtgcccagct
 V  K  P  Y  I  K  R  F  Q  L  N  Y  K  G  R  M  W  C  P  A
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 G  R  P  S  E  E  K  P  E  H  A  V  I  P  G  W  L  D  G  Q
cccaaaacttattcggaaactttccaaaaaggtttcatctctcaacagggggcccccaat
 P  K  T  Y  S  E  T  F  Q  K  G  F  I  S  Q  Q  G  A  P  N
ggctcacctcaaagt
 G  S  P  Q  S
```

**Fig.3.2. Nucleotide and amino acid sequences of ALF-1 (GU732814) from the haemocyte of the Indian white shrimp, *F. indicus***

**Fig.3.3. Structural model of ALF-1 (GU732814) in *F. indicus* created using SWISS-MODEL Server**

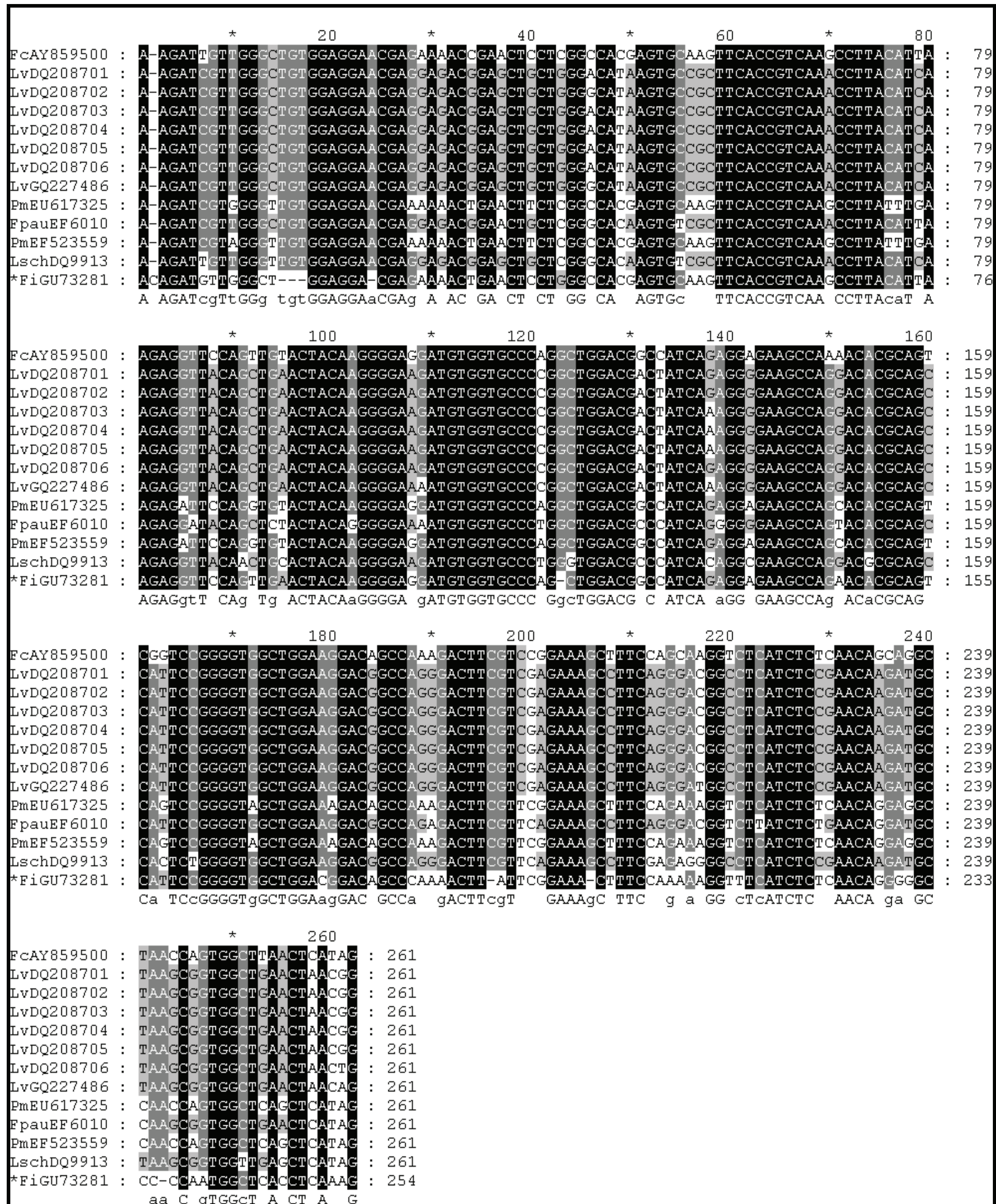


**Fig.3.4. Multiple alignment of nucleotide sequence of the *F. indicus* ALF-1 (GU732814) with other ALFs (*F. chinensis* AY859500, *L. vannamei* DQ208701, *L. vannamei* DQ208702, *L. vannamei* DQ208703, *L. vannamei* DQ208704, *F. paulensis* EF601054, *L. vannamei* DQ208705, *L. vannamei* DQ208706, *L. vannamei* GQ227486, *P. monodon* EU617325, *P. monodon* EF523559, *F. paulensis* EF601051, *L. schmitti* DQ991357) obtained using GeneDoc programme Version 2.7.0. Black and grey indicates conserved sequences.**

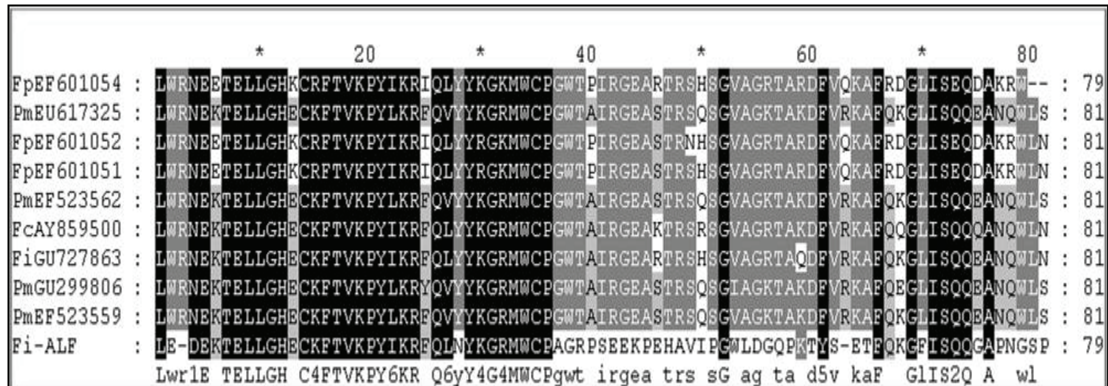



**Fig.3.5. Multiple alignment of deduced amino acid sequence of the *F. indicus* ALF-1 (GU732814) with other ALFs (*F. paulensis* EF601054, *P. monodon* EU617325, *F. paulensis* EF601051 , *F. paulensis* EF601052, *P. monodon* EF523559, *P. monodon* EF523562, *F. chinensis* AY859500, *P. monodon* GU299806, *F. chinensis* AY859500, *F. indicus* GU727863) obtained using GeneDoc programme Version 2.7.0. Black and grey indicates conserved sequences.**

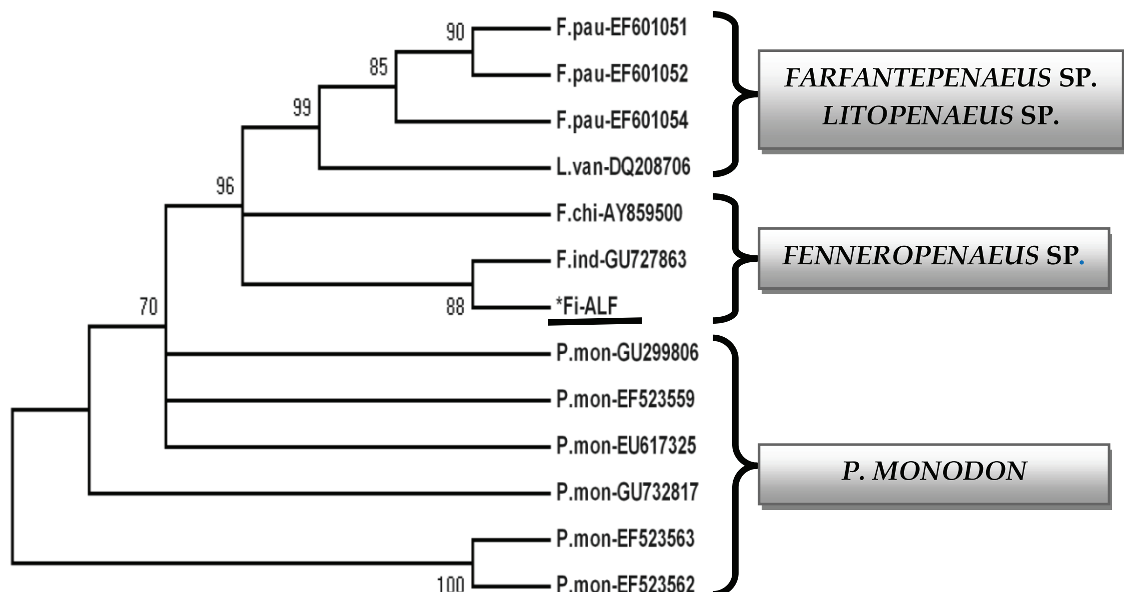



**Fig.3.6. A bootstrapped neighbour-joining tree obtained using MEGA version 4.0 illustrating relationships between the deduced amino acid sequence of the *F. indicus* ALF-1, (GU732814) with other ALFs of shrimps (*F. paulensis* EF601054, *F. paulensis* EF601051, *F. paulensis* EF601052, *L. vannamei* DQ208706, *F. chinensis* AY859500, *F. indicus* GU727863, *P. monodon* EU617325, *P. monodon* EF523559, *P. monodon* EF523562, *P. monodon* GU299806). Values at the node indicate the percentage of times that the particular node occurred in 1000 trees generated by bootstrapping the original deduced protein sequences.**

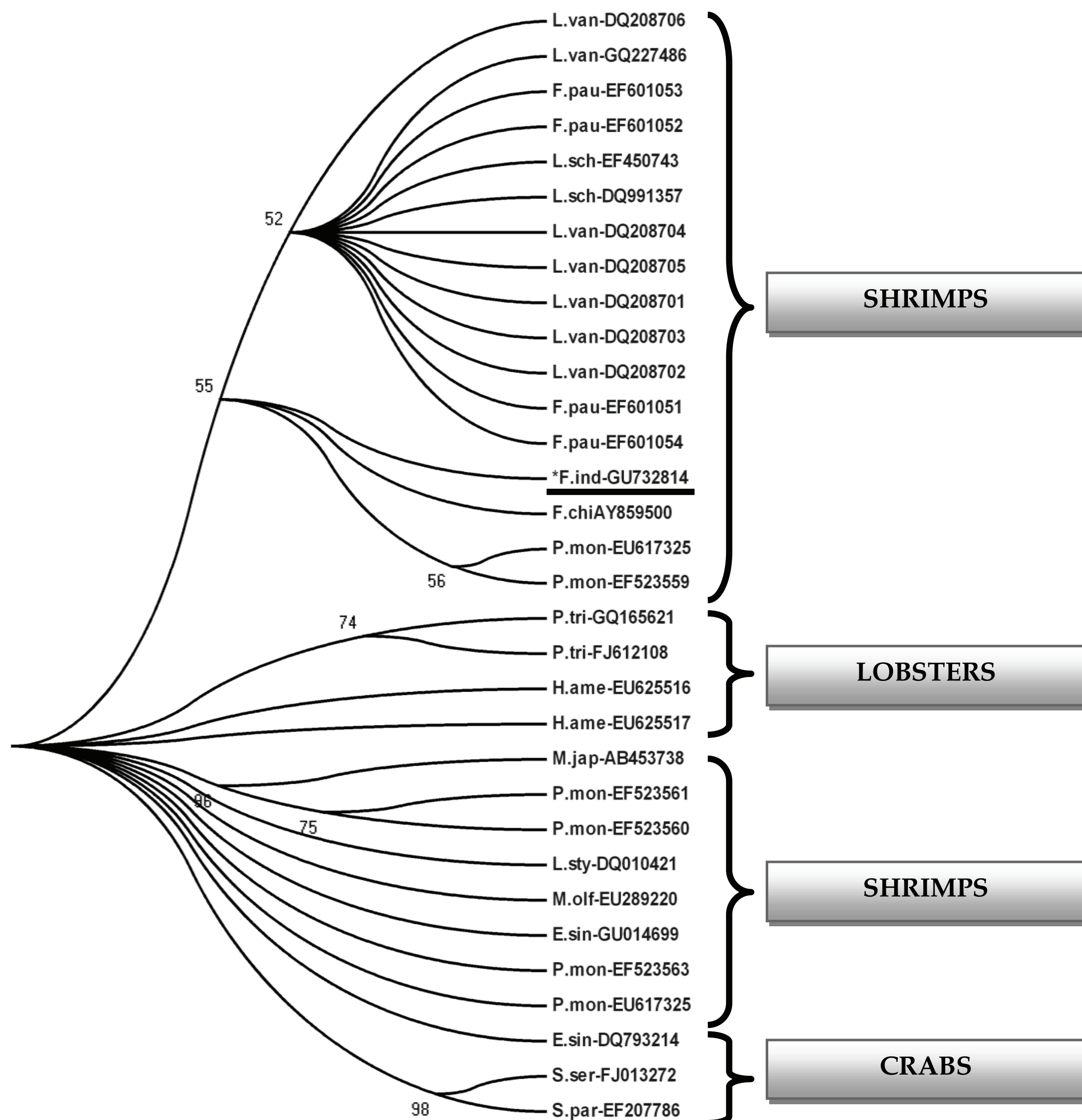



**Fig.3.7. A bootstrapped neighbour-joining tree obtained using MEGA version 4.0 illustrating relationships between the deduced amino acid sequence of the *F. indicus* ALF-1 (GU732814) with other ALFs of decapod crustaceans (*F. chinensis* AY859500, *L. vannamei* DQ208701, *L. vannamei* DQ208702, *L. vannamei* DQ208703, *L. vannamei* DQ208704, *L. vannamei* DQ208705, *L. vannamei* DQ208706, *L. stylirostris* DQ010421, *P. trituberculatus* GQ165621, *E. sinensis* DQ793214, *S. serrata* FJ013272, *P. trituberculatus* FJ612108, *L. vannamei* GQ227486, *M. japonicus* AB453738, *H. americanus* EU625516, *H. americanus* EU625517, *P. monodon* EU617325, *E sinensis* GU014699, *P. monodon* EF523563, *P. monodon* EF523559, *P. monodon* EF523561, *P. monodon* EF523560, *M. olfersii* EU289220, *F. paulensis* EF601054, *F. paulensis* EF601053, *F. paulensis* EF601052, *F. paulensis* EF601051, *L. schmitti* DQ991357, *L. schmitti* EF450743, *S. paramamosain* EF207786). Values at the node indicate the percentage of times that the particular node occurred in 1000 trees generated by bootstrapping the original deduced protein sequences.**

**Fig.3.8. Nucleotide and amino acid sequences of ALF-2 (HM588914) from the haemocyte of the Indian white shrimp, *F. indicus***

**Fig.3.9. Structural model of ALF-2 (HM588914) in *F. indicus* created using SWISS-MODEL Server**

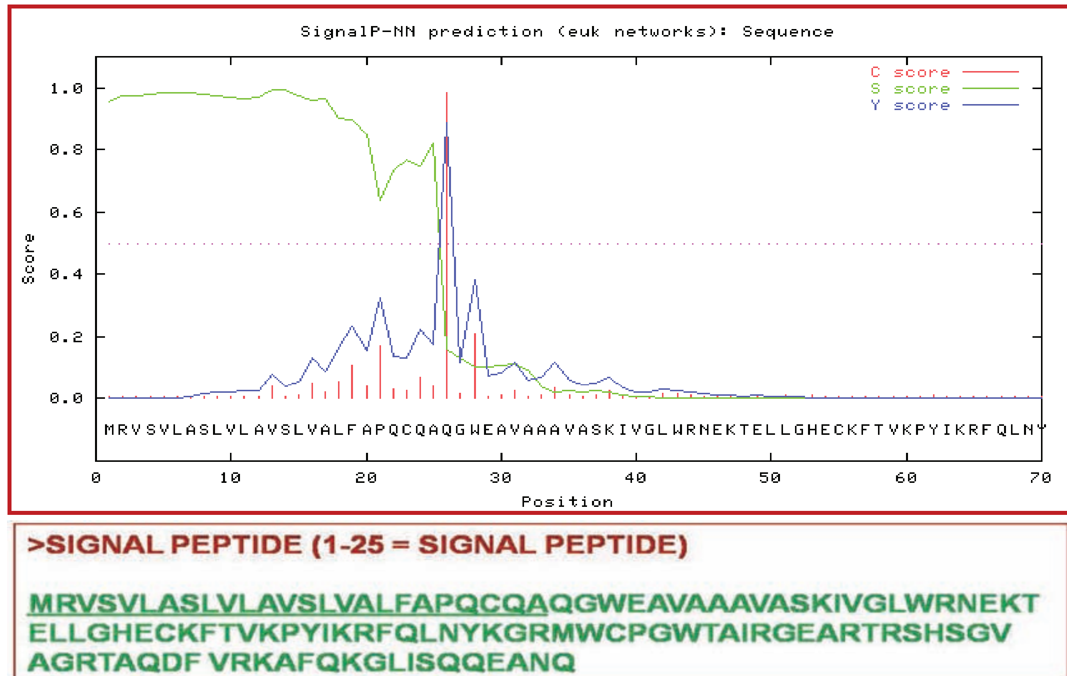



**Fig.3.10 Signal peptide analysis of ALF-2 (HM366921) in *F. indicus* as predicted by the SignalP 3.0 server. The underlined amino acid residues indicate a putative signal sequence.**

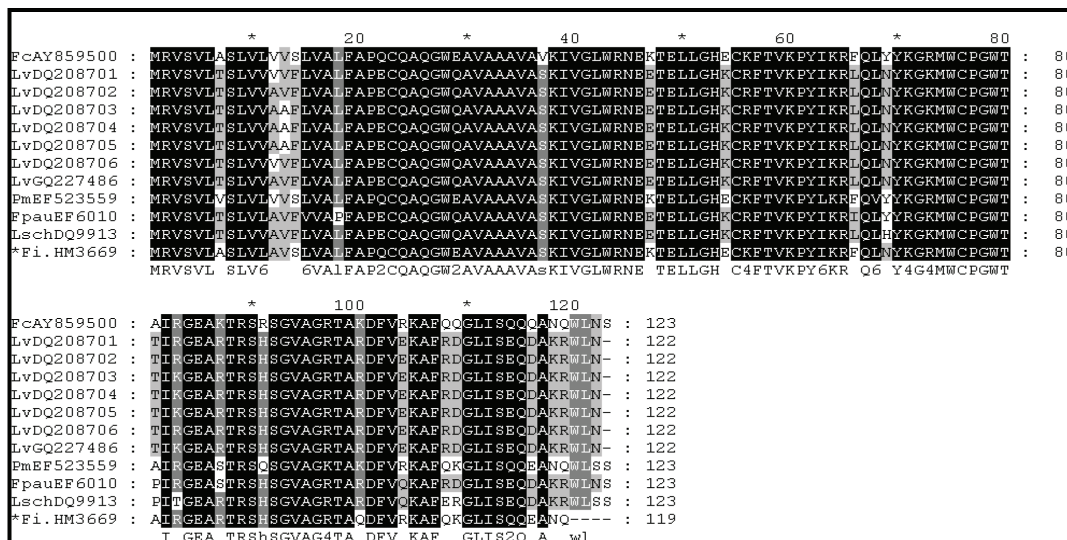


**Fig.3.11. Multiple alignment of the deduced amino acid sequence of the *F. indicus* ALF-2 (HM366921) with other ALFs (*F. chinensis* AY859500, *L. vannamei* DQ208701, *L. vannamei* DQ208702, *L. vannamei* DQ208703, *L. vannamei* DQ208704, *L. vannamei* DQ208705, *L. vannamei* DQ208706, *L. vannamei* GQ227486, *P. monodon* EF523559, *F. paulensis* EF601051, *L. schmitti* DQ991357) obtained using GeneDoc programme Version 2.7.0. Black and grey indicates conserved sequences.**

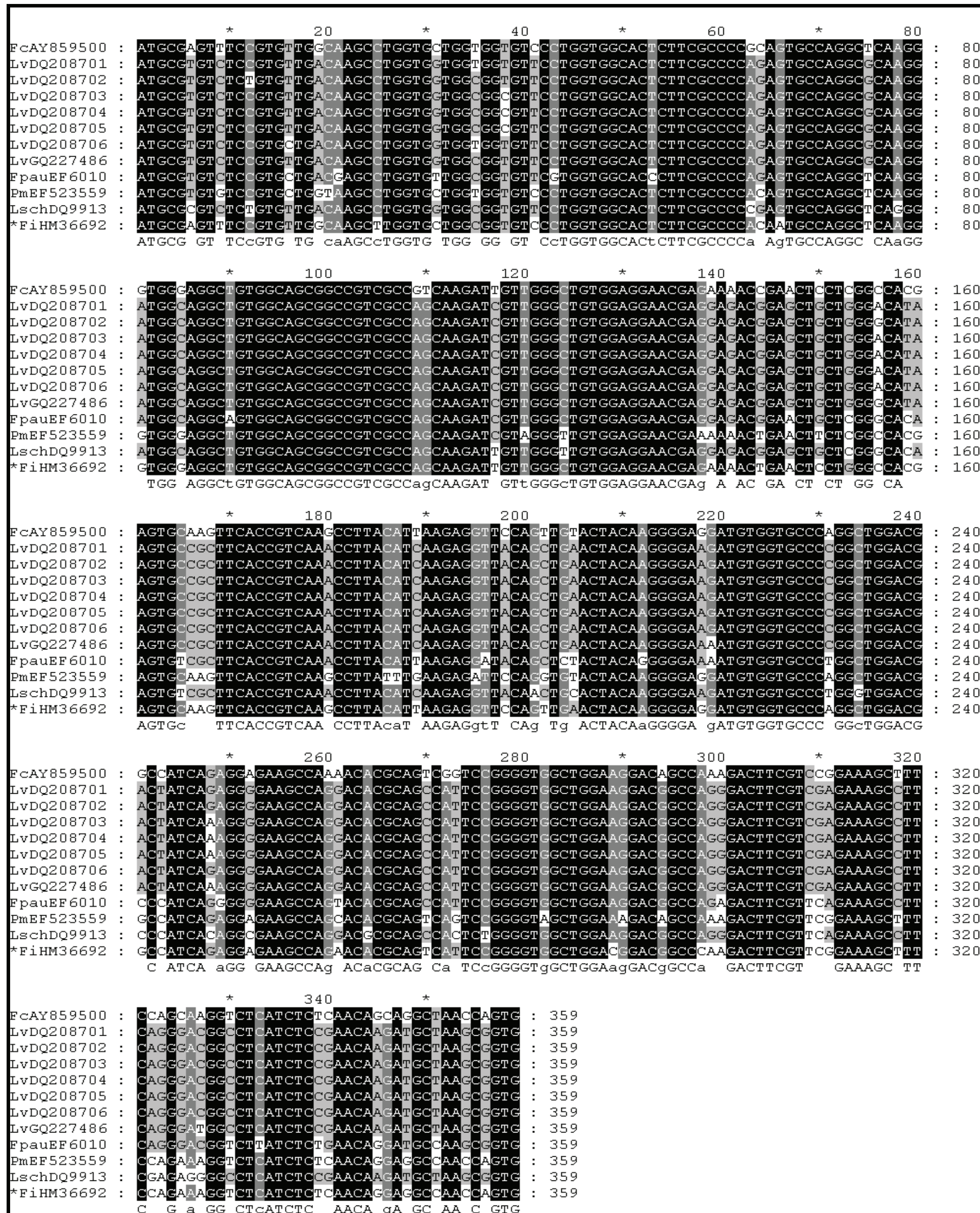


**Fig.3.12. Multiple alignment of the nucleotide sequence of the *F. indicus* ALF-2 (HM366921) with other ALFs (*F. chinensis* AY859500, *L. vannamei* DQ208701, *L. vannamei* DQ208702, *L. vannamei* DQ208703, *L. vannamei* DQ208704, *L. vannamei* DQ208705, *L. vannamei* DQ208706, *L. vannamei* GQ227486, *P. monodon* EF523559, *F. paulensis* EF601051, *L. schmitti* DQ991357) obtained using GeneDoc programme Version 2.7.0. Black and grey indicates conserved sequences.**

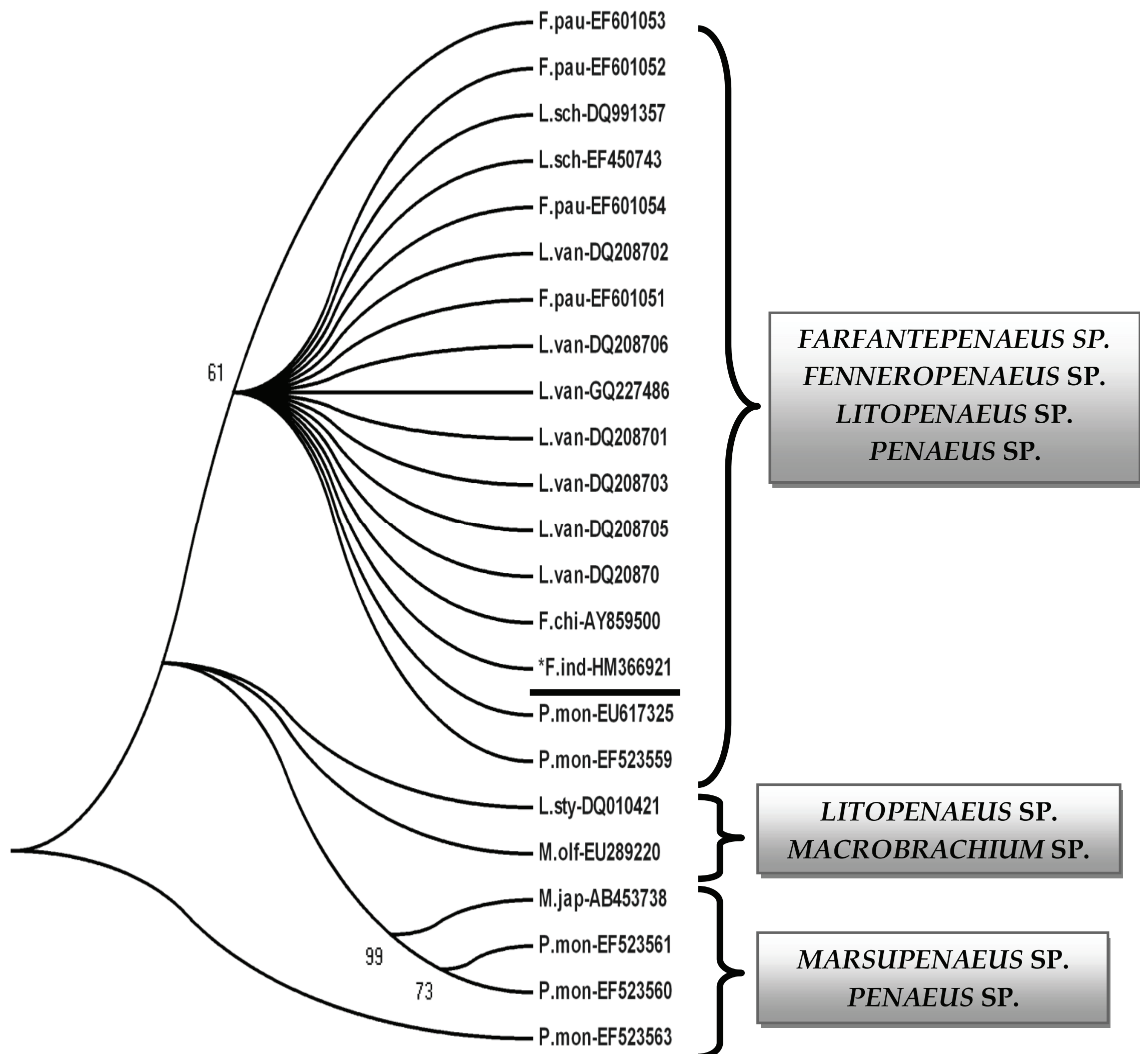



**Fig.3.13. A bootstrapped neighbour-joining tree obtained using MEGA version 4.0 illustrating relationships between the deduced amino acid sequence of the *F. indicus* ALF-2, (HM588914) with other ALFs of shrimps (*L. schmitti* EF450743, *L.schmitti* DQ991357, *F. paulensis* EF601051 , *F. paulensis* EF601052, *F. paulensis* EF601053, *F. paulensis* EF601054, *M. olfersii* EU289220, *P. monodon* EF523560, *P. monodon* EF523561, *P. monodon* EF523559, *P. monodon* EF523563, *P. monodon* EU617325, *M. japonicus* AB453738, *L. vannamei* GQ227486, *L. stylirostris* DQ010421, *L. vannamei* DQ208706, *L. vannamei* DQ208705, *L. vannamei* DQ208704, *L. vannamei* DQ208703, *L. vannamei* DQ208702, *L. vannamei* DQ208701, *F. chinensis* AY859500). Values at the node indicate the percentage of times that the particular node occurred in 1000 trees generated by bootstrapping the original deduced protein sequences.**

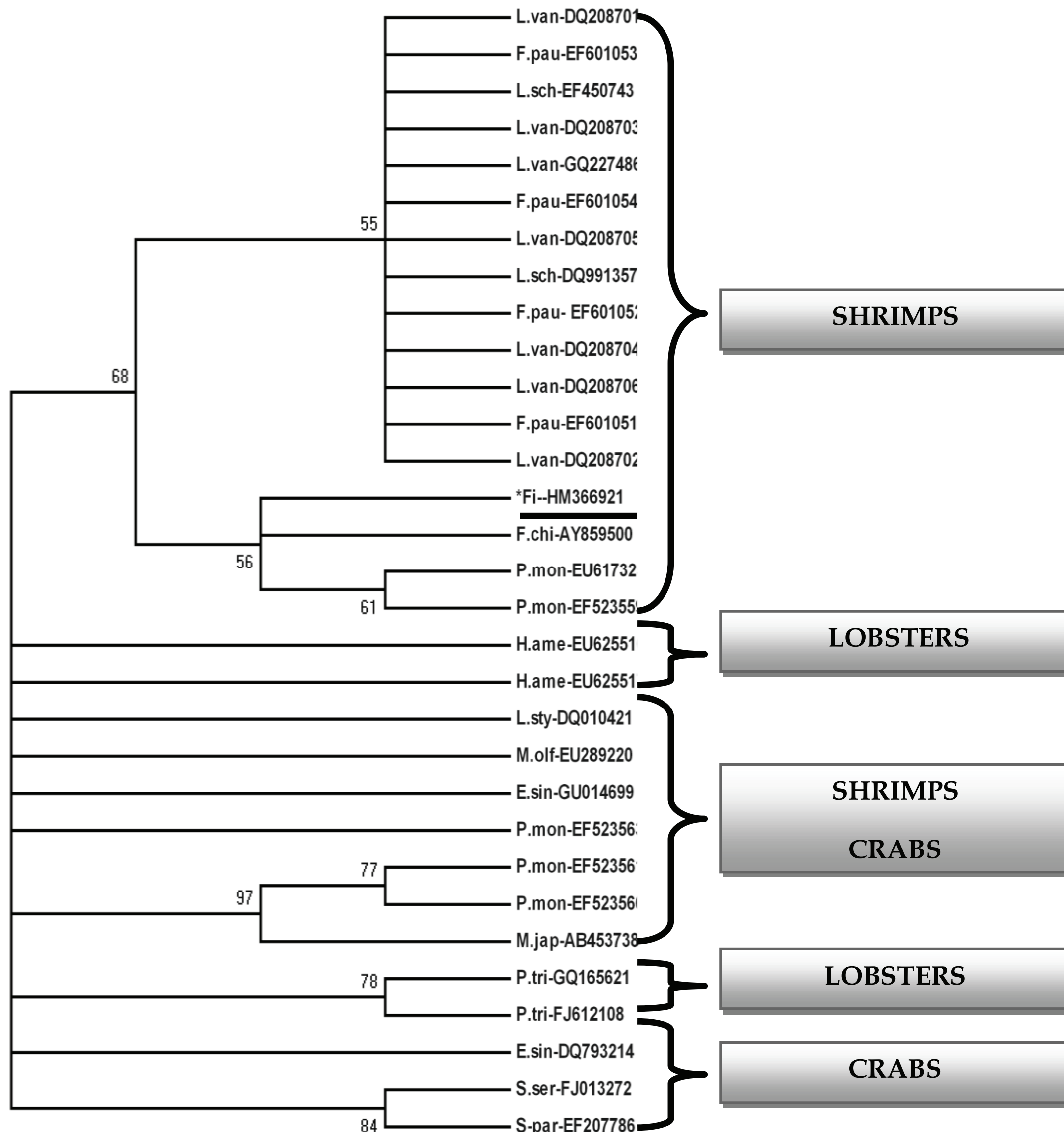
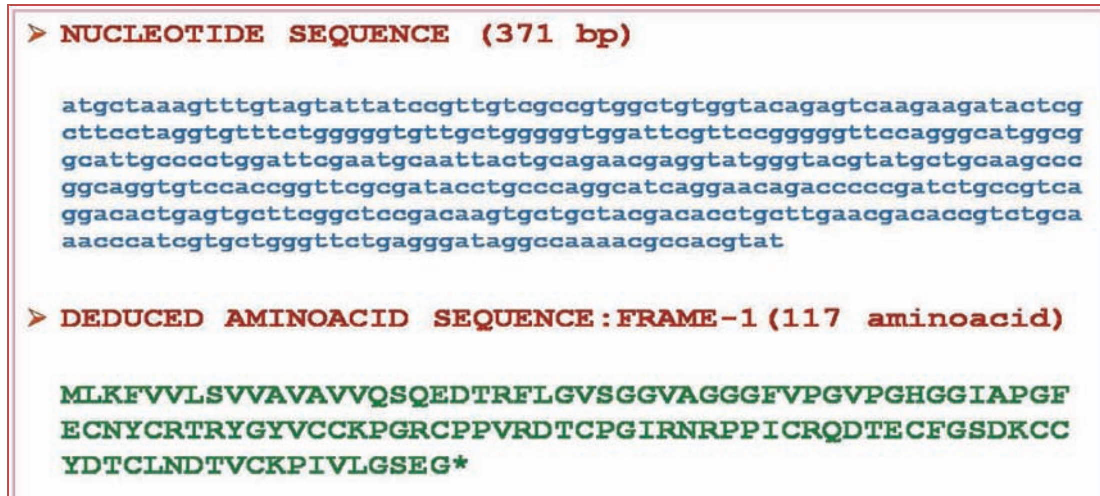



**Fig.3.14. A bootstrapped neighbour-joining tree obtained using MEGA version 4.0 illustrating relationships between the deduced amino acid sequence of the *F. indicus* ALF-2 (HM588914) with other ALFs of decapod crustaceans (*F. chinensis* AY859500, *L. vannamei* DQ208701, *L. vannamei* DQ208702, *L. vannamei* DQ208703, *L. vannamei* DQ208704, *L. vannamei* DQ208705, *L. vannamei* DQ208706, *L. stylirostris* DQ010421, *P. trituberculatus* GQ165621, *E. sinensis* DQ793214, *S. serrata* FJ013272, *P. trituberculatus* FJ612108, *L. vannamei* GQ227486, *M. japonicus* AB453738, *H. americanus* EU625516, *H. americanus* EU625517, *P. monodon* EU617325, *E. sinensis* GU014699, *P. monodon* EF523563, *P. monodon* EF523559, *P.monodon* EF523561, *P. monodon* EF523560, *M. olfersii* EU289220, *F.paulensis* EF601054, *F. paulensis* EF601053, *F. paulensis* EF601052, *F. paulensis* EF601051, *L. schmitti* DQ991357, *L. schmitti* EF450743, *S. paramamosain* EF207786). Values at the node indicate the percentage of times that the particular node occurred in 1000 trees generated by bootstrapping the original deduced protein sequences.**



➤ NUCLEOTIDE SEQUENCE (371 bp)

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ggacactgagtgcttcggctccgacaagtgctgctacgacacctgcttgaacgacaccgtctgca
aacccatcgtgctgggttctgagggataggccaaaacgccacgtat

➤ DEDUCED AMINOACID SEQUENCE:FRAME-1(117 aminoacid)

MLKFVVLSVVAVAVVQSQEDTRFLGVSGGVAGGGFVPGVPGHGGIAPGF
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YDTCLNDTVCKPIVLGSEG*

atgctaaagtttgtagtattatccgttgtcgccgtggctgtggtacagagtcaagaagat
M L K F V V L S V V A V A V V Q S Q E D
actcgcttcctaggtgtttctgggggtgttgctgggggtggattcgttccgggggttcca
T R F L G V S G G V A G G G F V P G V P
gggcatggcggcattgcccctggattcgaatgcaattactgcagaacgaggtatgggtac
G H G G I A P G F E C N Y C R T R Y G Y
gtatgctgcaagcccggcaggtgtccaccggttcgcgatacctgcccaggcatcaggaac
V C C K P G R C P P V R D T C P G I R N
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R P P I C R Q D T E C F G S D K C C Y D
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T C L N D T V C K P I V L G S E G * A K
acgccacgtat
T P R

**Fig.3.15. Nucleotide and amino acid sequences of Fi-crustin (GQ469987) from the haemocyte of the Indian white shrimp, *F. indicus*. The underlined amino acid residues indicate a putative signal sequence. Cysteine residues that participate in the formation of intramolecular disulphide bonds are bold printed. An asterisk is the stop codon.**

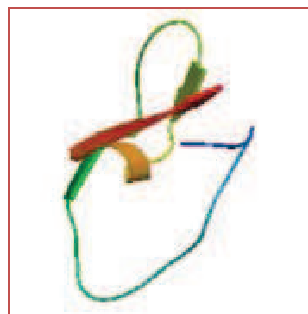


**Fig.3.16. Structural model of Fi-crustin (GQ469987) in *F. indicus* created uisng SWISS-MODEL Server**

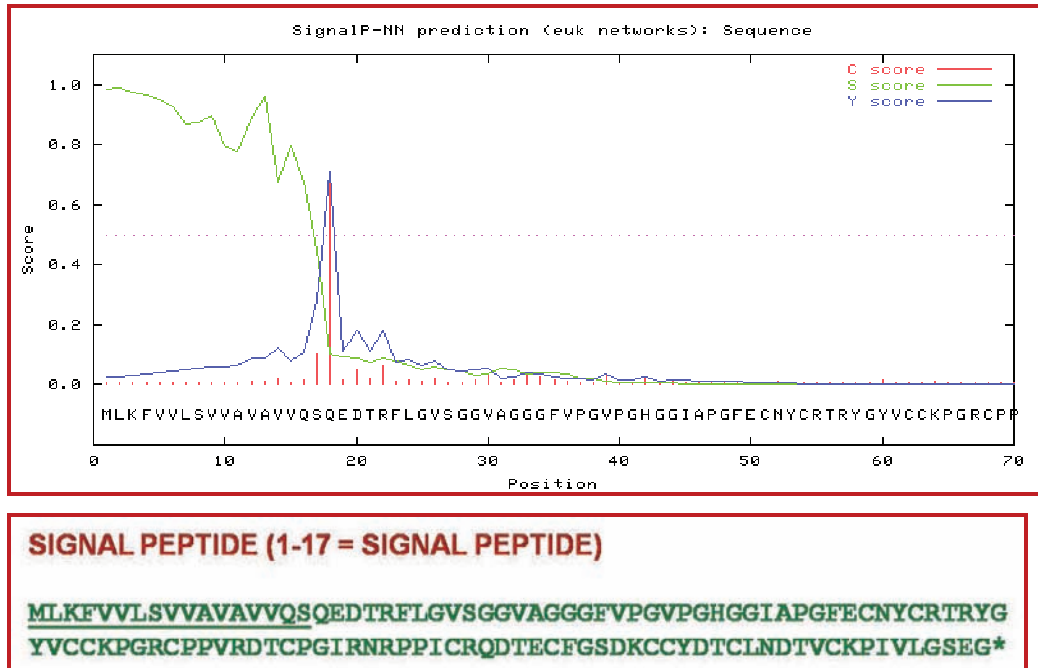



**Fig.3.17. Signal peptide analysis of Fi-crustin (GQ469987) in *F. indicus* as predicted by the SignalP 3.0 server. The underlined amino acid residues indicate a putative signal sequence.**

**Fig.3.18. ScanProsite analysis for the presence of conserved domain / pattern / motif in Fi-crustin (GQ469987) of *F. indicus*.**

**(A) WAP domain signature (B) Glycine and cysteine rich regions**

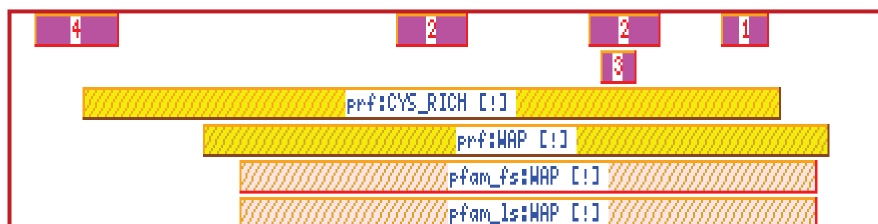



**Fig.3.19. Schematic representation of matches map and list of matches obtained for Fi-crustin (GQ469987) of *F. indicus* from the Motif Scan search**

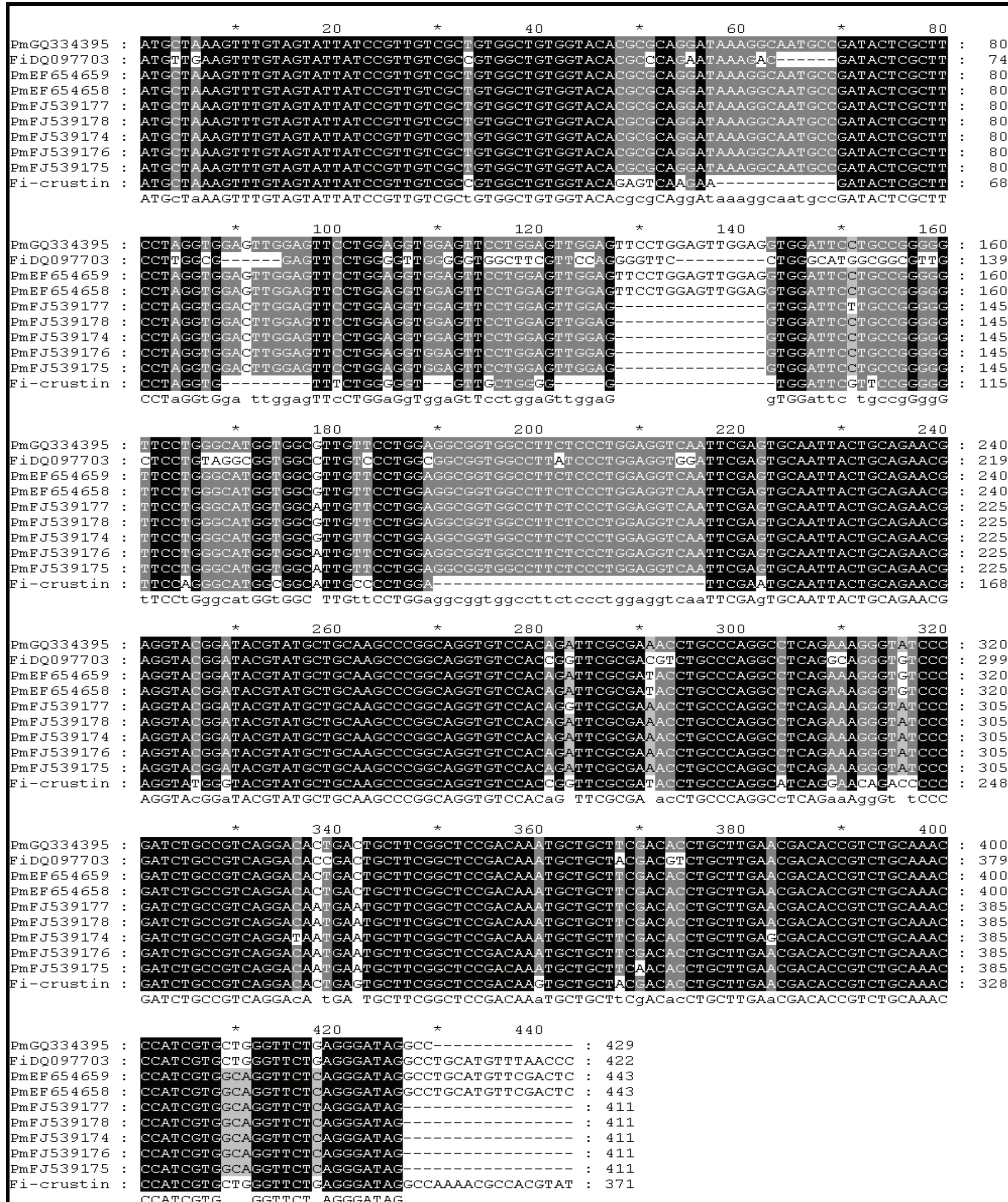


**Fig.3.20 Multiple alignment of nucleotide sequence of the *F. indicus* crustin-like antimicrobial peptide, Fi-crustin (GQ469987) with other shrimp crustins (*P. monodon* GQ334395, *F. chinensis* DQ097703, *P. monodon* EF654659, *P. monodon* EF654658, *P. monodon* FJ539177, *P. monodon* FJ539178, *P. monodon* FJ539174, *P. monodon* FJ539175, *P. monodon* FJ539176), obtained using GeneDoc programme Version 2.7.0. Black and grey indicates conserved sequences.**

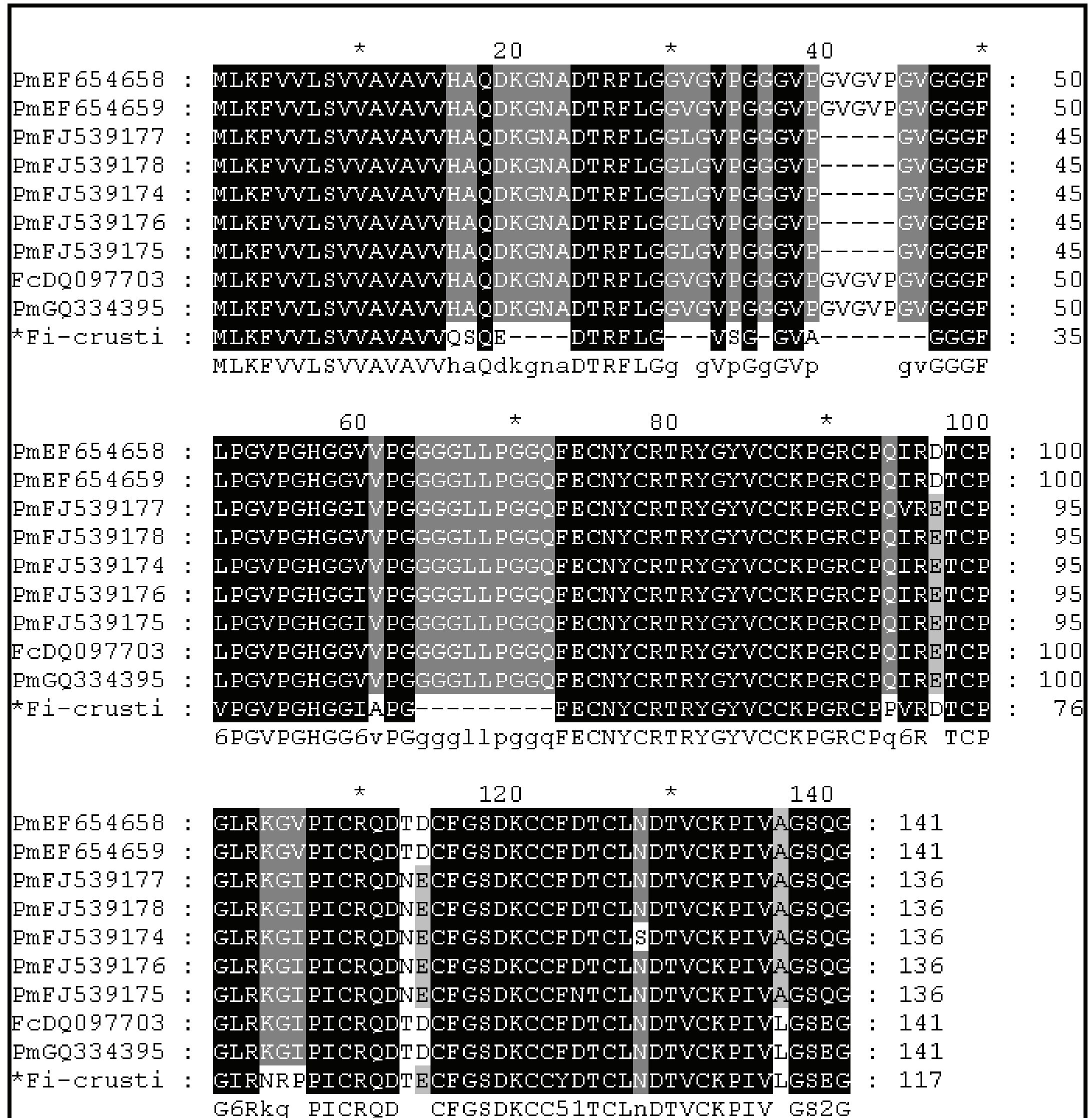



**Fig.3.21. Multiple alignment of deduced amino acid sequence of the *F. indicus* crustin-like antimicrobial peptide, Fi-crustin (GQ469987) with other shrimp crustins (*P. monodon* EF654658, *P. monodon* EF654659, *P. monodon* FJ539177, *P. monodon* FJ539178, *P. monodon* FJ539174, *P. monodon* FJ539175, *P. monodon* FJ539176, *P. monodon* GQ334395, *F. chinensis* DQ097703) obtained using GeneDoc programme Version 2.7.0. Black and grey indicates conserved sequences.**

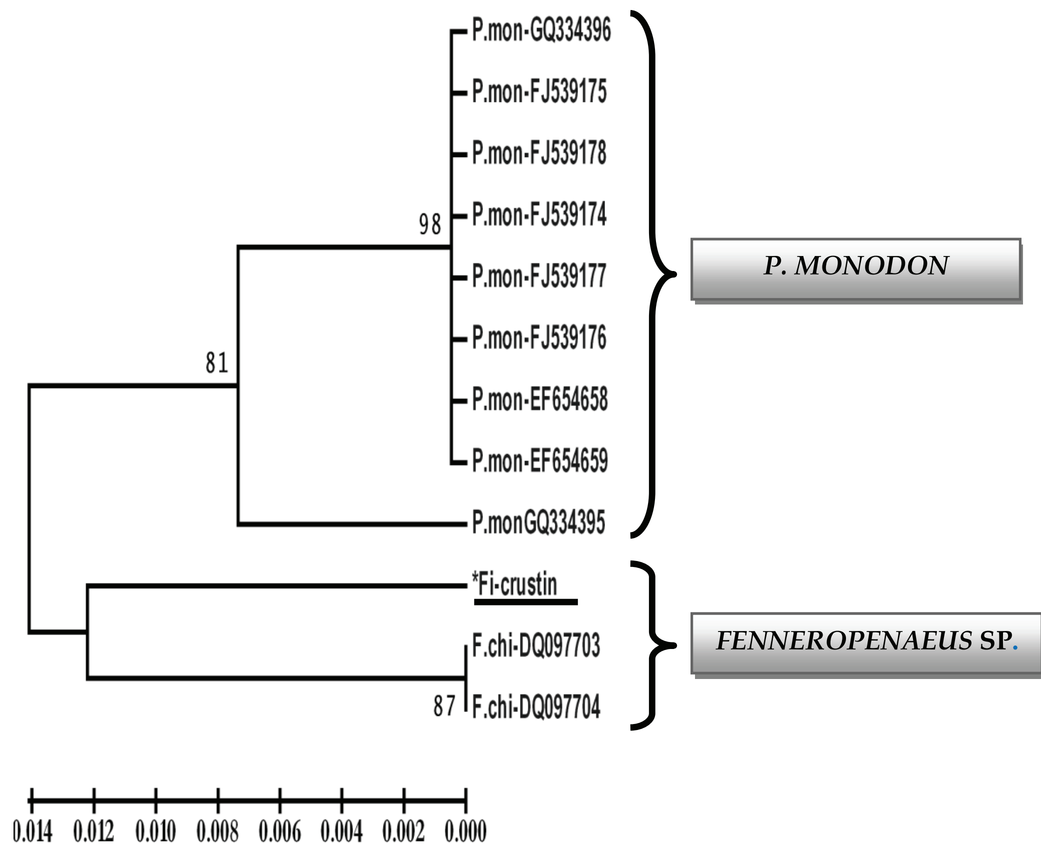



**Fig.3.22. A bootstrapped neighbour-joining tree obtained using MEGA version 4.0 illustrating relationships between the nucleotide sequence of the *F. indicus* crustin-like AMP, Fi-crustin (GQ469987) with other shrimp crustins (*P. monodon* GQ334395, *F. chinensis* DQ097703, *P. monodon* EF654659, *P. monodon* EF654658, *P. monodon* FJ539177, *P. monodon* FJ539178, *F. chinensis* DQ097704, *P. monodon* FJ539174, *P. monodon* FJ539175, *P. monodon* FJ539176)). Values at the node indicate the percentage of times that the particular node occurred in 1000 trees generated by bootstrapping the original deduced protein sequences.**

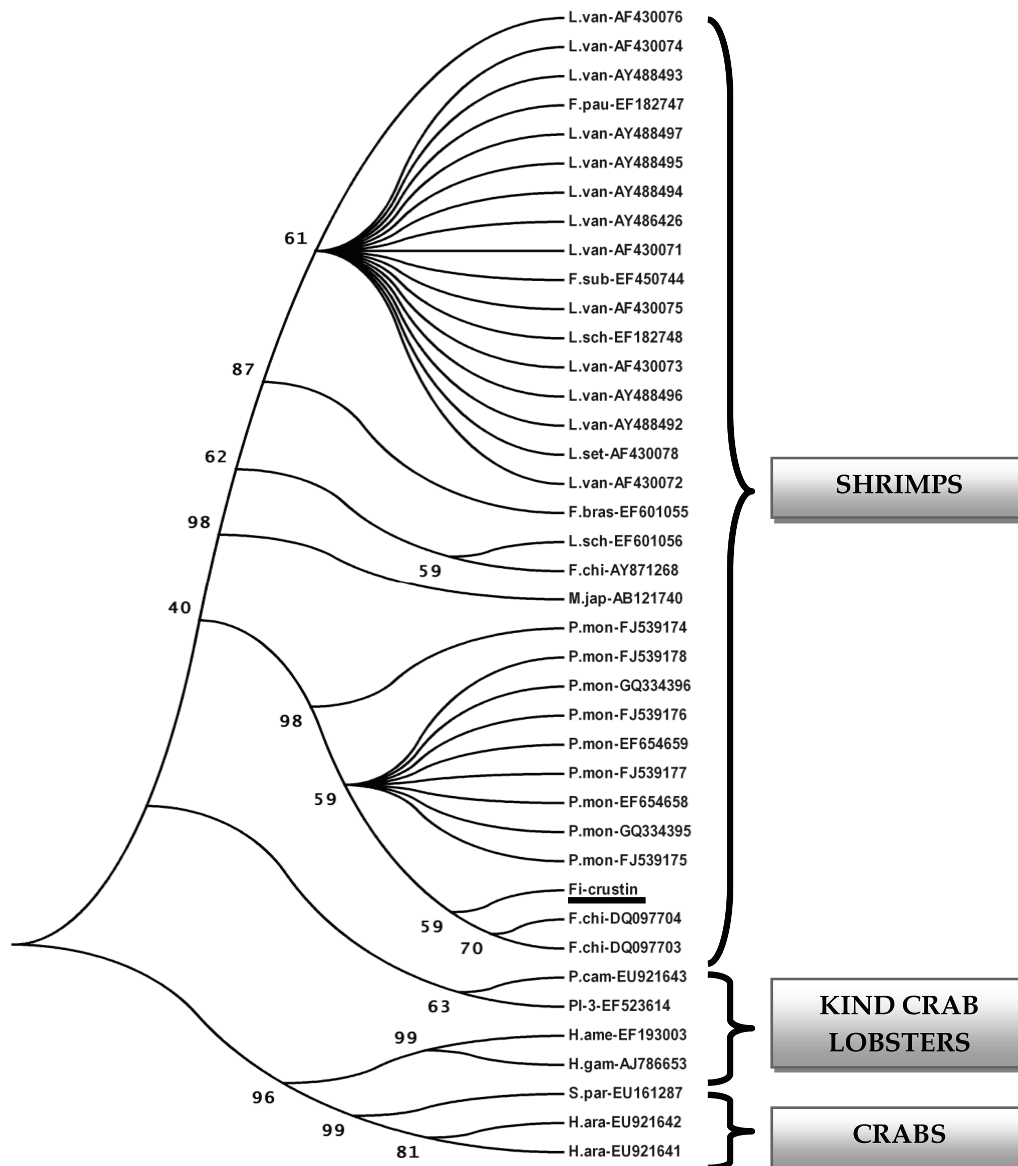



**Fig.3.23. A bootstrapped neighbour-joining tree obtained using MEGA version 4.0 illustrating relationships between the deduced amino acid sequence of the *F. indicus* crustin-like AMP, Fi-crustin (GQ469987) with other crustins of decapod crustaceans (*L. vannamei* AF430071, *L. vannamei* AF430074, *L. vannamei* AY488497, *L. vannamei* AY488492, *L. setiferus* AF430078, *F. paulensis* EF182747, *L. vannamei* AY488493, *L. vannamei* AF430073, *L. vannamei* AY486426, *L. vannamei* AF430072, *F. subtilis* EF450744, *L. schmitti* EF182748, *L. vannamei* AY488496, *L. vannamei* AY488495, *L. vannamei* AF430075, *L. vannamei* AF430076, *L. vannamei* AY488494, *F. brasiliensis* EF601055, *L. schmitti* EF601056, *F. chinensis* AY871268, *M. japonicus* AB121740, *P. monodon* FJ539174, *P.monodon* EF654659, *P. monodon* FJ539178, *P. monodon* EF654658, *P. monodon* GQ334395, *P. monodon* GQ334396, *P. monodon* FJ539175, *P. monodon* FJ539177, *F. chinensis* DQ097704, *F. chinensis* DQ097703, *P. camtschaticus* EU921643, *P. leniusculus* 1 EF523614, *H. americanus* EF193003, *H. gammarus* AJ786653, *S. paramamosain* EU161287, *H. araneus* EU921642, *H. araneus* EU921641). Values at the node indicate the percentage of times that the particular node occurred in 1000 trees generated by bootstrapping the original deduced protein sequences.**

➢ NUCLEOTIDE SEQUENCE (186 bp)

```
atgcgtctcgtgatctgcctggtcttcttggcctccttcgccctggtctg
ccaaggccacagggatacttatacttaccctttctctttatctagagcta
ctggacctctacctctttcacccactgtctgctcttcctcttcccttatt
atcattaatgacactgatgcttgtgcttgttgctga
```

➢ DEDUCED AMINOACID SEQUENCE:FRAME-1 (61 aminoacid)

```
MRLVICLVFLASFALVCQGHRDTYTYPFSLSRATGPLPLS
PTVCSSSSLIIINDTDACACC*
```

```
atgcgtctcgtgatctgcctggtcttcttggcctccttcgccctggtctgccaaggccac
 M  R  L  V  I  C  L  V  F  L  A  S  F  A  L  V  C  Q  G  H
agggatacttatacttaccctttctctttatctagagctactggacctctacctctttca
 R  D  T  Y  T  Y  P  F  S  L  S  R  A  T  G  P  L  P  L  S
cccactgtctgctcttcctcttcccttattatcattaatgacactgatgcttgtgcttgt
 P  T  V  C  S  S  S  S  L  I  I  I  N  D  T  D  A  C  A  C
tgctga
 C  *
```

**Fig.3.24. Nucleotide and amino acid sequences of Fi-penaeidin (HM243617) from the haemocyte of the Indian white shrimp, *F. indicus*. The underlined amino acid residues indicate a putative signal sequence. An asterisk is the stop codon.**

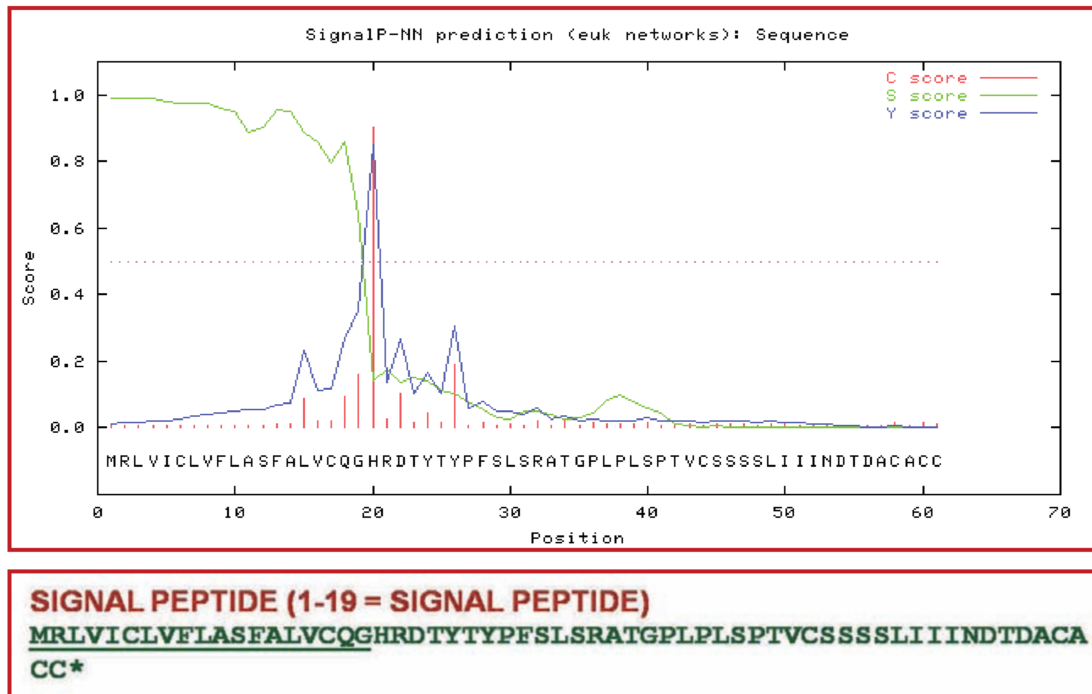



**Fig.3.25. Signal peptide analysis of Fi- penaeidin (HM243617) in *F. indicus* as predicted by the SignalP 3.0 server. The underlined amino acid residues indicate a putative signal sequence.**

**Fig.3.26. Schematic representation of matches map and list of matches obtained for Fi-penaeidin (HM243617) of *F. indicus* from the Motif Scan search**

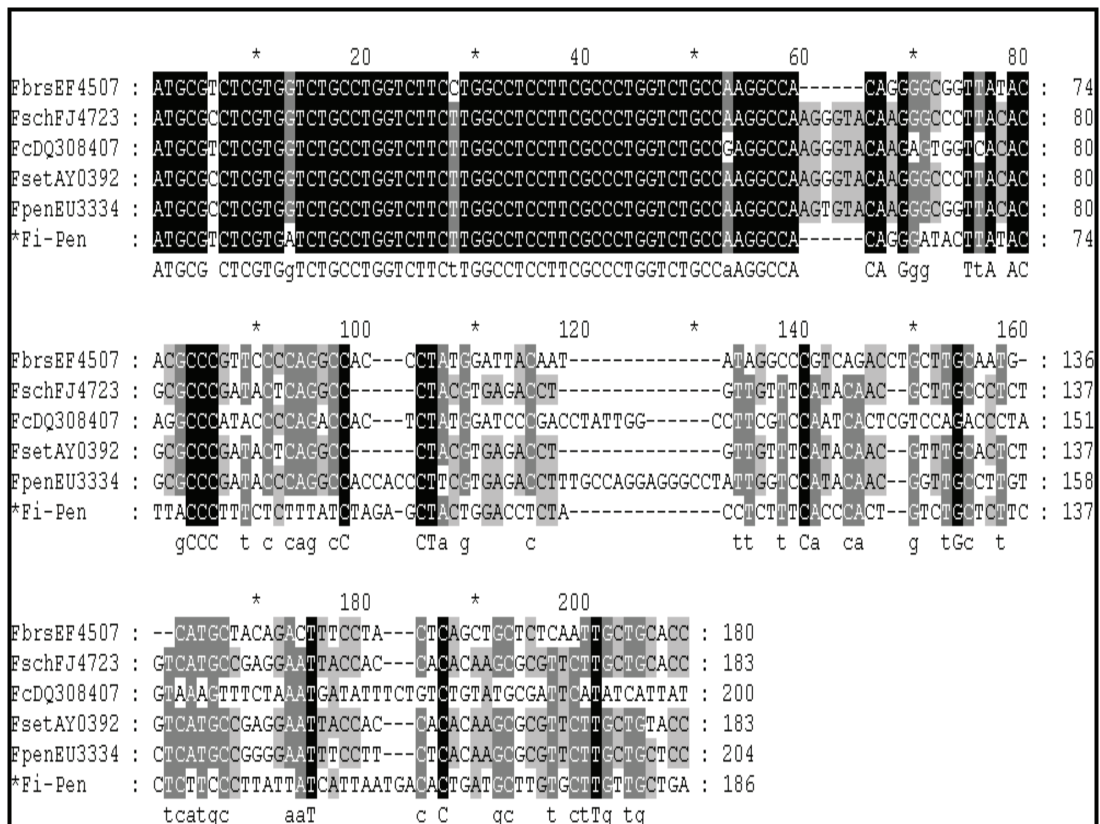



**Fig.3.27 Multiple alignment of nucleotide sequence of the *F. indicus* penaeidin-like antimicrobial peptide, Fi-penaeidin (HM243617) with other shrimp penaeidins (*F. brasiliensis* EF450745, *L. schmitti* FJ472344, *F. chinensis* DQ308407, *L. setiferus* AY039202, F. *penicillatus* EU333491) obtained using GeneDoc programme Version 2.7.0. Black and grey indicates conserved sequences.**

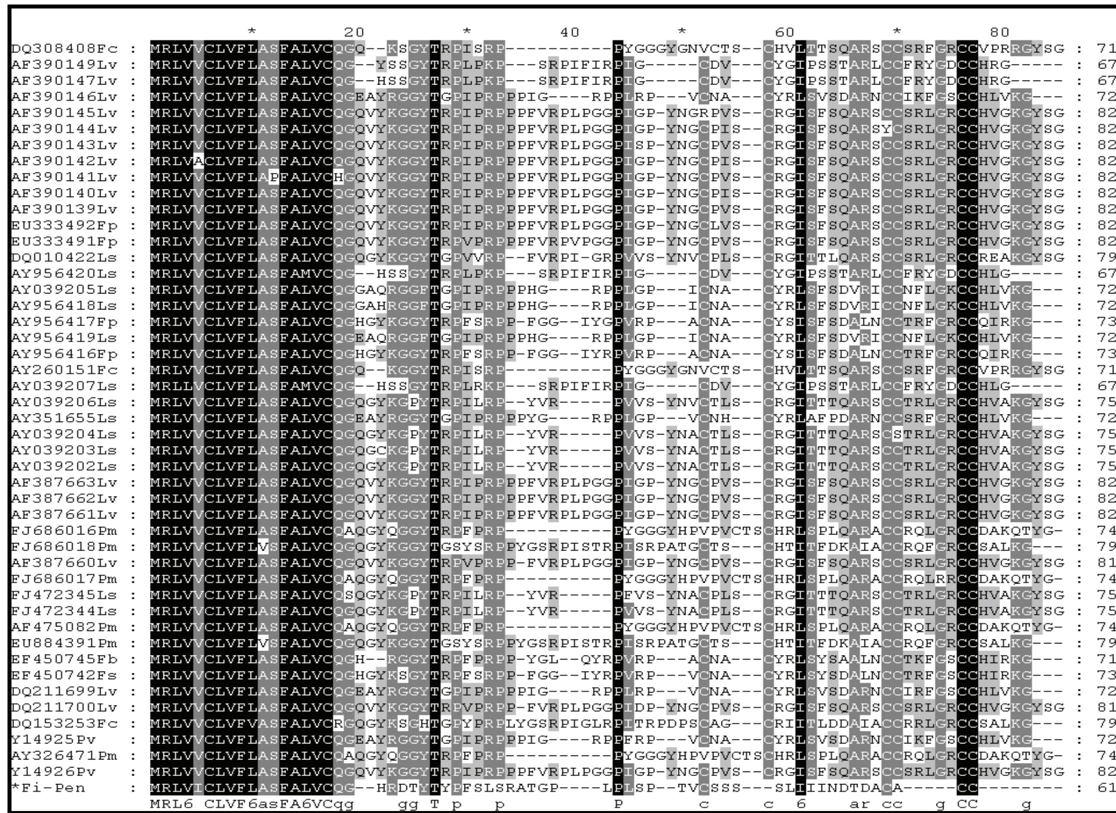


**Fig. 3.28. Multiple alignment of deduced amino acid sequence of the *F. indicus* penaeidin-like antimicrobial peptide, Fi-penaeidin (HM243617) with all known penaeidins (*L. schmitti* AY956418, *L. schmitti* AY956419, *L. setiferus* AY039205, *P. vannamei* Y14925, *L. vannamei* AF390146, *L. stylirostris* AY351655, *L. vannamei* DQ211699, *L. stylirostris* DQ010422, *L. stylirostris* AY351656, *F. chinensis* EU884391, *P. monodon* FJ686018, *P. monodon* DQ153253, *L. schmitti* AY956420, *L. setiferus* AY039207, *L. vannamei* AF390147, *L. vannamei* AF390149, *F. brasiliensis* EF450745, *P. monodon* FJ686017, *P. monodon* GQ334397, *P. monodon* FJ686016, *P. monodon* AF475082, *P. monodon* AY326471, *L. vannamei* DQ211700, *F. penicillatus* EU333491, *L. vannamei* AF387660, *L. vannamei* AF390145, *L. vannamei* AF387661, *L. vannamei* AF390140, *L. vannamei* AF390142, *F. penicillatus* EU333492, *P. vannamei* Y14926, *L. vannamei* AF387662, *L. vannamei* AF387663, *L. vannamei* AF390139, *L. vannamei* AF390143, *L. vannamei* AF390144, *L. vannamei* AF390141, *F. paulensis* AY956417, *F. paulensis* AY956416, *F. subtilis* EF450742, *F. chinensis* DQ308408, *F. chinensis* AY260151, *L. setiferus* AY039204, *L. setiferus* AY039203, *L. schmitti* FJ472344, *L. setiferus* AY039206, *L. setiferus* AY039202, *L. schmitti* FJ472345) obtained using GeneDoc programme Version 2.7.0. Black and grey indicates conserved sequences.**

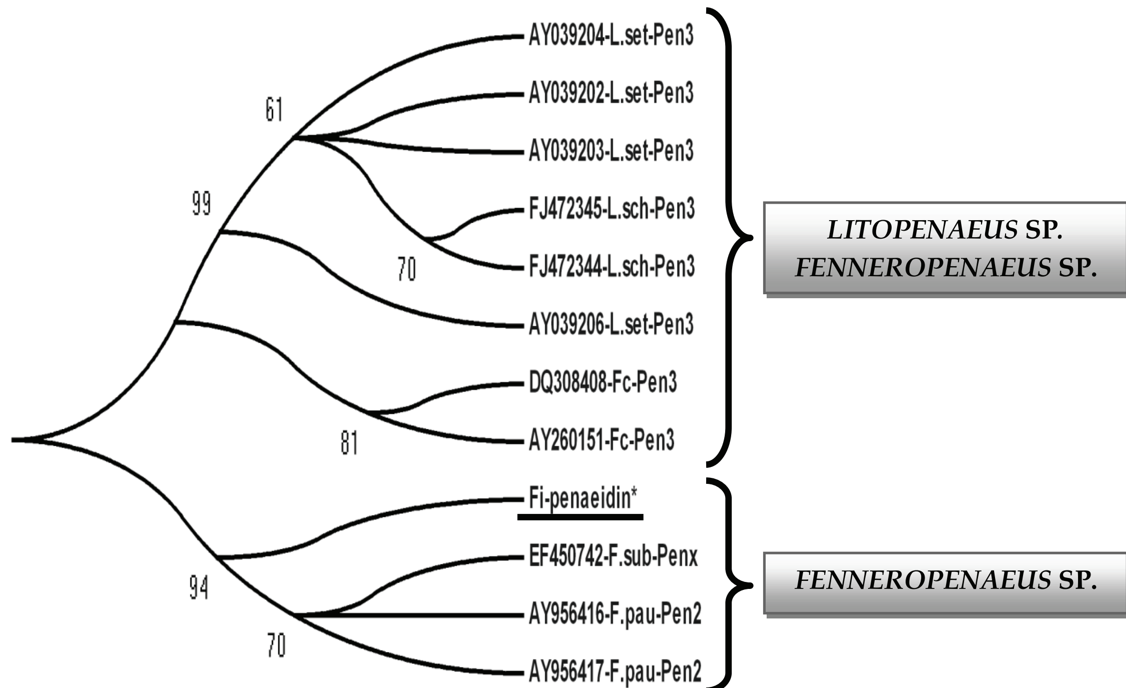



**Fig.3.29. Bootstrapped neighbor-joining tree obtained using MEGA version 4.0 illustrating relationships between the deduced amino acid sequence of the *F. indicus* penaeidin-like AMP, Fi-penaeidin (HM243617) with other penaeidins that showed maximum similarity during BLAST analysis (*L. setiferus* AY039204, *L. setiferus* AY039202, *L. setiferus* AY039203, *L. schmitti* FJ472345, *L. schmitti* FJ472344, *L. setiferus* AY039206, *F. chinensis* DQ308408, *F. chinensis* AY260151, *F. paulensis* AY956417, *F. paulensis* AY956416, *F. subtilis* EF450742). Values at the node indicate the percentage of times that the particular node occurred in 1000 trees generated by bootstrapping the original deduced protein sequences.**

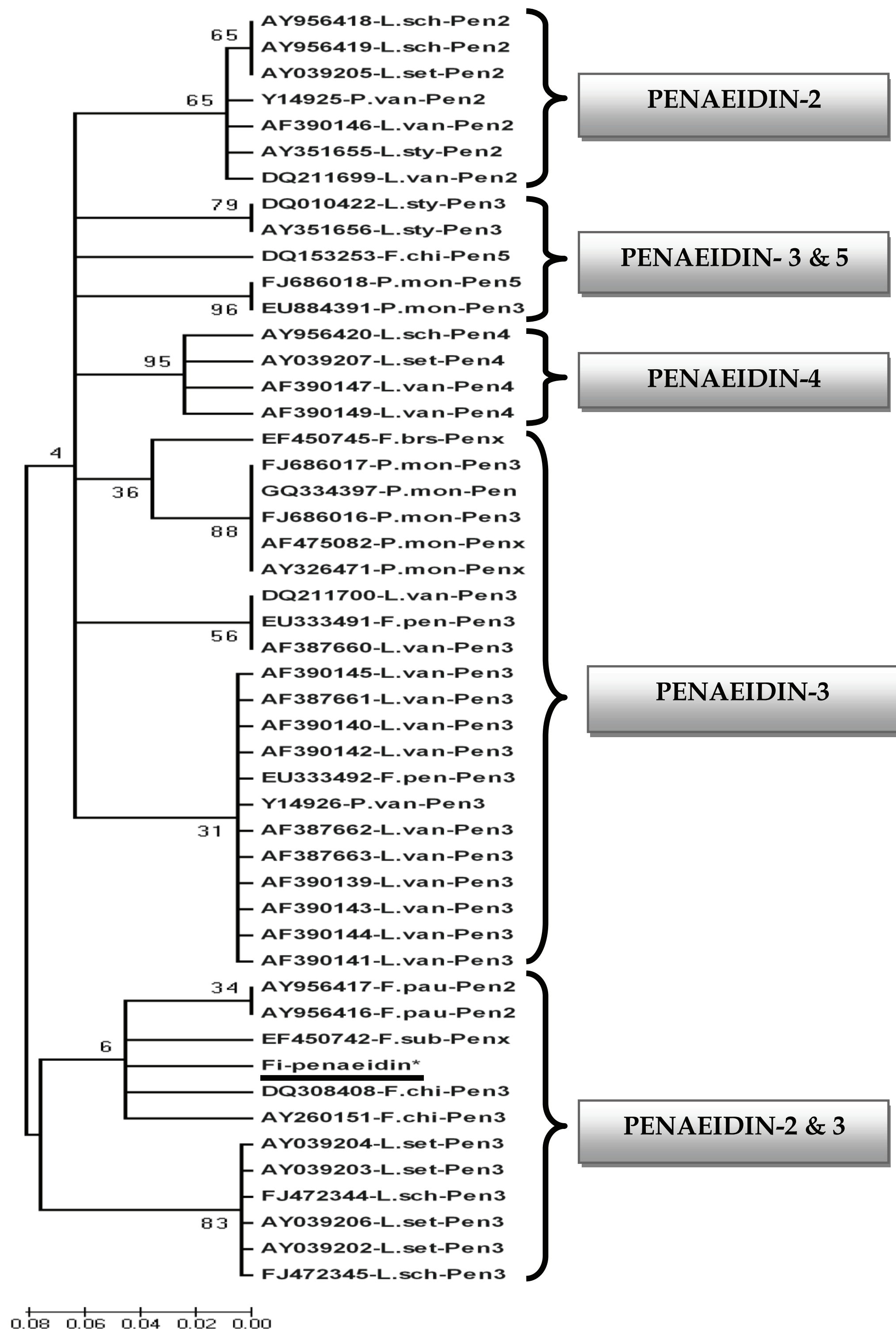



**Fig. 3.30. Bootstrapped neighbor-joining tree obtained using MEGA version 4.0 illustrating relationships between the deduced amino acid sequence of the *Fenneropenaeus indicus* penaeidin-like AMP, Fi-penaeidin (HM243617) with all known**

**penaeidins (*L. schmitti* AY956418, *L. schmitti* AY956419, *L. setiferus* AY039205, *P. vannamei* Y14925, *L. vannamei* AF390146, *L. stylirostris* AY351655, *L. vannamei* DQ211699, *L. stylirostris* DQ010422, *L. stylirostris* AY351656, *F. chinensis* EU884391, *P. monodon* FJ686018, *P. monodon* DQ153253, *L. schmitti* - 4 AY956420, *L. setiferus* AY039207, *L. vannamei* AF390147, *L. vannamei* AF390149, *F. brasiliensis* EF450745, *P. monodon* -3 FJ686017, *P. monodon* GQ334397, *P. monodon* -3 FJ686016, *P. monodon* AF475082, *P. monodon* AY326471, *L. vannamei* DQ211700, *F. penicillatus* EU333491, *L. vannamei* AF387660, *L. vannamei* AF390145, *L. vannamei* AF387661, *L. vannamei* AF390140, *L. vannamei* AF390142, *F. penicillatus* EU333492, *P. vannamei* Y14926, *L. vannamei* AF387662, *L. vannamei* AF387663, *L. vannamei* AF390139, *L. vannamei* AF390143, *L. vannamei* AF390144, *L. vannamei* AF390141, *F. paulensis* AY956417, *F. paulensis* AY956416, *F. subtilis* EF450742, *F. chinensis* DQ308408, *F. chinensis* AY260151, *L. setiferus* AY039204, *L. setiferus* AY039203, *L. schmitti* FJ472344, *L. setiferus* AY039206, *L. setiferus* AY039202, *L. schmitti* FJ472345). Values at the node indicate the percentage of times that the particular node occurred in 1000 trees generated by bootstrapping the original deduced protein sequences.**

➢ NUCLEOTIDE SEQUENCE (320 bp)

```
atacctggacggaagggcctgtcaagttttattttatatgttggcatgtggtatgtatgt
gcacaaatccgtgtttatccgtcaatgtgggatgtatgtgcacagtccttcagtcactgg
ttgctcttcatgccgccttattaccttcaattacgctgttgcttgttgctggcggttagg
acgctgttgttctgcagagaggattatctggccgatggagagacaacgaaaccctggctt
tgcaacgtgttatttggactcatatatcaagagactgcaaccttgtttgaactgtatttt
ctcgttcaatttttttttttt
```

➢ DEDUCED AMINOACID SEQUENCE:FRAME-2 (106 aminoacid)

```
YLDGRACQVLFYMLACGMYVHKSVFIRQCGMYVHSPSVTGCSSC
RLITFNYAVACCWRLGRCCSAERIIWPMERQRNPGFATCYLDSY
IKRLQPCLNCIFSFNFFF
```

```
atacctggacggaagggcctgtcaagttttattttatatgttggcatgtggtatgtatgtg
 Y  L  D  G  R  A  C  Q  V  L  F  Y  M  L  A  C  G  M  Y  V
cacaaatccgtgtttatccgtcaatgtgggatgtatgtgcacagtccttcagtcactggt
 H  K  S  V  F  I  R  Q  C  G  M  Y  V  H  S  P  S  V  T  G
tgctcttcatgccgccttattaccttcaattacgctgttgcttgttgctggcggttagga
 C  S  S  C  R  L  I  T  F  N  Y  A  V  A  C  C  W  R  L  G
cgctgttgttctgcagagaggattatctggccgatggagagacaacgaaaccctggcttt
 R  C  C  S  A  E  R  I  I  W  P  M  E  R  Q  R  N  P  G  F
gcaacgtgttatttggactcatatatcaagagactgcaaccttgtttgaactgtattttc
 A  T  C  Y  L  D  S  Y  I  K  R  L  Q  P  C  L  N  C  I  F
tcgttcaatttttttttttt
 S  F  N  F  F  F
```

**Fig.3.31. Nucleotide and amino acid sequences of Penaeidin-5 (HM243616) from the haemocyte of the Indian white shrimp, *F. indicus*.**

**Fig.3.32. Schematic representation of matches map and list of matches obtained for Penaeidin-5 (HM243616) of *F. indicus* from the Motif Scan search**

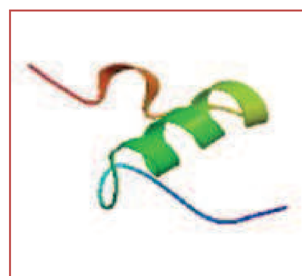


**Fig.3.33. Structural model of Penaeidin-5 (HM243616) in *F. indicus* created using SWISS-MODEL Server**

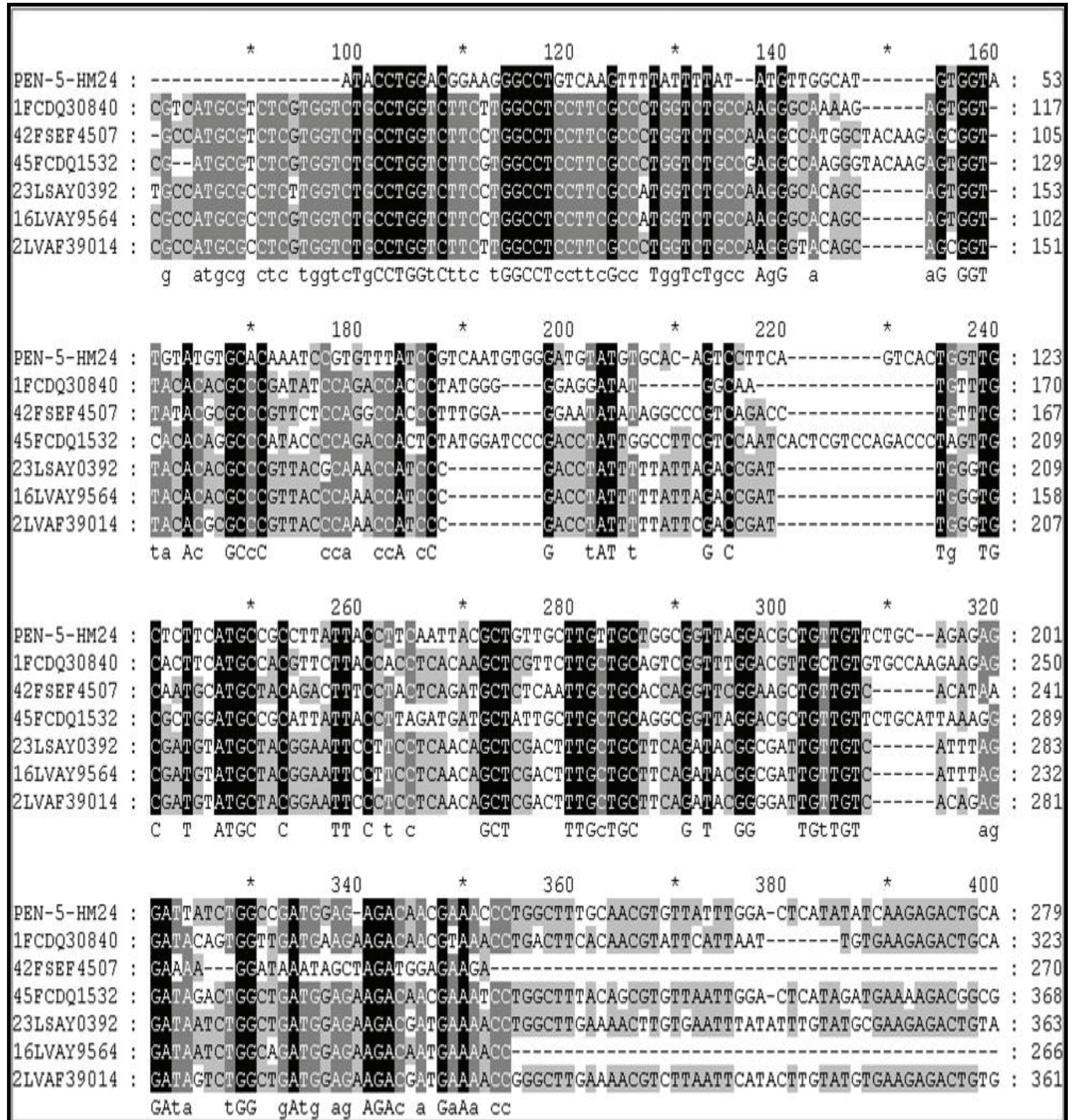


**Fig.3.34. Multiple alignment of deduced amino acid sequence of the *F. indicus* penaeidin-5 (HM243616) with other shrimp penaeidins (*F. chinenis* DQ308407, *P. monodon* FJ686018, *P. monodon* EU884391, *L. setiferus* AY039207, *L. vannamei* DQ206401, *F. brasiliensis* EF450745, *L. schmitti* FJ472344, *L. setiferus* AY039206, *F. paulensis* EU333492) obtained using GeneDoc programme Version 2.7.0. Black and grey indicates conserved sequences.**

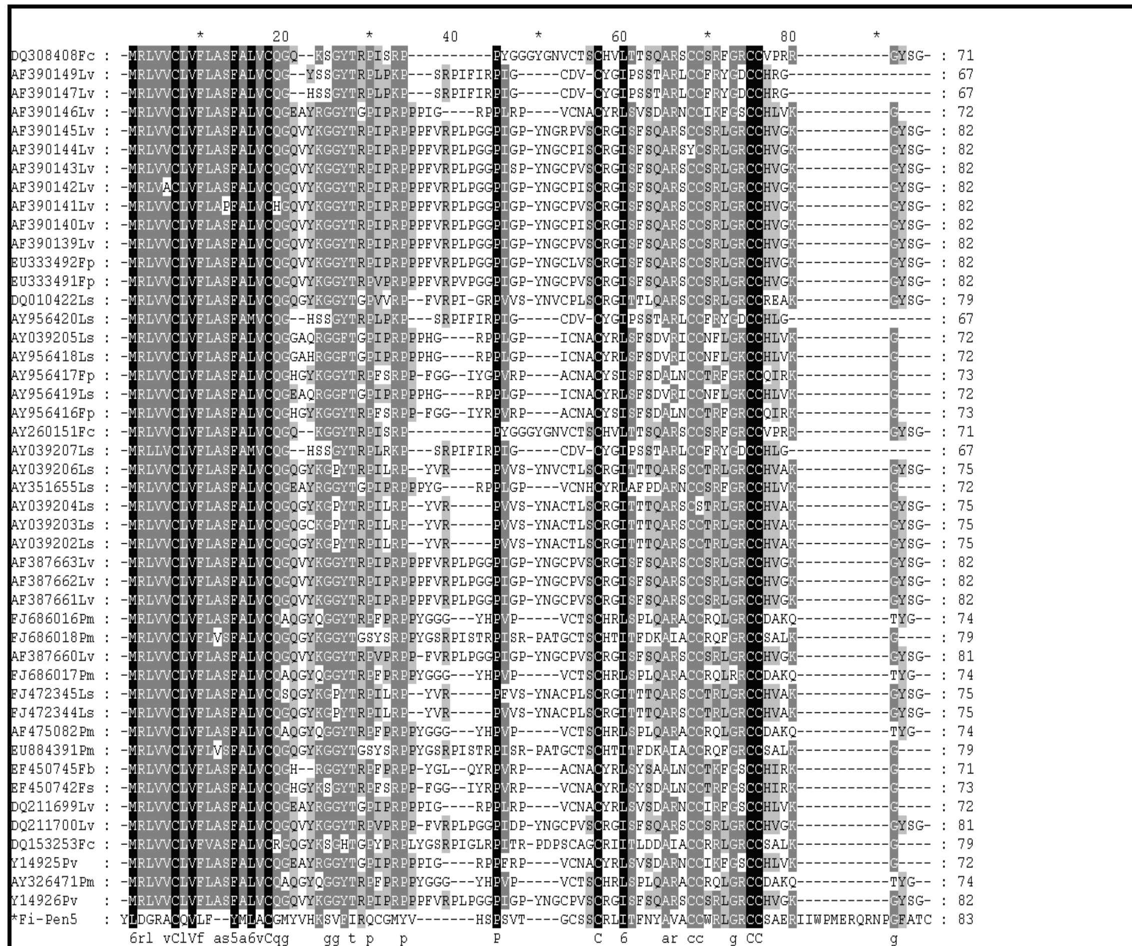


**Fig.3.35. Multiple alignment of nucleotide sequence of the *F. indicus* penaeidin-5 (HM243616) with other shrimp penaeidins (*F. chinenis* DQ308408, *L. vannamei* AF390149, *L. vannamei* AF390147, *L. vannamei* AF390146, *L. vannamei* AF390145, *L. vannamei* AF390144, *L. vannamei* AF390143, *L. vannamei* AF390142, *L. vannamei* AF390141, *L. vannamei* AF390140, *L. vannamei* AF390139, *F. paulensis* EU333492, *F. paulensis* EU333491, *L. stylirostris* DQ010422, *L. schmitti* AY956420, *L. setiferus* AY039205, *L. schmitti* AY956418, *F. paulensis* AY956417, *L. schmitti* AY956419, *F. paulensis* AY956416, *F. chinenis* AY260151, *L. setiferus* AY039207, *L. setiferus* AY039206, L. stylirostris AY351655, *L. setiferus* AY039204, *L. setiferus* AY039203, *L. setiferus* AY039202, *L. vannamei* AF387663, *L. vannamei* AF387662, *L. vannamei* AF387661, *P. monodon* FJ686016, *P. monodon* FJ686018, *L. vannamei* AF387660, *P. monodon* FJ686017, *L. schmitti* FJ472345, *L. schmitti* FJ472344, *P. monodon* AF475082, P. *monodon* EU884391, *F. brasiliensis* EF450745, *F. subtilis* EF450742, *L. vannamei* DQ211699, *L. vannamei* DQ211700, *F. chinenis* DQ153253, *P. vannamei* Y14925, *P. monodon* AY326471, *P. vannamei* Y14926) obtained using GeneDoc programme Version 2.7.0. Black and grey indicates conserved sequences.**

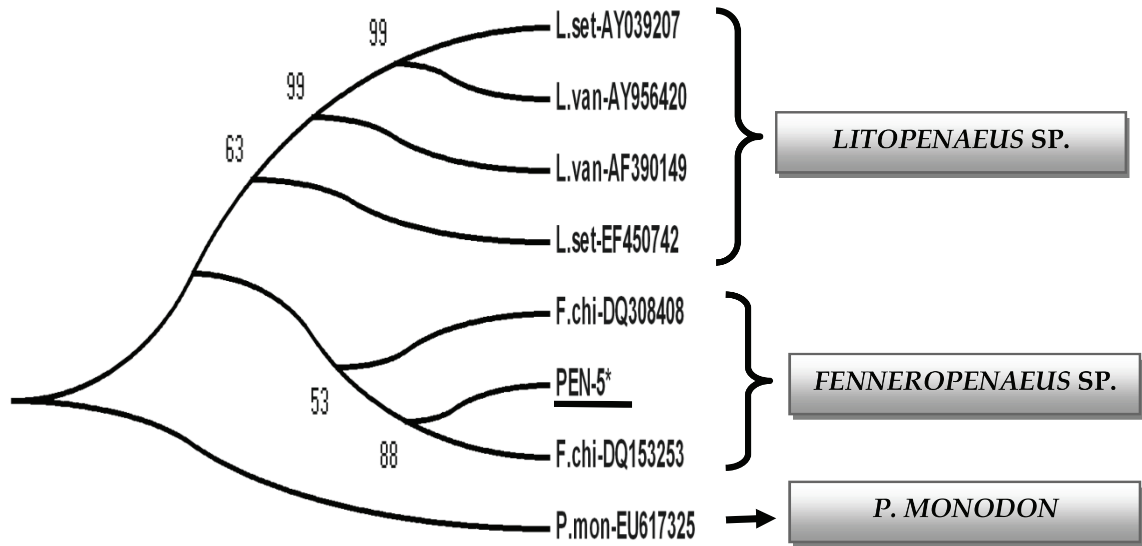



**Fig.3.36 Bootstrapped neighbor-joining tree obtained using MEGA version 4.0 illustrating relationships between the deduced amino acid sequence of the *Fenneropenaeus indicus* penaeidin-5 AMP, Fi-penaeidin (HM243616) with other penaeidins that showed maximum similarity during BLAST analysis (*L. setiferus* AY039207, L. *vannamei* AY956420, *L. vannamei* AF390149, *L. setiferus* EF450742, *F. chinensis* DQ308408, *F. chinensis* DQ153253, *P. monodon* EU617325) . Values at the node indicate the percentage of times that the particular node occurred in 1000 trees generated by bootstrapping the original deduced protein sequences.**

➢ NUCLEOTIDE SEQUENCE (547 bp)

tttcagtcggcttcgctggtgacgatgcaccacgagccgtgttcccctccatcgtcggccgacc
ccgtcatcagggtgtgatggtcggcatgggccagaaggactcgtacgtcggcgacgaggcccag
agcaagcgaggtatcctcaccctgaaataccccatcgagcacggcatcgtcaccaactgggacg
acatggagaagatctggcatcataccttctacaacgagctccgcgtggcccccgaggagcaccc
cgtcctgctgaccgaggctcccctcaaccccaaggccaaccgcgagaagatgacacagatcatg
ttcgagacgttcaacaccccccgccatgtacgtggccatccaggccgtgctgtccctgtacgcct
ccggccgtaccaccggcatcgtgctggactccggcgacggcgtgtcccacaccgtgcccatcta
cgagggctacgccctgccccacgccatcctgcgtctggacttggccggccgcgacctcacagac
tacctgatgaagatcctgaccgagcgtggctactc

➢ DEDUCED AMINOACID SEQUENCE:FRAME-3

SVGFAGDDAPRAVFPSIVGRPRHQGVMVGMGQKDSYVGDEAQSKRGILTLK
YPIEHGIVTNWDDMEKIWHHTFYNELRVAPEEHPVLLTEAPLNPKANREKM
QIMFETFNTPAMYVAIQAVLSLYASGRTTGIVLDSGDGVSHTVPIYEGYAL
PHAILRLDLAGRDLTDYLMKILTERGYS

**Fig.3.37 Nucleotide and amino acid sequences of β-actin (GU732815) from the haemocyte of the Indian white shrimp, *F. indicus***

➢ NUCLEOTIDE SEQUENCE (234 bp)

cactgaggtaagtctgtggagatgcaccacgaagctcttaccgaggctgt
ccctggtgacaacgttggcttcaacgtgaagaacgtgtccgtgaaggacc
tgaagcgtggcttcgtcgcttccgactcgaagaacgacccagccaaggaa
gctgctgacttcaccgcccaggtgatcgtcctcaaccaccctggccagat
ccaggctggctactcacctgtgcttgattgccac

➢ DEDUCED AMINOACID SEQUENCE:FRAME-1

MHHEALTEAVPGDNVGFNVKNVSVKDLKRGFVASDSKNDPAKEA
ADFTAQVIVLNHPGQIQAGYSPVLDCH

**Fig.3.38 Nucleotide and amino acid sequences of Elongation factor (GU732816) from the haemocyte of the Indian white shrimp, *F. indicus***

# CHAPTER-4

## Time-Course of Antimicrobial Peptide Gene Expression in Response to White Spot Syndrome Virus Challenge

## 4.1. Introduction

The aquaculture of penaeid shrimp has grown from its experimental beginnings roughly three decades ago into a major industry which, on a worldwide basis, provides not only employment to hundreds of thousands of skilled and unskilled workers, but also billions of U.S. dollars in revenue, and a high quality food product (FAO, 2006). Shrimp farms and hatcheries sprang up overnight and whole regions were often developed seemingly without any plan. Almost from the beginning disease was recognized as a biological threat to the shrimp culture industry, and some diseases caused serious economic losses (Lightner and Redman, 1998). Conventional methods for controlling aquatic animal pathogens, such as chemotherapy, appear less effective in managing newly emerging pathogens. Thus, molecular biotechnology has an increasingly important role for application in screening and detection of pathogens, elucidation of pathogenicity, development of effective control and preventive measures, and treatment of diseases. The improvement of diagnostic methodologies for detection and identification of pathogens using immunological and/or nucleic acid probes for prevention, management, and control of disease in cultured shrimp is one of the most important applications of biotechnology. Unlike traditional chemotherapeutic methods which have been plagued by development of pathogen resistance, these new technologies provide an opportunity for prophylactic intervention to minimize disease outbreaks (Subasinghe et al., 1998).

Cultured shrimps are often affected by different pathogens, creating a serious economic problem for shrimp farming in many parts of the world (Flegel, 1997). The diseases of cultured penaeid shrimp include syndromes

with infectious and noninfectious etiologies. Included among the infectious diseases of economic importance to cultured shrimps are those with viral, rickettsial, bacterial, fungal, protistan and metazoan etiologies (Lightner, 1988, 1993, 1996; Brock and Lightner, 1990; Fulks and Main, 1992). A number of noninfectious diseases are also of importance to the industry, and included among these are diseases due to environmental extremes, nutritional imbalances, toxicants, and genetic factors (Lightner, 1988, 1996; Brock and Lightner, 1990).

White spot syndrome virus (WSSV) is a major pathogen causing up to 100% mortality within 7-10 days in commercial shrimp farms, resulting in large economic losses to the shrimp farming industry (Lightner, 1996; Yang et al., 2001). A recent major outbreak of WSSV infection in China, Japan, Taiwan, Bangladesh, Thailand, India (Cen, 1998; Zhan and Wang, 1998; Pongmaneerat et al., 2001; Otta et al., 2003), resulted in a high reduction in shrimp production, has raised major concerns in aquaculture around the world. WSSV is an enveloped, double-stranded DNA (dsDNA) virus recently assigned to its own new genus, *Whispovirus*, and family, *Nimaviridae* (Mayo, 2002a, 2002b). The WSS virion is a non-occluded ellipsoid or bacilliform shaped enveloped particle about 275 nm in length and 120 nm in width, with a distinctive tail-like appendage at one end, and can be found throughout the body of infected shrimp. White spot syndrome virus has a large circular double-stranded DNA genome ranging from 292 kbp to 307 kbp in size covering approximately 185 open reading frames (ORFs) (van Hulten et al., 2001; Yang et al., 2001; Chen et al., 2002a). It is ovoid to bacilliform in shape with a tail like extension at one end (van Hulten et al., 2001; Yang et al., 2001) (Fig. 4.1A). The virions are 70-138 nm x 240-340 nm in diameter. It contains a rod shaped nucleocapsid of 70-90 x 200-350 nm (Wang et al., 2000). The virions contain one nucleocapsid with a typical striated

appearance and have major and at least other 50 structural proteins (van Hulten et al., 2000a, 2000b, 2002; Huang et al., 2002; Liu et al., 2006).

The complete viral genome has been known and available since 2001 (van Hulten et al., 2001; Yang et al., 2001). Viral proteins have been characterized and, in some cases, their role in pathogenesis elucidated (van Hulten et al., 2000a, 2000b, 2001, 2002; Tsai et al., 2004; Witteveldt et al., 2005; Wu et al., 2005). Though the genome of WSSV has recently been sequenced (van Hulten et al., 2001; Yang et al., 2001), little is known about the molecular mechanisms underlying the WSSV life cycle and its modes of infectivity.

**Fig. 4.1A. White Spot Syndrome Virus**

White spot disease (WSD) caused by WSSV emerged in East Asia in 1992-93 (Takahashi et al., 1994; Chou et al., 1995) and is currently the most serious viral pathogen of shrimps worldwide. WSSV has a wide host range among decapod crustaceans (Lo et al., 1996; Flegel, 1997; Flegel and Alday-Sanz, 1998), and is potentially lethal to most of the commercially cultivated penaeid shrimp species (OIE, 2003). The presence of WSSV has been described in a wide range of captured and cultured crustaceans and other arthropods, including wild crab (*Calappa lophos*, *Portunus sanguinolentus*, *Charybidis* sp., *Helice tridens*), wild lobster (*Panulirus* sp.), palamonidae pest shrimp (*Exopalamon orientalis*), copepods, pupae of Ephydried insects, cray fish (*Orconectes punctimans, Procambus clarkia*) pest crab (*Sesarma pictum*), mud crab (*Scylla serrata*), krill (*Acetes* sp.) and many other marine crustaceans (Lo et al., 1996; Chang et al., 1998; Supamattaya et al., 1998; Hossain et al., 2001). Also, WSSV causes high mortality in many cultured shrimp species

including *Penaeus monodon*, *P. semisulcatus*, *P. vannamei, P. stylirostris, P. indicus, P. merguiensis, P. setiferus, Marsupenaeus japonicus*, *Fenneropenaeus chinensis*, *F. penicillatus*, *Metapenaeus ensis*, *Macrobrachium rosenbergii* and *Exopalaemon orientis* (Lightner, 1996; Lo et al., 1997).

WSSV infected cells have been detected in tissues of ectodermal and mesodermal origin in both naturally and experimentally infected shrimp. WSSV replication occurs in the nucleus of the cells (Wongteerasupaya et al., 1995; Wang et al., 1999). Qualitative (Chang et al., 1996; Lo et al., 1997) and quantitative pathogenic analyses (Tan et al., 2001; Durand and Lightner, 2002; Escobedo-Bonilla et al., 2007) have shown that the major target tissues for WSSV replication are gills, stomach cuticular epithelium, cuticular epithelium of the body wall, hematopoietic tissues, antennal glands and lymphoid organ. WSSV infections have been detected in haemolymph, gills, stomach and body cuticular epithelium, hematopoietic tissues, lymphoid organ, antennal glands, connective tissues, muscle tissues, hepatopancreas, heart, midgut, hindgut, nervous tissues, compound eyes, eye stalks, pleopods, pereiopods, testes and ovaries of naturally and experimentally infected shrimp (Wongteerasupaya et al., 1995; Chang et al., 1996; Lo et al., 1997; Yoganandhan et al., 2003b; Escobedo-Bonilla et al., 2007). Quantitative pathogenic analyses suggest that the major target tissues for replication are gills, stomach and body cuticular epithelium, hematopoietic tissues, lymphoid organ and antennal glands (Tan et al., 2001; Durand and Lightner, 2002; Escobedo-Bonilla et al., 2007). Major target tissues for WSSV replication in shrimp are shown in Figure 4.1B. Hepatopancreas and heart were infected only in the connective tissues (Chang et al., 1996; Lo et al., 1997). Infected cells could not be found in the midgut cecum (Escobedo-Bonilla et al., 2007). At a terminal stage of infection, the epithelial cells, hematopoietic tissues, tubules of antennal gland become degenerative and lysed (Chang et al., 1996; Lo et al., 1997). The route of WSSV entry and spreading mechanism among

the tissues has recently been shown by Escobedo-Bonilla et al. (2007). Gills and cuticular epithelium of foregut in *P. vannamei* are portals of entry after oral inoculation of WSSV. After primary replication in these tissues, the virus crosses the basal membrane and reaches the associated haemal sinuses. Through haemolymph circulation, the virus is spread to internal organs where it causes a new wave of infections.

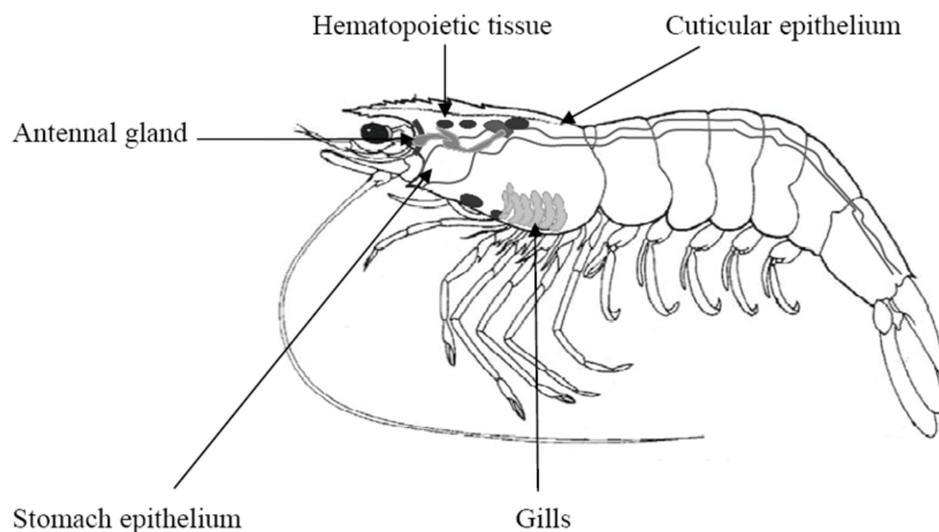



**Fig. 4.1B. Major target tissues of WSSV replication in shrimp (Adapted from Rahman, 2007)**

WSSV has been the subject of extensive and in-depth research by several researchers across the world. The various aspects of WSSV investigated include, WSSV isolation (Lightner, 1996; Flegel, 1997; van Hulten et al., 2001; Khadijah et al., 2003; Tsai et al., 2004; Witteveldt et al., 2004a, 2004b), virulence genes of WSSV (Lo et al., 1999; Yang et al., 2001; Marks et al., 2005), regulatory genes of WSSV (van Hulten et al., 2000b; Huang et al., 2002; Khadijah et al., 2003), structural genes of WSSV (van Hulten et al., 2000b, 2001; Yang et al., 2001; Xie et al., 2006), functional genes of WSSV (Tsai et al., 2000a, 2000b; Wang et al., 2004; Liu and Yang 2005), latency related gene of WSSV (Khadijah et al., 2003; Hossain et al., 2004),

Temporal regulatory genes of WSSV (Liu et al., 2005), epidemics of WSSV (Wu et al., 2001) various host species of WSSV (Jiravanichpaisal et al., 2001; Rodriguez et al., 2003) WSSV pathology (Wang et al., 1995, 2002; Lightner 1996), WSSV transmission (Chang et al., 1996; Lightner et al., 1997) diagnostic methods for WSSV (Liu et al., 2002; Okumura et al., 2004) and control of WSSV (Dupuy et al., 2004; Park et al., 2004; Witteveldt et al., 2004b).

Although considerable progress has been made in the characterization of WSSV, little work has been done on the host immune response particularly by haemocytes in response to the viral infection at the molecular level (Soderhall and Cerenius, 1992; Wongpanya et al., 2007). Also, little is known about the molecular mechanisms underlying the WSSV infection and host immune response. To develop effective infection controls, more information about the mode of infection and interaction between the virus and its host is needed. Knowledge of the immune system in shrimp is necessary to develop methods to control and minimize the loss of production due to infectious diseases. Larger numbers of immune-related genes need to be identified and functionally characterized to better understand shrimp immunity.

### *4.1.1. Antiviral immune response*

The molecular mechanisms that underlie the crustacean antiviral immune responses are still unknown and only in the early stages of investigation. Recently, studies using different techniques have been carried out on host-WSSV interactions in crustaceans (Lan et al., 2006; Liu et al., 2006; Wang et al., 2006a, 2007b; Zhao et al., 2007). It has been reported that virus-inhibiting proteins could be produced and some genes were up-regulated upon viral infection in crustaceans (Pan et al,. 2000; Rojtinnakorn et al., 2002; Roux et al., 2002; Dhar et al., 2003; He et al., 2005; Pan et al., 2005). Genes induced by viral infections and genes whose expression is associated

with the ability of shrimp to survive from viral infections have been widely reported. Crayfish ALF was shown to inhibit WSSV replication both *in vivo* and *in vitro* (Liu et al., 2006). The mechanism of this inhibition still needs further investigations.

A Lipopolysaccharide and β-1, 3-glucan binding protein (LGBP) gene was up-regulated in WSSV infected shrimp suggesting that shrimp LGBP is an inducible acute-phase protein that may play a critical role in shrimp-WSSV interaction (Roux et al., 2002). *Pm*AV was found in virus resistant shrimp which has a C-type lectin-like domain (CTLD). Recombinant *Pm*AV protein displayed a strong antiviral activity in inhibiting virus-induced cytopathic effect in fish cells invitro. Further experiments showed that *Pm*AV did not bind to the WSSV implying that the antiviral mechanism of this protein was not due to inhibition of the attachment of virus to target host cell (Luo et al., 2003). A β-integrin was found to interact with a WSSV envelope protein VP187 containing the RGD motif. Soluble integrin, integrin-specific antibody and an RGD containing peptide could block the WSSV infection invivo and invitro. Silencing of β-integrin efficiently inhibited the virus infection. These data suggest that this β-integrin may function as a cellular receptor for WSSV infection (Li et al., 2007). A syntenin and its protein partner α-2-macroglobulin co-precipitated with each other and both of them were up-regulated in the acutephase of a WSSV infection (Tonganunt et al., 2005). A chitin-binding protein (*Pm*CBP) interacted with a WSSV067C protein and showed up-regulation at the late stage of WSSV infection (Chen et al. 2007). Actin microfilaments were shown to interact with VP26 in shrimp (Xie and Yang 2005). VP28 of WSSV was suggested to bind to shrimp cells as an attachment protein and could help the virus to enter into the cytoplasm (Yi et al., 2004). Moreover, haemocyanin, the respiratory protein of arthropods and molluscs, was found to delay the infection of WSSV invivo in *P. japonicus* (Lei et al., 2008). Another C-type-lectin (*Lv*LT) was decreased

initially in the first 2 h and then increased to a much higher level after 4 h challenge of WSSV in shrimp (Ma et al., 2007). Some other genes were only found to be up-regulated as a response to WSSV infection in the animals but no mechanistic studies were performed. These genes include a Ras-related nuclear protein (Ran protein) gene (Han and Zhang, 2007), a caspase-3 like gene (Wongprasert et al., 2007), calreticulin (Luana et al., 2007), a Rab GTPase gene (Wu and Zhang, 2007), manganese superoxide dismutase, *Fc*lectin (Liu et al., 2007), and a syntenin-like protein gene (Bangrak et al., 2002). These genes might also play certain roles in antiviral responses and more investigations are needed to elucidate the details.

Viral entry into host cells needs endocytosis machineries of the host. Importantly, *Pm*Rab7, a shrimp small GTPase protein binding directly to VP28 of WSSV, was suggested to be involved in WSSV infection (Sritunyalucksana et al., 2006). Silencing of *Pm*Rab7 dramatically inhibited WSSV-VP28 RNA and protein expression. Further, the silencing of *Pm*Rab7 also inhibited yellow head virus (YHV) replication in the YHV-infected shrimp suggesting that *Pm*Rab7 is a common cellular factor required for WSSV or YHV replication in shrimp (Ongvarrasopone et al., 2008). Besides, another RabGTPase from *P. japonicus* was reported to regulate shrimp haemocytic phagocytosis through a proposed protein complex consisting of the *Pj*Rab, β-actin, tropomyosin, and an envelope protein VP466 of WSSV, indicating a possible role of phagocytosis involved in viral invasion in shrimp (Wu et al., 2008).

Apoptosis has been observed in viral target organs of WSSV-infected shrimp. When a shrimp *M. japonicus Pj*Caspase gene was silenced, the WSSV-induced apoptosis was significantly inhibited which resulted in an increase of viral copies, indicating that apoptosis played a key role in antiviral processes of shrimp (Wang et al., 2008). Whereas, knocking down caspase-3 by RNAi reduces mortality in Pacific white shrimp challenged

with a low dose of WSSV but not a high-dose of WSSV, suggesting that apoptosis may increase rather than decrease mortality in WSSV-challenged shrimp (Rijiravanich et al., 2008). Apoptosis was considered as a defense responsible for eliminating the virus (Anggraeni and Owens, 2000), however, the importance of this process for the mortality still needs further investigation (Wongprasert et al., 2003).

RNAi has been proven to be a natural antiviral mechanism in plants (Lecellier and Voinnet, 2004), fruit flies (Galiana-Arnoux et al., 2006; Wang et al., 2006b; Zambon et al., 2006), mosquitoes (Keene et al., 2004) and nematodes (Lu et al., 2005; Wilkins et al., 2005). RNAi technique now is explored as an alternative and more specific approach to counteract virus infections in shrimps. Injection of dsRNA/small interference RNA (siRNA) specific to viral genes can block viral disease progression. This effect has been confirmed with different unrelated viruses. Viral replication was efficiently suppressed with injection of WSSV-specific dsRNA/siRNA in penaeid shrimp (Robalino et al., 2005; Kim et al., 2007; Xu et al., 2007). Lower YHV replication was observed in shrimp primary cell cultures by transfecting the cells with dsRNA targeted to the viral nonstructural genes (Tirasophon et al., 2005). Inhibition of YHV replication by cognate dsRNA significantly resulted in lower mortality in the black tiger shrimp (Yodmuang et al., 2006; Tirasophon et al., 2007). Meanwhile, recent studies revealed the existence of both innate (non-sequence specific) and RNAi related (sequence specific) antiviral phenomena in a crustacean model (Robalino et al., 2004, 2005, 2007b; Westenberg et al., 2005). However, the protection induced by dsRNA could be overwhelmed by a higher dose (8-fold) of infectious virus. The mechanism of this difference between higher and lower amount of infectious virus remains unknown. The protective efficiency for WSSV infection by specific dsRNAs varied between different viral genes targeted and no reason has been addressed for these differences

in this study. It remains unclear whether the antiviral protection by virus specific long dsRNA is the result of RNAi mechanism alone or the combination of innate immune activation and RNAi. Further, the readout from these studies was mainly based on the cumulative mortalities and no mechanism so far is available for these antiviral phenomena. Importantly, these studies suggest a possible evolutionary link (recognition of dsRNA) between innate antiviral immunity in invertebrates and vertebrates. It would be interesting to elucidate the possible role, potency and application of dsRNA in crustacean antiviral immunity in future.

Some researchers suggested other approaches to protect the animals from infection of WSSV. For instance, a number of WSSV envelope proteins, such as VP28, have been proposed to be involved in viral infectivity based on the ability of specific antibodies to reduce WSSV-induced mortality. When injected intramuscularly or administered orally with VP28, the shrimps obtained a higher and prolonged survival rates after WSSV challenge (Witteveldt et al., 2004a, 2004b). Different protein/DNA vaccinations against WSSV infection were reported for the protection of shrimp/crayfish (Jha et al., 2006; Li et al., 2006; Vaseeharan et al., 2006; Witteveldt et al., 2006; Rout et al., 2007; Kumar et al., 2008). Other antibodies against WSSV envelope proteins, such as VP68, VP281 and VP466 were also shown to reduce and delay the mortality of shrimp challenged with WSSV (Wu et al., 2005). However, further investigations are necessary for the availability of these antibodies against different viral proteins. Some other immune stimulants have also been described such as: glucans derived from yeast (Huang and Song, 1999; Chang et al., 2003), LPS from bacteria (Takahashi et al., 2000), inactivated viruses (Singh et al., 2005; Melena et al., 2006), and dsRNA (Robalino et al., 2004, 2005; Yodmuang et al., 2006; Sarathi et al., 2008). Among these immune stimulants, viral proteins and dsRNA might be of particular interest for further investigations, because as virus-associated

molecules they are likely to be the targets of immune recognition in the context of natural viral infections (Robalino et al., 2007). Taken together, all these studies may help us to understand the virus-host interactions, which may provide further promising therapeutic treatment and prevention of serious aquaculture viral diseases.

AMPs constitute a primitive immune defense mechanism and are found in a wide range of eukaryotic organisms, from humans to plants (Lehrer and Ganz, 1999). In penaeid shrimps, three main families of AMPs have been currently described and characterized from the haemocytes: ALFs, crustins and penaeidins (Destoumieux et al., 1999; Bartlett et al., 2002; Somboonwiwat et al., 2005). Though AMPs have been well studied in the context of antibacterial (Khoo et al., 1999; Relf et al., 1999; Bartlett et al., 2002; Haug et al., 2002; Chen et al., 2004a, 2004b; Hauton et al., 2006; Christie et al., 2007; Amparyup et al., 2008; Brockton et al., 2008; Charnley et al., 2008) and antifungal responses (Destoumieux et al., 2001; Cabral et al., 2003; Sonesson et al., 2007; de la Vega et al., 2008); there is little published data on the possible involvement of AMPs in antiviral defense.

The present study was undertaken to study the differential expression of AMP genes in the haemocytes of *P.monodon* in response to WSSV infection on a time-course basis employing semi-quantitative RT-PCR. AMP gene expression in *P. monodon* haemocytes isolated from healthy shrimps and from those challenged/infected with WSSV were analyzed. Also, three widely used control genes have been evaluated in the present study so as to find out the best control gene for gene expression studies in WSSV challenged shrimps. Expression profiles of eight WSSV genes were also analyzed.

# 4.2. Materials and Methods

## *4.2.1. Experimental animals and rearing conditions*

Healthy adult *P. monodon* (45-50 g) were collected from a local shrimp farm in Vypeen, Kochi. They were transferred to aquarium tanks of 500 l capacity and acclimatized for 2 weeks under laboratory conditions (Fig. 4.2). Shrimps were fed standard feed (Higashi, India) twice daily *ad libitum*. Constant aeration was provided in all tanks during the experiment and bioreactor was set for maintaining water quality. Physico-chemical parameters such as salinity, pH, alkalinity, ammonia, nitrite, nitrate, dissolved oxygen and temperature were monitored daily following standard procedures (APHA, 1995) and maintained at optimal levels as given in Table 4.1.

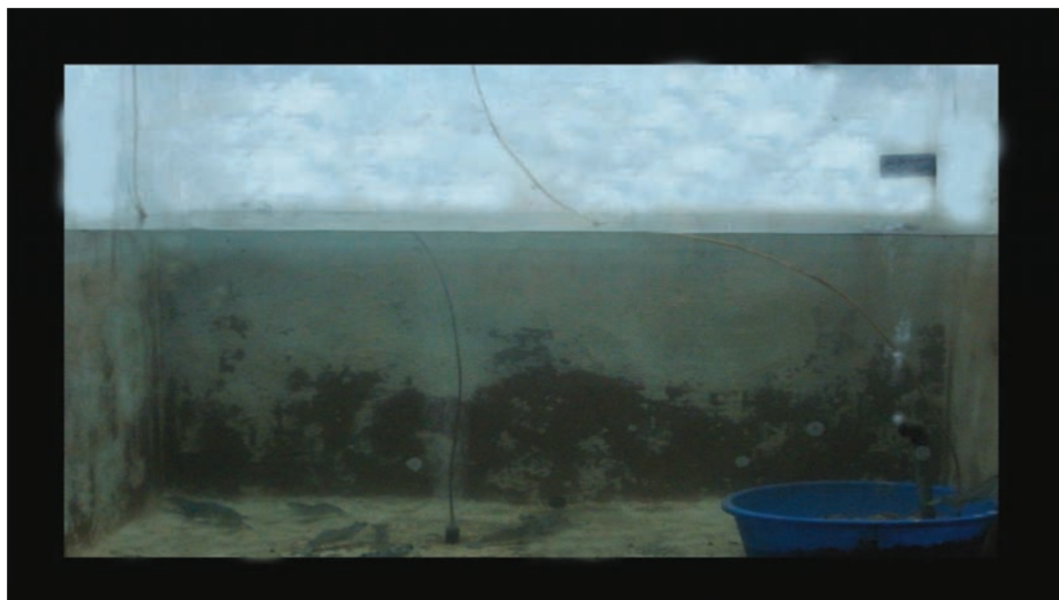


**Fig. 4.2. Experimental tank**

## *4.2.2. Sampling*

Six animals were sampled for the baseline data of AMP gene expression profile analysis. Thereafter the animals were maintained for a further period of four weeks (28 days) and again the animals were sampled

for understanding gene expression profile in healthy animals (Pre-challenge control).

### *4.2.3. WSSV challenge*

On the 29th day all the animals were challenged with WSSV by feeding WSSV infected frozen tissue at a rate of 1g/animal. Thereafter they were maintained on standard feed and sampling was performed on a time-course basis. Sampling was performed at one day interval for 10 days post-challenge WSSV. Six animals were sampled at every interval and only those in the intermoult stage were taken for analysis; since, immune states of penaeid shrimps are known to show variation in relation to the moult cycle (Hose et al., 1992, Liu et al., 2004). Mortality by WSSV infection was confirmed by checking the characteristic white spots on the carapace of infected shrimps.

### *4.2.4. Haemolymph collection*

Haemolymph was collected from the rostral sinus as described in section 2.2.3.

### *4.2.5. Total RNA isolation and reverse transcription*

Total RNA isolation and first strand cDNA synthesis were performed as described in sections 2.2.4, 2.2.5 and 2.2.6.

### *4.2.6. Semi-quantitative RT-PCR analysis of target gene expression*

Time-course analysis of target gene expression was determined by semi-quantitative RT-PCR analysis using 18S rRNA, β-actin and ELF as the internal control (Marone et al., 2003). cDNA was diluted 5 times and amplified using Taq polymerase. PCR amplification of 1µl of the diluted cDNA was performed in a 25 µl reaction volume containing 1x standard Taq buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3), 200 µM dNTPs, 0.4 µM each primer and 1U Taq DNA polymerase (New England Biolabs, USA).

Amplification was performed using AMP gene primers viz. ALF, crustin-1, crustin-2, crustin-3, penaeidin-3 and penaeidin-5 primers (Table

4.2). Control genes viz. 18S rRNA, β-actin and ELF were also amplified (Table 4.2). Amplification of eight WSSV genes viz. DNA polymerase, endonuclease, immediate early gene, latency related gene, protein kinase, ribonucleotide reductase, thymidine kinase and VP28 were also analyzed (Table 4.2). The thermal profile used was an initial denaturation at 94 $^0$C for 2 min followed by 27-30 cycles of denaturation at 94 $^0$C for 15 sec, extension at 68 $^0$C for 30 sec and a final extension at 68 $^0$C for 10 min for the target genes. Annealing temperature, final $MgCl_2$ concentration and number of PCR cycles varied for the different target genes as given in Table 4.2. The cycling number of the PCR had been optimized when the target genes and housekeeping genes were amplified at logarithmic phase. The PCR reaction of each sample was carried out in triplicate. PCR product was analyzed by electrophoresis in 1.5% agarose gel in TBE buffer, stained with ethidium bromide and visualized under UV light. The intensity of the gel bands were measured using Image J analysis software.

## 4.3. Results

### *4.3.1. Expression profile of control genes in the haemocytes of P. monodon in response to WSSV challenge*

Expression profile of control genes viz. 18S rRNA, β-actin and ELF were found to vary for the various experimental samples analyzed. Among the three control genes analyzed, the expression profile of β-actin was found to vary the most and ELF was found to be the most stable (Fig. 4.3. to 4.5.). Expression profile of 18S rRNA was found to vary during the experimental period both during pre- and post-challenge (Fig.4.3). β-actin also showed variation in the expression profile during the course of the experiment (Fig.4.4). Expression profile of ELF was found to be the best among the three control genes studied. Though very slight variations could be noticed, the overall profile of expression was found to stable in case of ELF (Fig. 4.5).

### *4.3.2. Expression profile of AMP genes in the haemocytes of P. monodon in response to WSSV challenge*

The AMP gene (ALF, crustin-1, crustin-2, crustin-3, penaeidin-3 and penaeidin-5) expression was found to be different in *P. monodon* pre- and post-challenge WSSV. Also, differential expression could be noticed for the various AMP genes studied (Fig.4.6 to 4.11).

#### *Expression profile of ALF gene*

ALF gene revealed differential expression during WSSV challenge. ALF was found to be up-regulated from early (day 1) to late period (day 10) of WSSV challenge. ALF was found to be up-regulated during both early and late hours of WSSV (Fig.4.6).

#### *Expression profile of crustin-1 gene*

Not much variation in the expression profile of crustin-1 could be detected in the haemocytes during initial hours of challenge; except for a very slight increase on PCD 3. Though in between a variation in gene expression could be noticed; the crustin-1 gene was found to down-regulate considerably during late hours of WSSV infection i.e. from post-challenge day 4 onwards (Fig.4.7).

#### *Expression profile of crustin-2 gene*

In case of crustin-2 gene also not much variation in the expression profile could be detected during initial hours of WSSV challenge. However, crustin-1 was found to down-regulate considerably during late hours of WSSV infection i.e. from post-challenge day 4 onwards (Fig.4.8).

#### *Expression profile of crustin-3 gene*

Expression profile of crustin-3 was worth noticeable. Except for some slight down-regulations of the gene during the 2$^{nd}$ and 5$^{th}$ PCD; the crustin-3 gene was found to up-regulate considerably from PCD 3 to PCD 10 especially during late hours of WSSV infection (Fig.4.9).

### *Expression profile of penaeidin-3 gene*

Expression profile of penaeidin-3 gene was worth noticeable. The gene was found to up-regulate considerably on WSSV challenge, especially during the 1st two days of WSSV challenge; thereafter showing a variation in gene expression pattern during the late hours of infection. During 10th PCD, the gene was found to be considerably down-regulated (Fig.4.10).

### *Expression profile of penaeidin-5 gene*

Not much variation in the expression profile of penaeidin-5 could be noticed during early hours of WSSV challenge, except for a very slight increase in the expression level during 1st to 3rd PCD. The gene was found to slightly down-regulate during the 5th PCD; came back to normal expression level by 6th PCD. However, during the late hours of WSSV infection, i.e. from 7th PCD onwards, penaeidin-5 was found to be down-regulated considerably (Fig.4.11).

### *4.3.3. Expression profile of WSSV genes in the haemocytes of P. monodon in response to WSSV challenge*

All WSSV gene transcripts could be detected within 48 hrs of WSSV challenge and revealed an almost similar pattern of expression for all the genes analyzed. None of the WSSV genes could be detected in the baseline and 28th day of samples confirming the animals used for the study to be free of WSSV infection (Fig. 4.12 to 4.19).

### *Expression profile of DNA polymerase gene*

Transcripts of DNA polymerase gene could be detected from PCD 1 onwards. However, only very low level of expression of the DNA polymerase gene could be detected until 4th PCD. After that, the gene transcripts were found to be increased considerably during the experimental period (Fig. 4.12).

### *Expression profile of endonuclease gene*

Endonuclease gene could also be detected from 1st PCD onwards. When compared to DNA polymerase the level of expression of endonuclease gene was found to be high during the 2nd to 4th PCD. After that, the gene transcripts were found to be increased considerably during the late hours of WSSV challenge (Fig 4.13).

### *Expression profile of immediate early gene*

Transcripts of immediate early gene could be detected only from post-challenge day 2 onwards. The level of gene expression was found to be high during the initial hours of WSSV challenge. From 5th PCD onwards the gene transcripts were found to increase considerably until the last day of experiment (Fig. 4.14).

### *Expression profile of latency related gene*

Expression of latency related gene could also be detected only from post-challenge day 2 onwards. The level of gene expression was found to be very high during the initial hours of WSSV challenge i.e. during 2nd, 3rd and 4th PCD. From 5th PCD onwards the gene transcripts were found to up-regulate considerably until 10th PCD (Fig. 4.15).

### *Expression profile of protein kinase gene*

Protein kinase gene could be detected from 1st PCD onwards. The level of expression was found to be very low during the 2nd PCD. However, the gene was found to be up-regulated during 2nd to 4th PCD. 5th PCD onwards, the gene was found to be up-regulated considerably until the last day of the experiment (Fig 4.16).

### *Expression profile of ribonucleotide reductase gene*

Transcripts of ribonucleotide reductase gene were present from PCD 2 onwards and only a low level of expression of the gene could be detected until 4th PCD. After that, from 5th PCD onwards, the ribonucleotide reductase

gene transcripts were found to increase considerably during the experimental period (Fig. 4.17).

***Expression profile of thymidine kinase gene***

Endonuclease gene could also be detected from 2nd PCD onwards. From 5th PCD onwards the gene transcripts were found to increase considerably during the late hours of WSSV challenge (Fig 4.18).

***Expression profile of VP28 gene***

Transcripts of VP28 gene could be detected from PCD 1 onwards. However, only very low level of expression of the DNA polymerase gene could be detected until 4th PCD. After that, the gene transcripts were found to be increased considerably during the experimental period (Fig. 4.19).

## 4.4. Discussion

WSSV is considered to be the most devastating viral disease affecting the shrimp industry around the world. This disease has caused huge production losses to most of the shrimp producing countries of the world (Nakano et al., 1994; Chou et al., 1995; Huang et al., 1995; Wang et al., 1995; Wongteerasupaya et al., 1995; Flegel, 1997; Lu et al., 1997; Lo et al., 2003). Although extensive research and considerable progress has been made in the characterization of WSSV, little work has been done on the host immune response at the molecular and cellular level particularly by haemocytes in response to the viral infection (Soderhall and Cerenius, 1992; Wongpanya et al., 2007). Also, little is known about the molecular mechanisms underlying the WSSV infection and host immune response. Therefore, there is an urgent need to understand the molecular basis of WSSV pathogenesis in shrimp, which may be helpful in developing strategies for management of the disease and for long-term sustainability of penaeid shrimp farming worldwide.

Crustacean response to viral infection may share some of the same pathways in the innate immune system already explored for defense against

bacterial and fungal infections. Indeed, this appears to be the case with respect to WSSV infection in shrimp, as there are published reports, where a gene normally associated with bacterial infection, appears to be up-regulated in response to the presence of WSSV (Roux et al., 2002; Bartlett et al., 2002; Rojtinnakorn et al., 2002; O'Leary and Gross, 2006). Innate immunity in multicellular organisms is the first line of defense against invading microbes such as bacteria, fungi and viruses. In crustaceans, only innate immunity without immunological memory exists (Lee and Soderhall, 2002). In the past decade , a number of proteins involved in innate immunity in crustaceans have been characterized at both the protein and molecular level such as members of the proPO activating system, AMPs and lectins (Gross et al., 2001; Cuthbertson et al., 2002; Rojtinnakorn et al., 2002; Roux et al., 2002; Cerenius and Soderhall, 2004). However these factors are aimed at bacteria, fungi or parasites rather than viruses and the host immune mechanisms against viruses are not well understood. Thus though the invertebrate immune system has been well studied in the context of antibacterial and antifungal responses; there is little published data on the possible involvement of AMPs in antiviral defense. Immune genes differentially expressed during WSSV infection are considered of special interest as they could represent a potential target for the development of antiviral strategies. Since little information is available on bio-defense genes of shrimp, especially those related to viruses, the present study was undertaken to study the differential expression of AMP genes in response to WSSV infection and thereby to analyze the antiviral property of AMPs.

Under natural and culturing conditions, WSSV infection is thought to occur mainly via ingestion of WSSV-infected tissues. Hence in the present study WSSV challenge was performed orally so as to mimic the natural mode of WSSV infection. Semi-quantitative RT-PCR was employed to examine changes in AMP gene expression in the haemocytes of the black

tiger shrimp *P. monodon* challenged with WSSV. Gene expression analysis using semi-quantitative RT-PCR is an easy, sensitive and reproducible method of detecting and quantifying an mRNA transcript. The method evaluates the product accumulation during the exponential phase of the reaction, has a large dynamic range and a high throughput capacity (Marone et al., 2001).

Expression profile of three widely used control genes viz. 18S rRNA, β-actin and ELF were analyzed in the present study to find out the best control gene for gene expression studies in shrimps. Internal reference genes such as β-actin, ELF and 18S rRNA are routinely used as internal controls in a variety of experimental conditions (Suzuki et al., 2000). However, in recent years it has been reported that the expression of many reference genes including β–actin and 18S rRNA vary depending on tissue, developmental stages and experimental conditions. Variations have been reported in quantitative studies related to normal and malignant tissues taken from patients undergoing surgery for breast or colorectal carcinoma (Tricarico et al., 2002), liver cirrhosis and hepatocellular carcinoma in human (Kim and Kim., 2003) and in different tissues of healthy and infectious salmon anemia virus infected Atlantic salmon, *Salmo salar* (Jorgensen et al., 2006; Ingerslev et al., 2006). The authors concluded that the use of a single housekeeping gene is inappropriate (Tricarico et al., 2002) and emphasized the need to search for stable reference genes for each experimental system.

However, in shrimps, only three studies have been so far addressed on the use of appropriate reference genes for expression studies in shrimps (Dhar et al., 2002; Sellars et al., 2007; Dhar et al., 2009). In one study, the expression of β-actin and ELF was compared while measuring the titer of RNA viruses, Taura syndrome virus (TSV) and yellow head virus (YHV) (Dhar et al., 2002). The ELF expression was found to be more reliable than β-actin and it was concluded that ELF is a better internal control while

measuring TSV and YHV load in shrimp tail muscle (Dhar et al., 2002). More recently, Sellars et al. (2007) examined the efficacy of using 18S rRNA and β-actin as reference genes when measuring the expression of target genes in a diverse set of kuruma shrimp samples representing embryonic, larval, post-larval, and gonad mRNAs. In this case, investigators found absolute quantification procedures to be superior to relative quantification when comparing transcript levels across tissues of different morphological developmental stages. Different housekeeping genes such as β-actin, ELF, 18S rRNA and ribosomal protein gene (R40s) have been used to quantify different immune genes in shrimp (Roux et al., 2002; Liu et al., 2006; Sritunyalucksana et al., 2006). Dhar et al. (2009) have studied the transcriptional stability of four internal control genes, β-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ELF, and 18S ribosomal RNA (18S rRNA) while measuring the mRNA expression of a gene encoding a pattern recognition protein, lipopolysaccharide and glucan binding protein (LGBP) gene, in healthy and WSSV infected shrimp (*P. stylirostris*) before and after (4, 8, 16 and 32 h) challenge using real-time RT-PCR. The results showed that among the four internal control genes examined, shrimp ELF gene was found to be the most suitable control gene while measuring the expression of moderate and highly expressed genes, whereas GAPDH was found to be a better control for the low expressed genes (Dhar et al., 2009). However, so far no time-course analysis has been carried out to compare the expression of different housekeeping genes in *P. monodon* while measuring the expression of immune genes in response to WSSV infection.

In order to identify suitable reference control genes for immune gene expression studies in *P. monodon* in response to WSSV challenge, we examined the transcriptional stabilities of three candidate reference genes, viz. β-actin, ELF and 18S rRNA in healthy and WSSV infected shrimp on a

time-course basis. Analysis of the results showed that expression profile varied greatly for the three control genes studied (Fig. 4.3 to 4.5). β-actin was found to vary greatly among the three control genes followed by 18S rRNA. However ELF showed almost similar level of expression and was found to vary least among the different experimental samples. The study points to the significance of ELF as a better control gene compared to β-actin and 18S rRNA especially for gene expression studies in shrimps. These results are in agreement with previous works by Dhar and co-workers (2002, 2009) on *P. stylirostris*, proving ELF as the best control gene for gene expression studies in shrimps.

Although AMPs have been well studied in the context of antibacterial and antifungal responses, there is little published data on the possible involvement of AMPs in viral infection, except for a few reports depicting the activity of AMPs against different viruses (Wachinger et al., 1998; Dupuy et al., 2004; Liu et al., 2006; Roch et al., 2007). Analysis of the results revealed that AMP genes were differentially expressed in the experimental samples (Fig. 4.6 to 4.11). Expression profile of AMP genes during various hours of WSSV challenge was unique and noteworthy. The AMP gene (crustin-1, crustin-2 and penaeidin-5) expression did not vary much during early hours of WSSV challenge, but showed a noticeable down-regulation during late hours of WSSV infection. The antibacterial activity of crustins and penaeidins have been studied and published by earlier researchers (Destoumieux et al., 1997, 2000b; Jimenez-Vega et al., 2004; Chiou et al., 2005; Cuthbertson et al., 2006; Hauton et al., 2006; Hu et al., 2006; Amparyup et al., 2008; Brockton et al., 2008; Supungul et al., 2008; Shockey et al., 2009). Difference in expression profiles of AMPs between bacterial and WSSV challenges may be due to different regulatory mechanisms responding to bacteria (gram-positive or -negative) or virus infection in shrimp, and as such is likely to be ecologically influenced and relevant (Amparyup et al., 2008).

Of particular interest here is the response shown by ALF, crustin-3 and penaeidins. The results indicated that ALF, crustin-3 and penaeidins respond to WSSV invasion. While both the penaeidins were found to be up-regulated during early hours of WSSV infection, ALF and crustin-3 were found to be up-regulated from early (day 1) to late period (day 10) of WSSV infection. The enhanced productions of these AMP genes during WSSV infection indicate their possible role in antiviral defense. ALF, initially characterized in horseshoe crab, is one of the proteins secreted in haemolymph upon infection. This protein was shown to bind bacterial LPS and to inhibit the LPS-mediated coagulation cascade of the haemolymph (Liu et al., 2005). The capacity of ALF-derived peptides to bind LPS and neutralize its toxic effects had been reported by several authors (Battafarano et al., 1995; Ried et al., 1996; Vallespi et al., 2003; Andra et al., 2004). The apparent ability of ALF to so strongly interfere with viral replication has been explored by Liu et al., (2006). Studies by Liu et al. (2006), on ALF, RNA interference (RNAi) experiments in whole animals and in cell cultures indicated that ALF can protect against WSSV infection, since knockdown of ALF by RNAi specifically resulted in higher rates of viral propagation. ALF has been reported to have a specific role in reducing viral replication (Liu et al., 2006). The role for ALF in viral propagation is intriguing, as its removal by RNAi results in a significant enhancement of viral replication. The ability of ALF to interfere with viral replication warrants further investigation. In the present study ALF was found to be up-regulated during the initial hours of WSSV challenge and even during the late hours of infection, when other AMP gene tend to down-regulate. A noticeable up-regulation could be observed for ALF. Indisputably this sheds light on the involvement of ALF in the host defense mechanisms especially against WSSV.

Crustins, on the other hand, are cationic, cysteine-rich AMP, of 7 to 14 KDa, with a characteristic WAP domain expressed by the circulating

haemocytes of crustaceans (Bartlett et al., 2002; Smith et al., 2008; Amparyup et al., 2008). Crustin types II and III have been isolated from shrimp (Smith et al., 2008). In shrimps, there are six possible crustin isoforms with antimicrobial activity against gram-positive and gram-negative bacteria, but the mechanisms are unknown (Vargas-Albores et al., 2004). Crustin-3 was found to belong to single WAP domain (SWD) crustin type III family. Amparyup et al. (2008) have studied the expression profile of SWD crustin in response to WSSV challenge via injection during the 6$^{th}$, 24$^{th}$ and 48$^{th}$ hour of post-challenge. It was found that crustin gene was up-regulated at 6$^{th}$ hour followed by a down-regulation at 24$^{th}$ hour and by 48$^{th}$ hour the expression level returned back to normal. The results of the present study are in agreement with these results for SWD crustin-3. A similar expression pattern has been observed in the double-WAP domain protein of the kuruma shrimp, *Marsupenaeus japonicus*, in which a rapid increase in the number of transcripts were detected during the early hours (6 h) of WSSV infection (Chen et al., 2008b). Despite the report of the sequences of several shrimp SWD proteins, no biological function has yet been ascribed to these proteins. The WAP domain has been described in proteins with diverse functions (Moreau et al., 2008) including antiproteinase and antimicrobial activities (Hagiwara et al., 2003). Our identification of crustin-3 over expression in WSSV -challenged *P. monodon* indicates that these molecules might have antiviral activity. Nevertheless, more functional and proteomic studies are needed to elucidate the bioactivity of these molecules.

Penaeidins were the first AMPs to be reported from penaeid shrimps (Destoumieux et al., 1997). Penaeidins are usually composed of a C-terminal domain containing six cysteine residues engaged in the formation of three intramolecular disulfide bridges (Destoumieux et al., 1997; Kang et al., 2004).The AMP penaeidin family is characterized by a proline-rich amino terminal region and a cysteine carboxyl-terminal domain. The diversity,

large distribution and abundance of these AMP within penaeid shrimp as well as their multiple and complementary properties, suggests that they have an essential role in the defense against a wide variety of pathogens (Destoumieux et al., 1997; O'Leary and Gross, 2006; Cuthbertson et al., 2008). However, antiviral activities of penaeidins have rarely been investigated. Each class of penaeidin possesses isoform diversity, which increases the structural complexity of these molecules and could account for differences in potency and specificity (O'Leary and Gross, 2006). Although penaeidin functions are not fully understood, these peptides bind to the cell membranes facilitating phagocytosis by opsonization (Cuthbertson et al., 2008). Penaeidins and crustins have been reported to be differentially expressed in the WSSV-infected pre-challenged organisms and are believed to directly involve in the immune response of invertebrates (Bartlett et al., 2002; Rojtinnakorn et al., 2002; O'Leary and Gross, 2006). Interestingly, the cysteine-rich AMP, mytilin has been shown to interact with WSSV, preventing viral replication and increasing palaemonid shrimp survival. This antiviral activity may result from its binding to the WSSV envelope, because contact of mytilin with viral particles for only one min reduced shrimp mortality up to 40% in 3.5 days (Dupuy et al., 2004). The identification of penaeidin-5 over expression in WSSV-challenged *P. monodon* indicates that these molecules might possess antiviral activity. However, the penaeidins got down-regulated during late hours of WSSV infection. Further investigation is needed to understand their role in the shrimp antiviral defense.

WSSV genes analyzed in the present study include both the functional and structural genes of WSSV. Of this DNA polymerase, endonuclease, protein kinase, ribonucleotide reductase and thymidine kinase belongs to functional genes. VP28 is a major envelope protein that has an important role in the infection process, and is required for the attachment of the virus to

shrimp cells and its complete internalization (Yi et al. 2004). This protein seems to participate in viral attachment to shrimp cells and plays a key role during the initial step of cell infection (van Hulten et al., 2001; Wu et al., 2005). Latency genes show maximal rate 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 are present. Viral immediate early genes are expressed immediately after primary infection, or as a result of the reactivation of a virus. This class of genes is defined experimentally by their ability to produce transcripts even in the presence of inhibitors of protein synthesis (Liu et al., 2005). The expression of viral immediate early genes depends on the host cell machinery and occurs independently of any viral de novo protein synthesis, which means that the immediate early genes are especially important in determining host range (Friesen, 1997). 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 (Liu et al., 2005).

In the present study, all WSSV genes were found to amplify within 48 hrs of WSSV challenge (Fig. 4.12 to 4.19). Pattern of expression was almost similar for all the eight WSSV genes studied, except that four of the WSSV genes viz. DNA polymerase, endonuclease, protein kinase and VP28 genes could be detected from 24 hours of WSSV challenge onwards; whereas other four WSSV genes viz. immediate early gene, latency related gene, ribonucleotide reductase and thymidine kinase were detected only from 48 hours of challenge. The results showed that only a small amount of transcripts of DNA polymerase, ribonucleotide reductase and VP28 could be detected during early hours of WSSV challenge and that the expression level remained unchanged until post-challenge day 4. Whereas, in case of other

WSSV genes viz. endonuclease, immediate early gene, latency related gene, protein kinase, and thymidine kinase higher levels of transcripts could be detected. However, in these samples also the expression levels remained more or less similar until post-challenge day 4. After that, the amount of all WSSV gene transcripts dramatically increased from 5$^{th}$ day post-challenge and continued to increase during the experimental period. The expression kinetics of the various WSSV gene transcripts were thus found to be quite similar in that all genes were found to be expressed during 1$^{st}$ or 2$^{nd}$ day post-challenge onwards and were found to be expressed at maximum level at the later stages of WSSV infection i.e. from 5$^{th}$ day of post-challenge onwards. This in turn shows that WSSV infection has reached its peak from 5$^{th}$ day onwards.

The present results are in agreement with previous works by Liu et al. (2005) in which, expression profile of DNA polymerase and VP28 was studied for 36 hours post-challenge WSSV. Lin et al. (2002) has studied the time-course of ribonucleotide reductase for 84 hrs of post-WSSV challenge. Lu et al. (2005) have studied the time-course analysis of immediate early gene for 48 hrs post-challenge WSSV injection. The results showed that the WSSV gene were transcribed as early as 2 hours post-challenge and after that, the amount transcripts continued to increase until the end of the analysis. Similar results have been reported by earlier researchers for VP35 gene (Chen et al., 2002a), DNA polymerase gene (Chen et al., 2002b), protein kinase gene (Liu et al., 2001), ribonucleotide reductase gene (Tsai et al., 2000a) and thymidine kinase gene (Tsai et al., 2000b). Viral replication could be the predominant process in the haemocytes of WSSV-infected shrimp, allowing them to overcome the expression of shrimp immune related genes (Chen et al., 2008b). In the present study also, viral replication was found to overcome immune response in the experimental animals during late hours of WSSV infection, except for ALF and crustin-3.

Taken together, the present results show that WSSV infection modulates AMP gene expression in haemocytes of WSSV challenged *P. monodon*. The over expression of AMP genes, such as ALF and crustin-3 in WSSV-challenged animals, strongly suggests that these genes could be postulated as potential candidates that are involved in anti-WSSV defense in shrimps by being over expressed on WSSV infection. This implies that these peptide molecules are involved in defense mechanisms in *P. monodon* to WSSV invasion. This basic knowledge will provide information on AMP genes involved in bio-defence of shrimps against virus invasion. Detailed study on these molecules will allow further exploration of their particular role in the anti-WSSV response and hopefully lead to a better understanding of the disease process and survival in these animals. The information may also be of great value for a novel prophylactic strategy in shrimp culture.

**Table 4.1. Rearing conditions and water quality parameters**

| | |
|---|---|
| **Tank capacity** | 500 l |
| **Stocking density** | 15 Nos. |
| **Feeding level** | 4-6 % of body weight |
| **Feeding frequency** | Twice daily |
| **Water temperature** | 24-27 $^{0}$C |
| **pH** | 7.5-8 |
| **Salinity** | 15-18 ‰ |
| **Ammonia** | 0.01-0.02 mg $l^{-1}$ |
| **Nitrite** | 0.00-0.01 mg $l^{-1}$ |
| **Nitrate** | Below detectable level |
| **Alkalinity** | 50-60 mg/l $CaCO_3$ |
| **Dissolved oxygen** | 6-7 mg $l^{-1}$ |

**Table 4.2. Primers used for the study**

| Target Gene | Primer Sequence (5' - 3') | Product Size (bp) | Annealing Temp. ($^0C$) | Final $MgCl_2$ Conc. (mM) | Reference |
|---|---|---|---|---|---|
| **CONTROL GENES** | | | | | |
| 18s rRNA | F- ttgtacgaggatcgagtgga<br>R- atgctttcgcagtaggtcgt | 350 bp | 52 | 2 | Bustin et al., 2002 |
| β-actin | F- cttgtggttgacaatggctccg<br>R-tggtgaaggagtagccacgctc | 520 bp | 60 | 1.5 | Supungul et al., 2004 |
| ELF | F- atggttgtcaactttgcccc<br>R- ttgacctccttgatcacacc | 600 bp | 60 | 1.5 | Loongyai et al., 2007 |
| **AMP GENES** | | | | | |
| ALF | F- caagggtgggaggctgtgg<br>R- tgagctgagccactggttgg | 300 bp | 62 | 1.5 | Tharantada et al., 2008 |
| Crustin-1 | F- cgcacagccgagagaaacactatcaagat<br>R- ggcctatccctcagaacccagcacg | 456 bp | 55 | 3.5 | Supungul et al., 2004 |
| Crustin-2 | F- tgttcccacgacttcaagtgtgc<br>R- caaagattcaactaaataaacag | 299 bp | 60 | 3.5 | Chen et al., 2004a |
| Crustin-3 | F- tccctggaggtcaattgagtg<br>R- agtcgaacatgcaggcctatcc | 233 bp | 60 | 1.5 | Jimenez-Vega et al., 2004 |
| Penaeidin-3 | F- aggatatcatccagttcctg<br>R- acctacatcctttccacaag | 240 bp | 60 | 1.5 | Jiravanichpaisal et al., 2007 |
| Penaeidin-5 | F- acctgaccctcacctgcagaggcc<br>R- ttcgttgtcttctccatcaacc | 300 bp | 60 | 1.5 | Chen et al., 2004b |
| **WSSV GENES** | | | | | |
| DNA polymerase | F- tgggaagaaagatgcgagag<br>R- rccctccgaacaacatctcag | 586 bp | 54 | 1.5 | Liu et al., 2005 |
| Endonuclease | F- tgacgaggaggattgtaaag<br>R- ttatggttctgtatttgagg | 408 bp | 50 | 1.5 | Liu et al., 2005 |
| Immediate Early Gene | F- gactctacaaatctctttgcca<br>R- ctacctttgcaccaattgctag | 502 bp | 54 | 1.5 | Liu et al., 2005 |
| Latency Related Gene | F- cttgtgggaaaagggtcctc<br>R- tcgtcaaggcttacgtgtc | 647 bp | 53 | 1.5 | Liu et al., 2005 |
| Protein Kinase | F- ggagggtggggaccaacggacaaaac<br>R- caaattgacagtagagaattttgcac | 512 bp | 55 | 1.5 | Liu et al., 2005 |
| Ribonucleotide Reductase | F- atctgctagtccctgcacac<br>R- aaagaggtggtgaaggcacg | 408 bp | 53 | 1.5 | Liu et al., 2005 |
| Thymidine Kinase | F- gagcagccatacgggtaaac<br>R- gcgagcgtctaccttaatcc | 412 bp | 54 | 1.5 | Liu et al., 2005 |
| VP 28 | F- ctgctgtgattgctgtattt<br>R- cagtgccagagtaggtgac | 555 bp | 54 | 1.5 | Liu et al., 2005 |

(A)

(B)

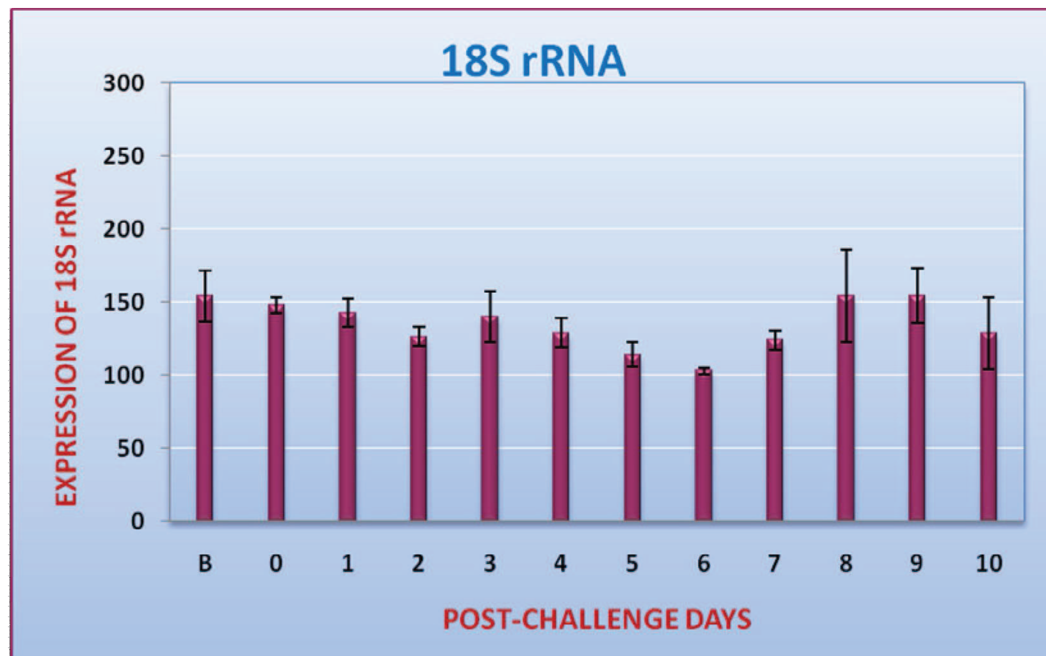


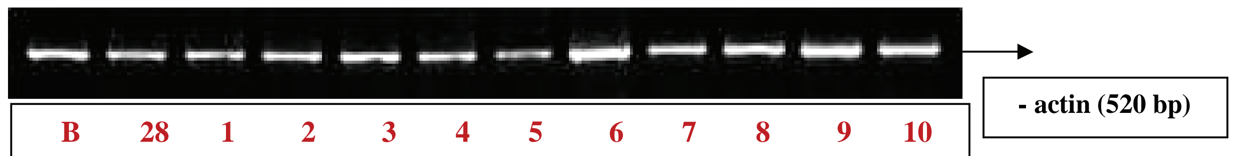

**Fig. 4.3. Time-course analysis of 18S rRNA (control gene) expression in the haemocytes of giant tiger shrimp, *P. monodon* in response to WSSV challenge**

**(A) Agarose gel electrophoretogram**

**(B) Graphical representation of the expression levels of 18S rRNA gene (x-axis = post-challenge days, y-axis = Expression levels of the 18S rRNA gene)**

*(B=Baseline, 28= 28th day pre-challenge WSSV, 1= post-challenge day 1, 2= post-challenge day 2, 3= post-challenge day 3, 4= post-challenge day 4, 5= post-challenge day 5, 6= post-challenge day 6, 7= post-challenge day7, 8= post-challenge day 8, 9= post-challenge day 9, 10= post-challenge day 10)*

(A)




(B)

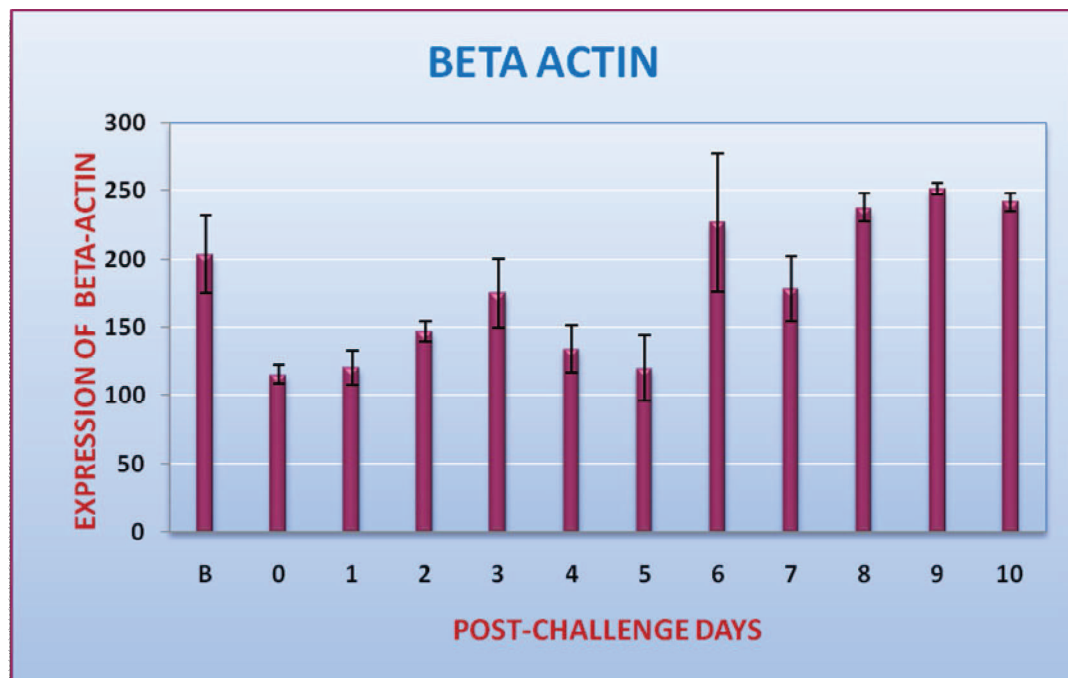



**Fig. 4.4. Time-course analysis of β- actin (control gene) expression in the haemocytes of giant tiger shrimp, *P. monodon* in response to WSSV challenge**

**(A) Agarose gel electrophoretogram**

**(B) Graphical representation of the expression levels of β- actin gene (x-axis = post-challenge days, y-axis = Expression levels of the β- actin gene)**

*(B=Baseline, 28= 28th day pre-challenge, 1= post-challenge day 1, 2= post-challenge day 2, 3= post-challenge day 3, 4= post-challenge day 4, 5= post-challenge day 5, 6= post-challenge day 6, 7= post-challenge day7, 8= post-challenge day 8, 9= post-challenge day 9, 10= post-challenge day 10)*

(A)

(B)

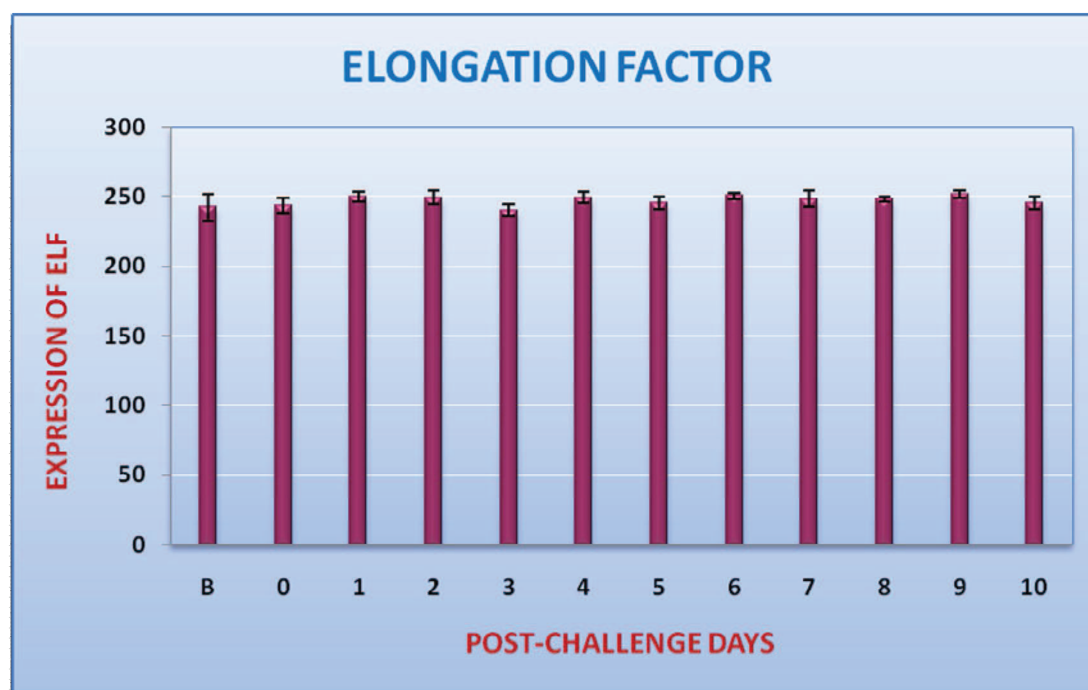



**Fig. 4.5. Time-course analysis of ELF (control gene) expression in the haemocytes of giant tiger shrimp, *P. monodon* in response to WSSV challenge**

**(A) Agarose gel electrophoretogram**

**(B) Graphical representation of the expression levels of ELF gene (x-axis = post-challenge days, y-axis = Expression levels of the ELF gene)**

*(B=Baseline, 28= 28th day pre-challenge, 1= post-challenge day 1, 2= post-challenge day 2, 3= post-challenge day 3, 4= post-challenge day 4, 5= post-challenge day 5, 6= post-challenge day 6, 7= post-challenge day7, 8= post-challenge day 8, 9= post-challenge day 9, 10= post-challenge day 10)*

(A)

(B)

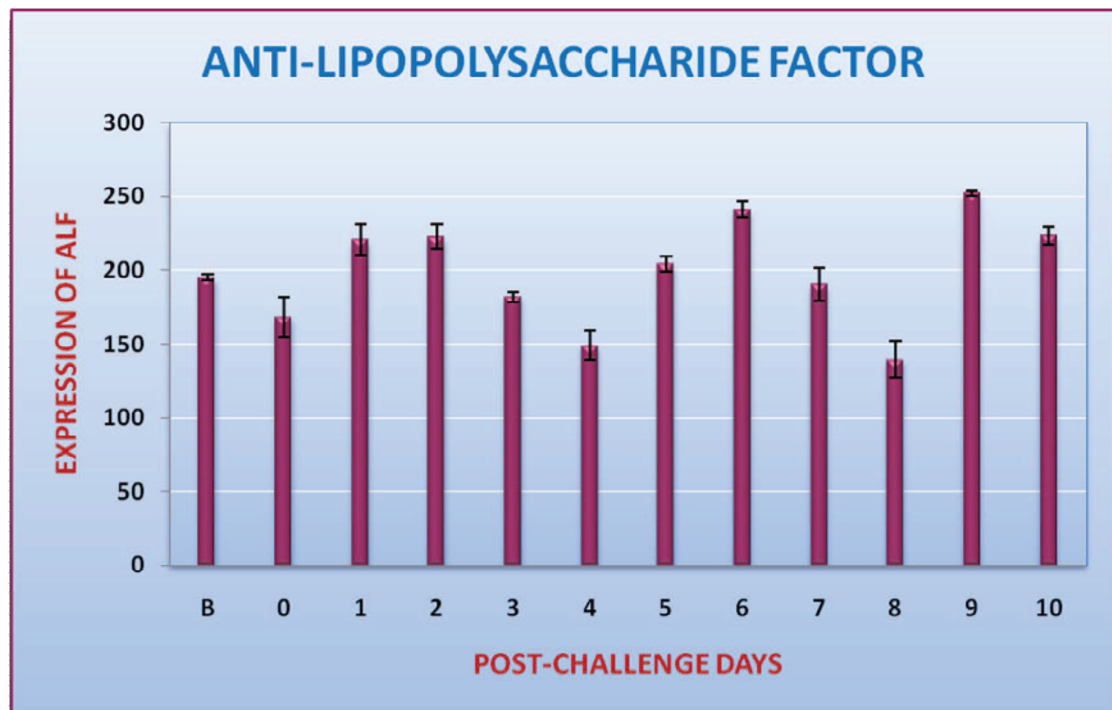



**Fig. 4.6. Time-course analysis of ALF (AMP gene) expression in the haemocytes of giant tiger shrimp, *P. monodon* in response to WSSV challenge**

**(A) Agarose gel electrophoretogram**

**(B) Graphical representation of the expression levels of ALF gene (x-axis = post-challenge days, y-axis = Expression levels of the ALF gene)**

*(B=Baseline, 28= 28th day pre-challenge WSSV, 1= post-challenge day 1, 2= post-challenge day 2, 3= post-challenge day 3, 4= post-challenge day 4, 5= post-challenge day 5, 6= post-challenge day 6, 7= post-challenge day7, 8= post-challenge day 8, 9= post-challenge day 9, 10= post-challenge day 10)*

(A)

(B)

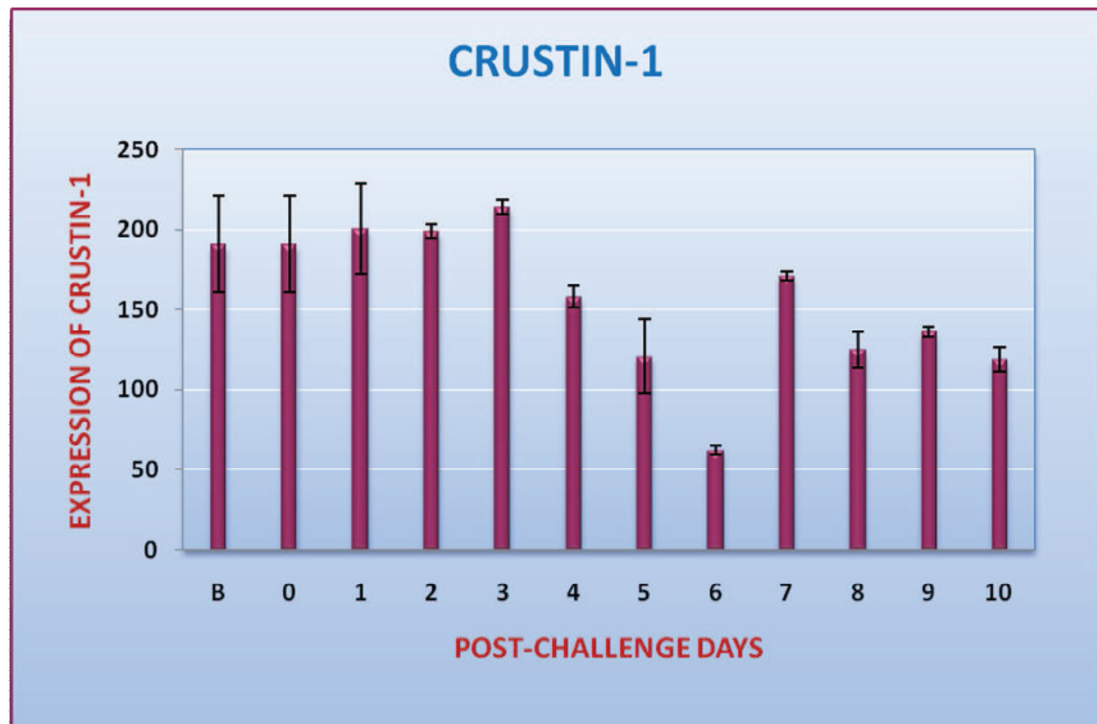


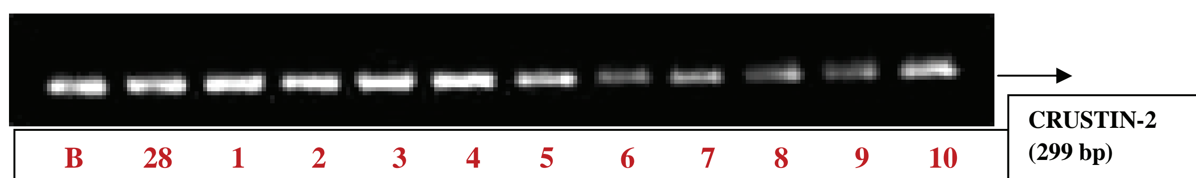

**Fig. 4.7. Time-course analysis of Crustin-1 (AMP gene) expression in the haemocytes of giant tiger shrimp, *P. monodon* in response to WSSV challenge**

**(A) Agarose gel electrophoretogram**

**(B) Graphical representation of the expression levels of Crustin-1 gene (x-axis = post-challenge days, y-axis = Expression levels of the Crustin-1 gene)**

*(B=Baseline, 28= 28th day pre-challenge WSSV, 1= post-challenge day 1, 2= post-challenge day 2, 3= post-challenge day 3, 4= post-challenge day 4, 5= post-challenge day 5, 6= post-challenge day 6, 7= post-challenge day7, 8= post-challenge day 8, 9= post-challenge day 9, 10= post-challenge day 10)*

(A)




(B)

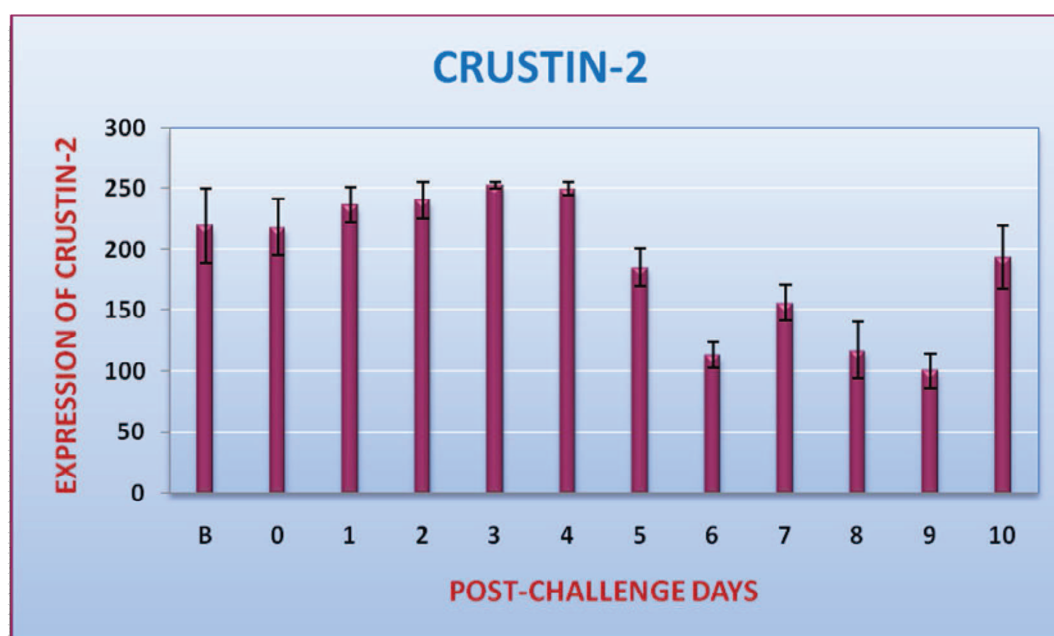



**Fig. 4.8. Time-course analysis of Crustin-2 (AMP gene) expression in the haemocytes of giant tiger shrimp, *P. monodon* in response to WSSV challenge**

**(A) Agarose gel electrophoretogram**

**(B) Graphical representation of the expression levels of Crustin-2 gene (x-axis = post-challenge days, y-axis = Expression levels of the Crustin-2 gene)**

*(B=Baseline, 28= 28th day pre-challenge WSSV, 1= post-challenge day 1, 2= post-challenge day 2, 3= post-challenge day 3, 4= post-challenge day 4, 5= post-challenge day 5, 6= post-challenge day 6, 7= post-challenge day7, 8= post-challenge day 8, 9= post-challenge day 9, 10= post-challenge day 10)*

(A)

(B)

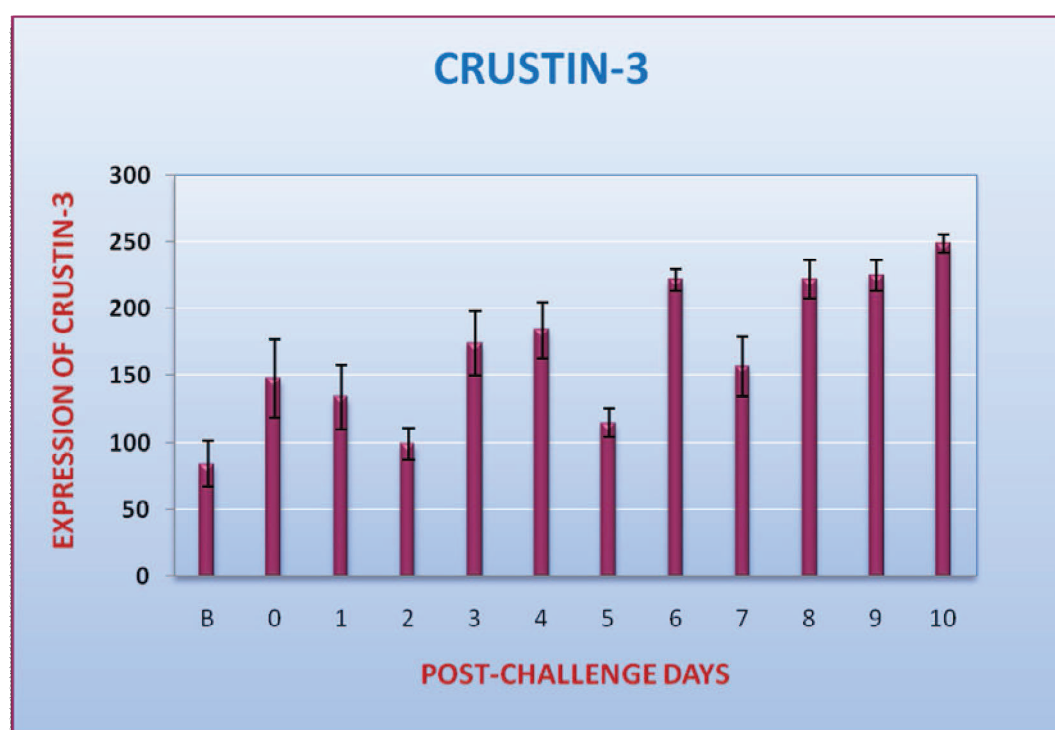


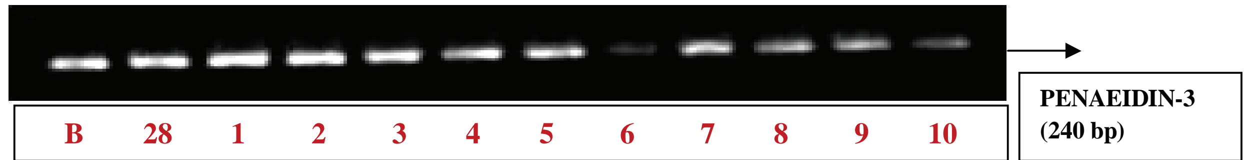

**Fig. 4.9. Time-course analysis of Crustin-3 (AMP gene) expression in the haemocytes of giant tiger shrimp, *P. monodon* in response to WSSV challenge**

**(A) Agarose gel electrophoretogram**

**(B) Graphical representation of the expression levels of Crustin-3 gene (x-axis = post-challenge days, y-axis = Expression levels of the Crustin-3 gene)**

*(B=Baseline, 28= 28th day pre-challenge WSSV, 1= post-challenge day 1, 2= post-challenge day 2, 3= post-challenge day 3, 4= post-challenge day 4, 5= post-challenge day 5, 6= post-challenge day 6, 7= post-challenge day7, 8= post-challenge day 8, 9= post-challenge day 9, 10= post-challenge day 10)*

(A)

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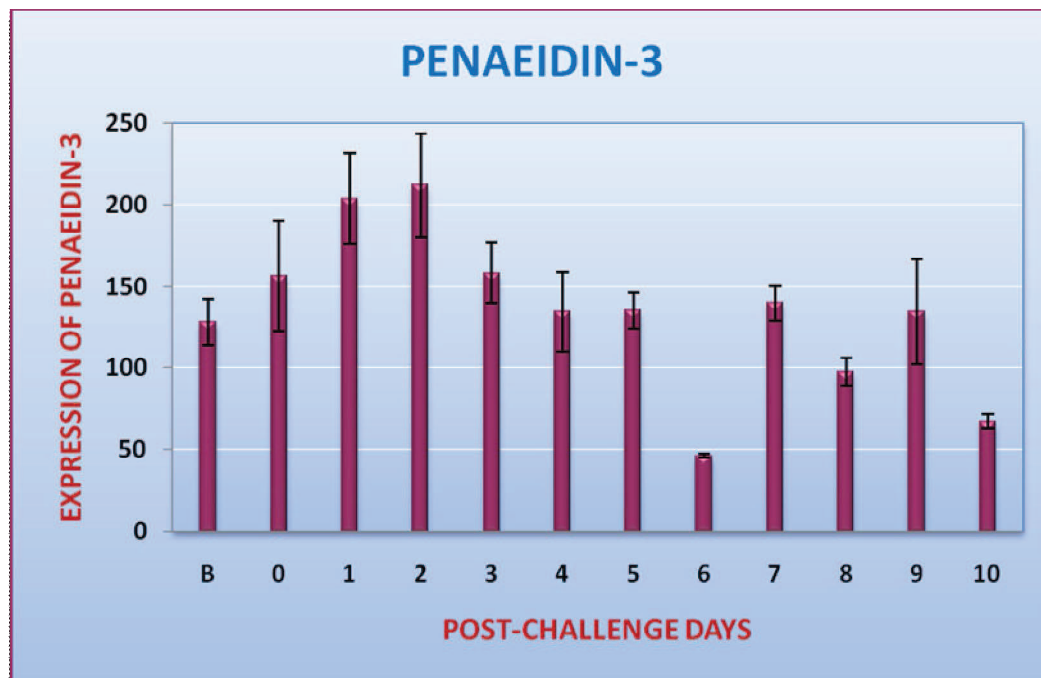


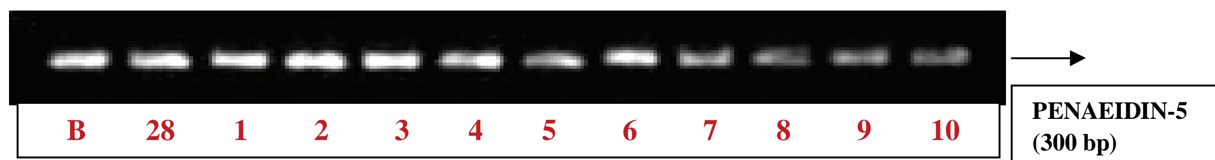

**Fig. 4.10. Time-course analysis of Penaeidin-3 (AMP gene) expression in the haemocytes of giant tiger shrimp, *P. monodon* in response to WSSV challenge**

**(A) Agarose gel electrophoretogram**

**(B) Graphical representation of the expression levels of Penaeidin-3 gene (x-axis = post-challenge days, y-axis = Expression levels of the Penaeidin-3 gene)**

*(B=Baseline, 28= 28$^{th}$ day pre-challenge WSSV, 1= post-challenge day 1, 2= post-challenge day 2, 3= post-challenge day 3, 4= post-challenge day 4, 5= post-challenge day 5, 6= post-challenge day 6, 7= post-challenge day7, 8= post-challenge day 8, 9= post-challenge day 9, 10= post-challenge day 10)*

(A)




(B)

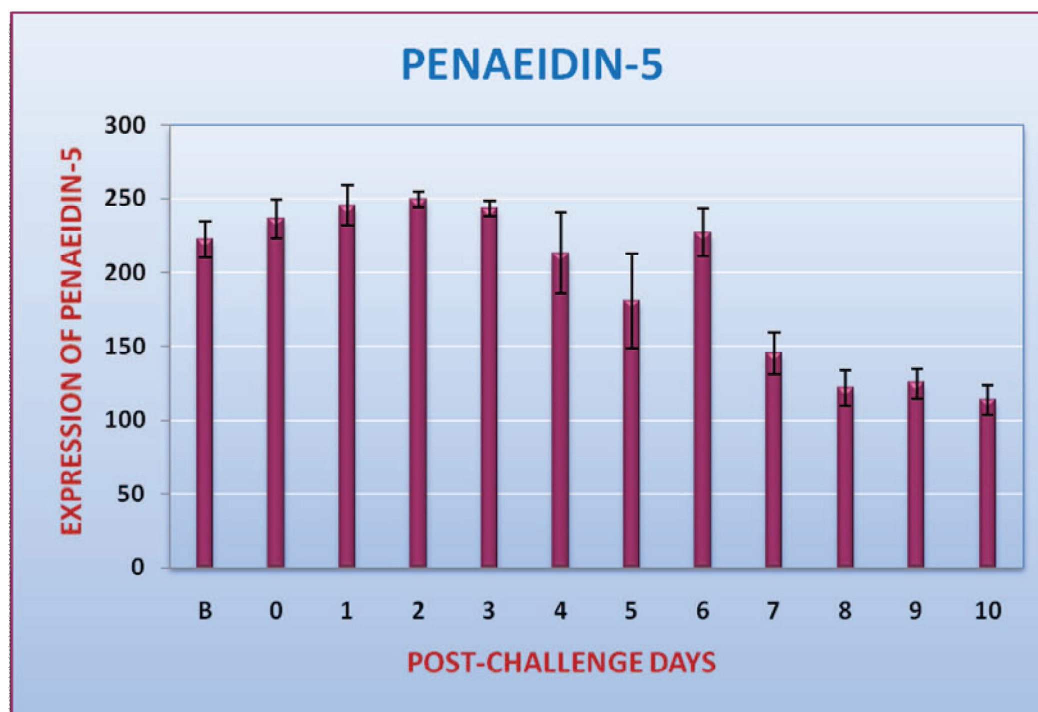


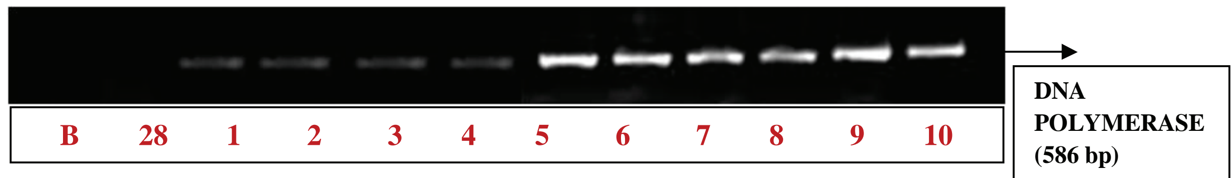

**Fig. 4.11. Time-course analysis of Penaeidin-5 (AMP gene) expression in the haemocytes of giant tiger shrimp, *P. monodon* in response to WSSV challenge**

**(A) Agarose gel electrophoretogram**

**(B) Graphical representation of the expression levels of Penaeidin-5 gene (x-axis = post-challenge days, y-axis = Expression levels of the Penaeidin-5 gene)**

*(B=Baseline, 28= 28$^{th}$ day pre-challenge WSSV, 1= post-challenge day 1, 2= post-challenge day 2, 3= post-challenge day 3, 4= post-challenge day 4, 5= post-challenge day 5, 6= post-challenge day 6, 7= post-challenge day7, 8= post-challenge day 8, 9= post-challenge day 9, 10= post-challenge day 10)*

(A)




(B)

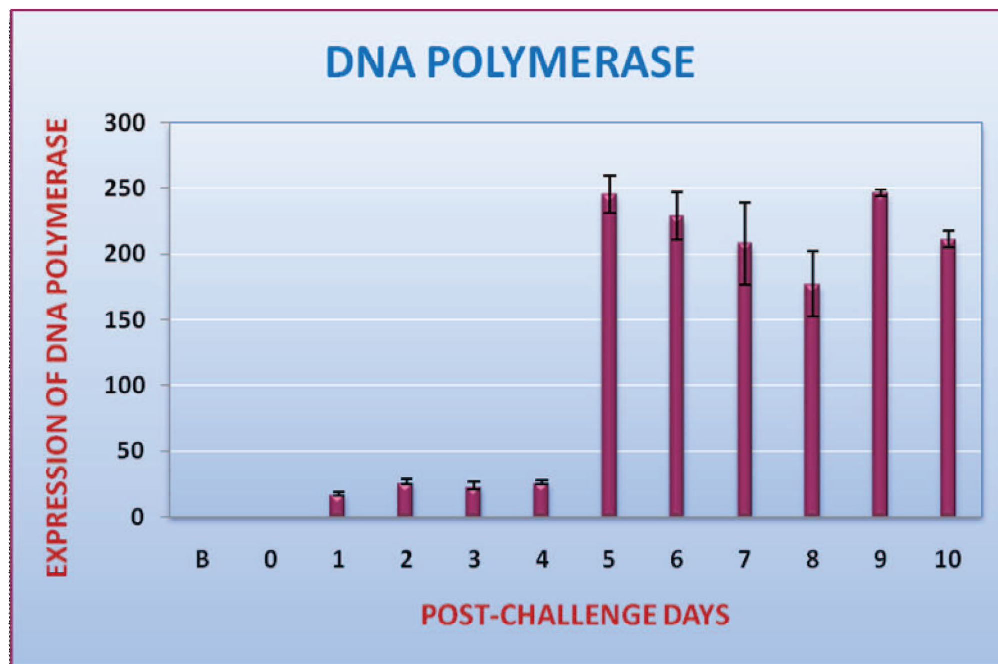


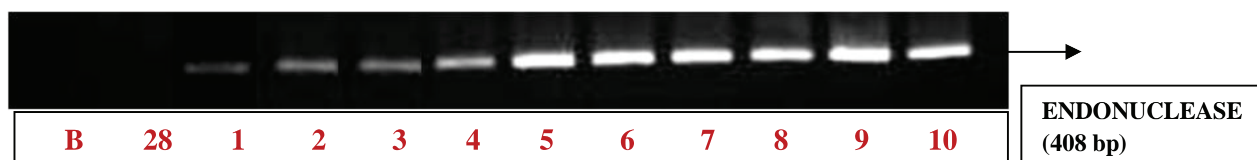

**Fig. 4.12. Time-course analysis of DNA polymerase (WSSV gene) expression in the haemocytes of giant tiger shrimp, *P. monodon* in response to WSSV challenge**

**(A) Agarose gel electrophoretogram**

**(B) Graphical representation of the expression levels of DNA polymerase gene (x-axis = post-challenge days, y-axis = Expression levels of the DNA polymerase gene)**

*(B=Baseline, 28= 28$^{th}$ day pre-challenge WSSV, 1= post-challenge day 1, 2= post-challenge day 2, 3= post-challenge day 3, 4= post-challenge day 4, 5= post-challenge day 5, 6= post-challenge day 6, 7= post-challenge day7, 8= post-challenge day 8, 9= post-challenge day 9, 10= post-challenge day 10)*

**(A)**




**(B)**

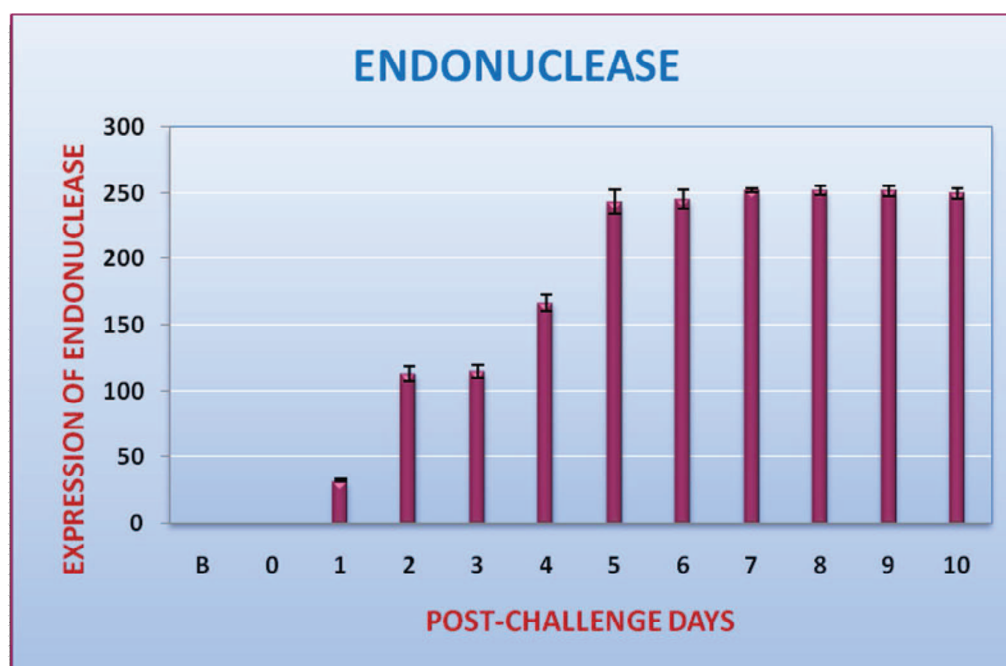


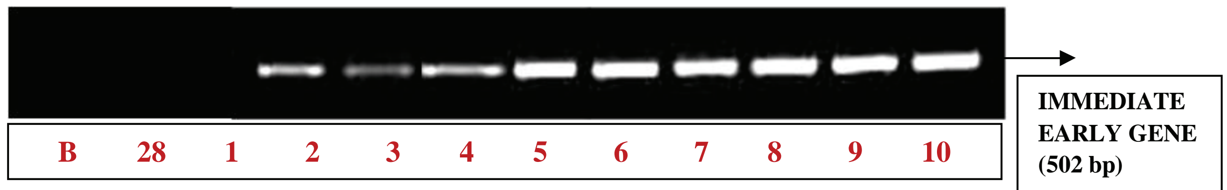

**Fig. 4.13. Time-course analysis of endonuclease gene (WSSV gene) expression in the haemocytes of giant tiger shrimp, *P. monodon* in response to WSSV challenge**

**(A) Agarose gel electrophoretogram**

**(B) Graphical representation of the expression levels of endonuclease gene (x-axis = post-challenge days, y-axis = Expression levels of the endonuclease gene)**

*(B=Baseline, 28= 28$^{th}$ day pre-challenge WSSV, 1= post-challenge day 1, 2= post-challenge day 2, 3= post-challenge day 3, 4= post-challenge day 4, 5= post-challenge day 5, 6= post-challenge day 6, 7= post-challenge day7, 8= post-challenge day 8, 9= post-challenge day 9, 10= post-challenge day 10)*

(A)




(B)

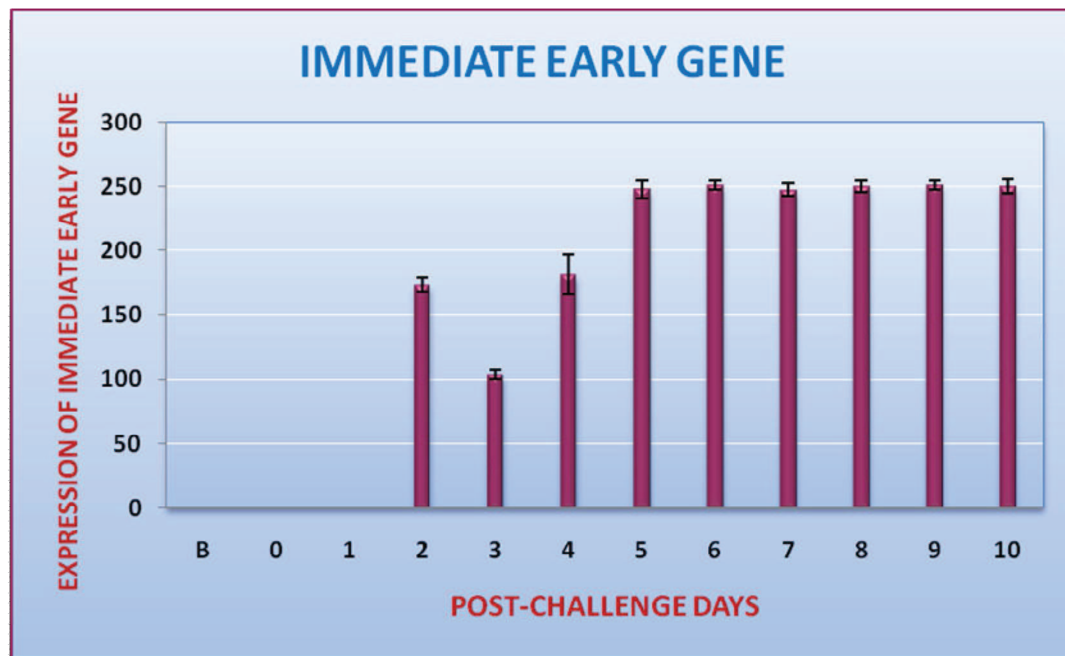



**Fig. 4.14. Time-course analysis of immediate early gene (WSSV gene) expression in the haemocytes of giant tiger shrimp, *Penaeus monodon* in response to WSSV challenge**

**(A) Agarose gel electrophoretogram**

**(B) Graphical representation of the expression levels of immediate early gene (x-axis = post-challenge days, y-axis = Expression levels of the immediate early gene)**

*(B=Baseline, 28= 28th day pre-challenge WSSV, 1= post-challenge day 1, 2= post-challenge day 2, 3= post-challenge day 3, 4= post-challenge day 4, 5= post-challenge day 5, 6= post-challenge day 6, 7= post-challenge day7, 8= post-challenge day 8, 9= post-challenge day 9, 10= post-challenge day 10)*

**(A)**

**(B)**

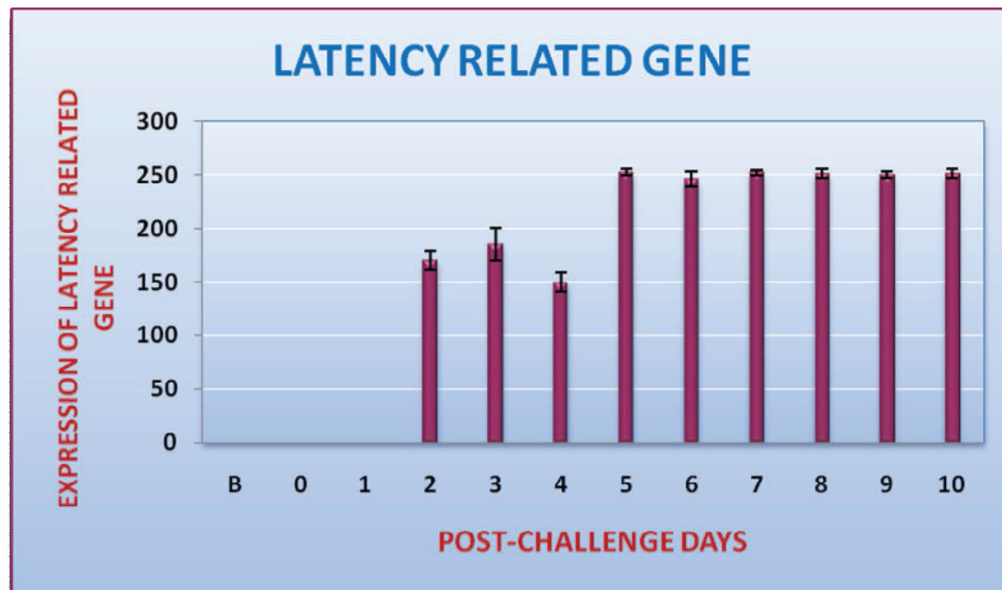



**Fig. 4.15. Time-course analysis of latency related gene (WSSV gene) expression in the haemocytes of giant tiger shrimp, *P. monodon* in response to WSSV challenge**

**(A) Agarose gel electrophoretogram**

**(B) Graphical representation of the expression levels of latency related gene (x-axis = post-challenge days, y-axis = Expression levels of the latency related gene)**

*(B=Baseline, 28= 28th day pre-challenge WSSV, 1= post-challenge day 1, 2= post-challenge day 2, 3= post-challenge day 3, 4= post-challenge day 4, 5= post-challenge day 5, 6= post-challenge day 6, 7= post-challenge day7, 8= post-challenge day 8, 9= post-challenge day 9, 10= post-challenge day 10)*

(A)

(B)

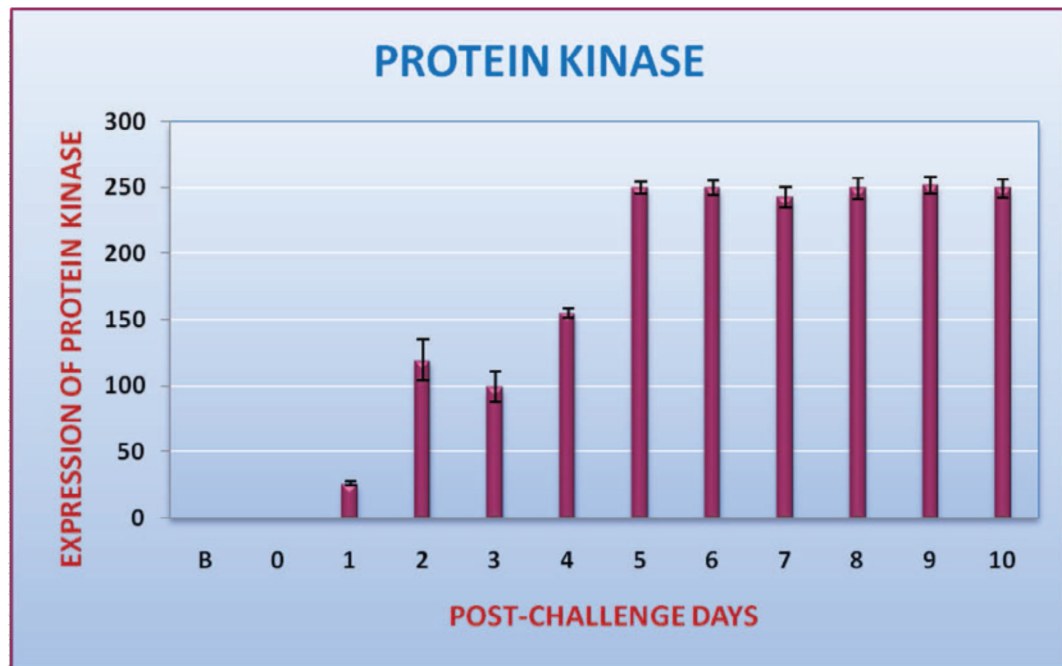



**Fig. 4.16. Time-course analysis of protein kinase gene (WSSV gene) expression in the haemocytes of giant tiger shrimp, *Penaeus monodon* in response to WSSV challenge**
**(A) Agarose gel electrophoretogram**
**(B) Graphical representation of the expression levels of protein kinase gene (x-axis = post-challenge days, y-axis = Expression levels of the protein kinase gene)**

*(B=Baseline, 28= 28$^{th}$ day pre-challenge WSSV, 1= post-challenge day 1, 2= post-challenge day 2, 3= post-challenge day 3, 4= post-challenge day 4, 5= post-challenge day 5, 6= post-challenge day 6, 7= post-challenge day7, 8= post-challenge day 8, 9= post-challenge day 9, 10= post-challenge day 10)*

(A)

(B)

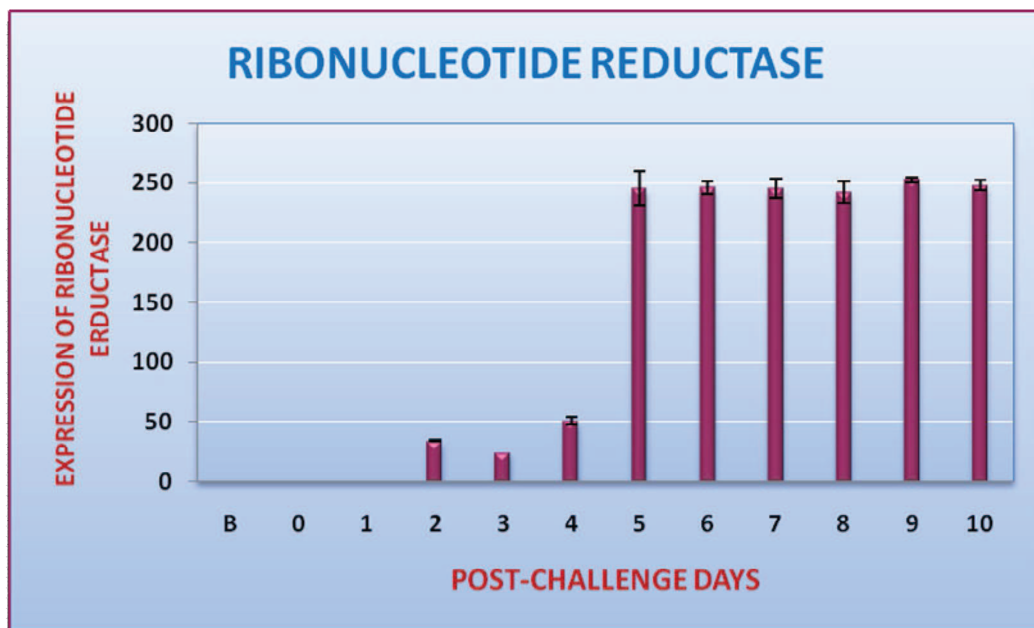


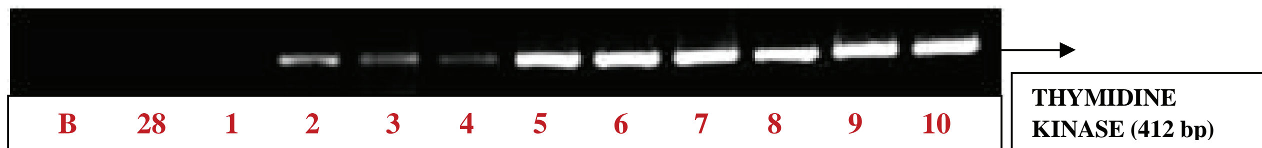

**Fig. 4.17. Time-course analysis of ribonucleotide reductase gene (WSSV gene) expression in the haemocytes of giant tiger shrimp, *P. monodon* in response to WSSV challenge**

**(A) Agarose gel electrophoretogram**

**(B) Graphical representation of the expression levels of ribonucleotide reductase gene (x-axis = post-challenge days, y-axis = Expression levels of the ribonucleotide reductase gene)**

*(B=Baseline, 28= 28th day pre-challenge WSSV, 1= post-challenge day 1, 2= post-challenge day 2, 3= post-challenge day 3, 4= post-challenge day 4, 5= post-challenge day 5, 6= post-challenge day 6, 7= post-challenge day7, 8= post-challenge day 8, 9= post-challenge day 9, 10= post-challenge day 10)*

(A)




(B)

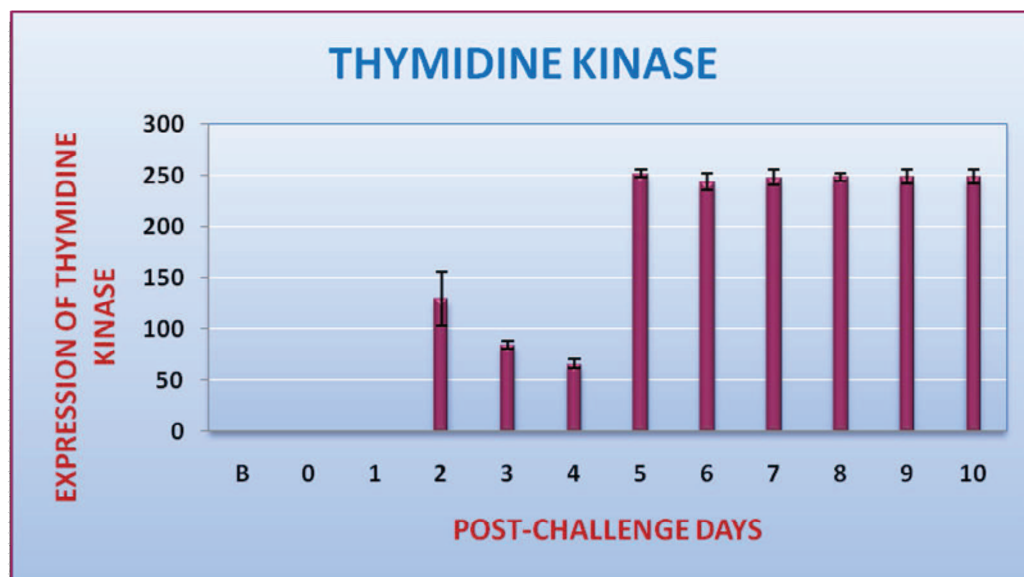


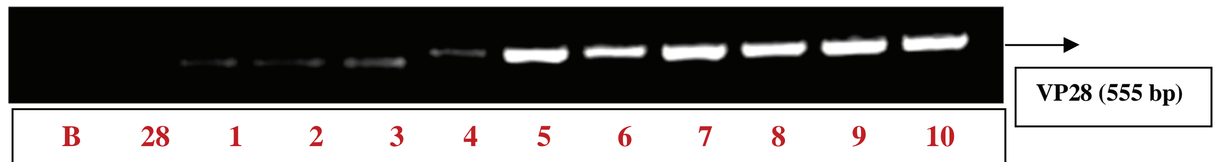

**Fig. 4.18. Time-course analysis of thymidine kinase gene (WSSV gene) expression in the haemocytes of giant tiger shrimp, *P. monodon* in response to WSSV challenge**

**(A) Agarose gel electrophoretogram**

**(B) Graphical representation of the expression levels of thymidine kinase gene (x-axis = post-challenge days, y-axis = Expression levels of the thymidine kinase gene)**

*(B=Baseline, 28= 28th day pre-challenge WSSV, 1= post-challenge day 1, 2= post-challenge day 2, 3= post-challenge day 3, 4= post-challenge day 4, 5= post-challenge day 5, 6= post-challenge day 6, 7= post-challenge day7, 8= post-challenge day 8, 9= post-challenge day 9, 10= post-challenge day 10)*

(A)




(B)

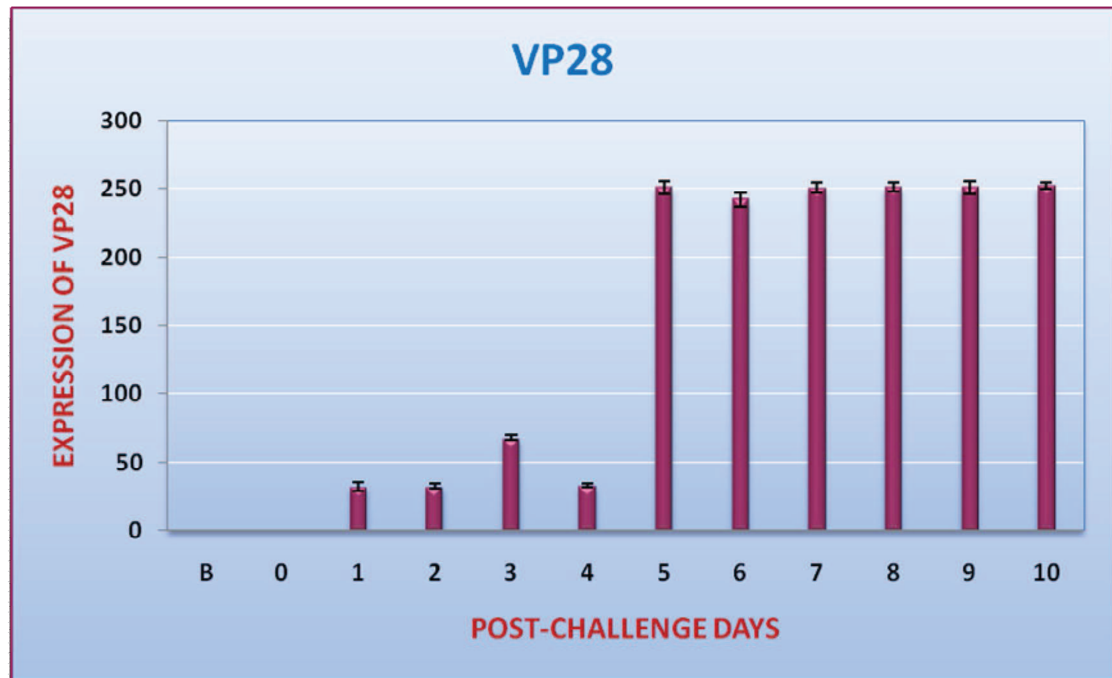



**Fig. 4.19. Time-course analysis of VP 28 gene (WSSV gene) expression in the haemocytes of giant tiger shrimp, *P. monodon* in response to WSSV challenge**

**(A) Agarose gel electrophoretogram**

**(B) Graphical representation of the expression levels of VP 28 gene (x-axis = post-challenge days, y-axis = Expression levels of the VP 28 gene)**

*(B=Baseline, 28= 28th day pre-challenge WSSV, 1= post-challenge day 1, 2= post-challenge day 2, 3= post-challenge day 3, 4= post-challenge day 4, 5= post-challenge day 5, 6= post-challenge day 6, 7= post-challenge day7, 8= post-challenge day 8, 9= post-challenge day 9, 10= post-challenge day 10)*

# CHAPTER-5

## Expression Profile of Antimicrobial Peptide Genes in Response to the Administration of Immunostimulants

## 5.1. Introduction

Penaeid shrimp is an interesting animal model to use for the study of defense mechanisms in crustaceans mainly because of its economical value, its short life-time, its ability to survive in microbe-rich marine environments without gross signs of disease and the high amount of samples that can easily be collected from shrimp farms (Sritunyalucksana and Soderhall, 2000). The giant tiger shrimp, *Penaeus monodon* is one of the major farmed penaeid species and accounts for most of the shrimp production in Asia. However, the shrimp aquaculture industry is suffering from diseases linked to infection by viral pathogens, which cause a drastic decrease in shrimp production, resulting in huge economic losses. In the last few years, many shrimp farms around the world have been badly hit by epidemics of WSSV (Krishna et al., 1997; Nair, 2000; Sanchez-Martinez et al., 2007). The disease attributed to this virus was first observed in East Asia as early as 1992–1993 (Inouye et al., 1993; Huang et al., 1995). It has rapidly spread, and by 1996, had severely infested most of the shrimp farming regions in Asia (Flegel, 1997). White spot syndrome virus (WSSV) causes high mortality in many cultured shrimp species including *P. monodon*, *P. semisulcatus*, *Marsupenaeus japonicus*, *Fenneropenaeus chinenis*, *F. penicillatus*, *Metapenaeus ensis*, *Macrobrachium rosenbergii* and *Exopalaemon orientis* (Lightner, 1996; Lo et al., 1997).

The application of antibiotics or other chemicals to culture ponds is expensive and undesirable as it risks contamination of both the environment and the final product (Grant and Briggs, 1998), besides causing impaired growth in juvenile stock (Stuck et al., 1992; Swastika et al., 1992) and drug resistance in pathogens (Brown, 1989; Juwana, 1990; Aoki, 1992; Smith et al.,

1994). The emergence of bacterial strains resistant to conventional antibiotics is a major cause of inefficient therapy and increased mortality from infections. To overcome this problem, studies should be focused on developing strategies of controlling viral diseases to ensure long-term survival of shrimp aquaculture. Thus, it is very important to find ways for enhancing natural immunity, especially towards major infectious agents like WSSV, in widely cultivated organisms, such as shrimps.

Currently the use of preventive approaches like vaccines, immunostimulants and probiotics, essential for further development of more sustainable aquaculture practices, are becoming increasingly important (Marques et al., 2006). Application of various immunostimulants to activate or boost the innate immune system has been widely accepted as a good alternative (Song et al., 2000). Also, in the aquaculture industry, increasing consideration has been given to the use of immunostimulants as adjuncts to vaccination and as a potential route to the reduction in the widespread use of antibiotics (Burrells et al., 2001a, 2001b). The word 'immunostimulant' refers to any substance that is used with an intent to boost immune reactivity and improve resistance to, or survival following infection by harmful micro-organisms (Smith et al., 2003). Immunostimulants have been reported to increase resistance to these infectious diseases in teleost fish and shellfish (Raa, 1996; Sakai, 1999) by enhancing the nonspecific immune system, the set of defenses directed against all potentially invasive, disease-causing organisms (Burgents et al., 2004).

Harnessing the hosts' specific and non-specific defense mechanisms for controlling diseases has considerable potential for health management in shrimp aquaculture. This will help reduce stress from handling (grading, manipulating stocking densities, removing mortalities etc.) and environmental manipulation (application of chemical treatments, pond drainage, etc.) in order to control disease manifestation under intensive culture conditions.

Important biotechnological interventions are being developed in the field of immunostimulants and modulators in an effort to reduce shrimp susceptibility to disease. Immunostimulants and nonspecific immune-enhancers are being incorporated into diets to provide added protection to the animals, even though our knowledge of shrimp immunity is limited at present, The large number of commercial immunostimulants available on the market reflects the interest of the industry in broadening the scope of tools available to manage shrimp diseases. However, the effectiveness of many of these products are yet to be established. Preliminary results from biological trials appear highly variable. Further research and field trials are clearly essential to determine the precise mechanisms of the action of these products and to evaluate their efficacy in commercial shrimp production (Flegel, 1996; Subasinghe et al., 1998).

In recent years, immunostimulants such as PG, LPS, β-1, 3 glucan and yeast cells, were administered orally, by injection or by immersion in a bath, to enhance the resistance of shrimp to bacterial and viral infections (Itami et al., 1996; Raa, 1996; Scholz et al., 1999; Takahashi et al., 2000; Sajeevan et al., 2006, 2009; Pillai, 2007; Shameeda, 2007). Yeasts have been proved to possess immunostimulatory properties by virtue of their complex carbohydrate and nucleic acid components (Anderson et al., 1995). Both cellular and humoral responses have been induced by dietary yeast, depending on the environmental conditions. Yeasts have been used to improve the growth rate of *L. vannamei* larvae (Intriago et al., 1998). Probiotic properties of yeasts like *Saccharomyces cerevisiae* have been reported and displayed as the ability to survive in the gastrointestinal tract and interact antagonistically with pathogens such as *E. coli*, *Shigella* and *Salmonella*. *S. boulardii* has been used for more than 50 years as a therapeutic agent for the treatment of a variety of gut disorders like diarrhea (Bekatorou et al., 2006). Other yeasts commonly used in animal feeds as probiotic additives are *C. pintolopesi* and *C. saitoana*

(Bovill et al., 2001). In fishes, dietary yeast stimulates metabolism and growth (Gatesoupe, 2007).

Even though, yeasts are widely used as source of immunostimulants, work on marine yeasts are very much limited. The terrestrial yeasts have been receiving great attention in science and industry for over hundreds of years because their potential has been very well established. However, only in recent years, it has been found that marine yeasts have wide applications in aquaculture (Sajeevan et al., 2006, 2009; Pillai, 2007; Shameeda, 2007). The halotolerant property of marine yeast is an added advantage when selected as a candidate species as feed supplement in aquaculture because, for baker's or brewer's yeast, the seawater or the rearing water for shrimp culture is hyperosmotic which cause cell rupture leading to water quality deterioration.

It has been well known that yeast cells have a rigid thick cell wall of about 200 nm thickness outside the plasma membrane (Ueda and Tanaka, 2000). The cell wall of yeast cells is mainly composed of mannoproteins, β-linked glucans (β-1,3- and β-1,6-linked glucose) and a fibrillar or brush-like outer layer predominantly of mannoproteins (Ueda and Tanaka, 2000). However, in general, the animals do not synthesize enzymes which hydrolyze mannoproteins and β-linked glucans. Therefore it is difficult for yeast cell wall to be attacked in the guts of animals, especially in guts of marine organisms. Most commonly employed method for glucan extraction is the alkali-acid hydrolysis method of Hassid (1941) later refined by Williams et al., (1991) according to which more than 97% pure form of glucan could be obtained. The ability of yeast cell wall glucans to stimulate the immune system (Williams et al., 1992) lowering serum cholesterol level (Robbins and Seeley, 1977), antitumour activity (Bohn and Be Miller, 1995) and their potential use in cosmetics (Donzis, 1996) are noteworthy. Yeast glucan is the most extensively studied of the glucans (Sakai et al., 1999).

However, very few works have been reported depicting the effect of these immunostimulants in the expression profile of AMP genes.

Administration of peptidoglycan (PG) in *M. japonicus* has shown to increase the expression of antibacterial peptide, crustin and antimicrobial proteins like lysozymes. Earlier works by Rattanachai and co-workers (2004) have also showed that PG feeding of *M. japonicus* increased the expression level of crustin-like peptide mRNA. Administration of sodium alginate in *P. monodon* has also proved to up-regulate the expression of penaeidin-5 and single whey acidic protein domain (Liu et al., 2006). Contrasting reports have been reported by Okumura (2007) where a down-regulation of the AMP genes, including penaeidin-2, penaeidin-3, penaeidin-4 and crustin was noticed when *L. vannamei* was injected with LPS. However, till date the molecular mechanisms by which immunostimulants affect the expression of each bio-defense gene are unclear.

Therefore, the present study was conducted to analyze the expression profile of AMP genes (ALF, Crustin-1, Crustin-2, Crustin-3, Penaeidin-3, Penaeidin-5) in *P. monodon* fed marine yeast (*Candida haemulonii* S27 and *C. sake* S165) and β-glucan (extracted from *Candida haemulonii* S27 and *C. sake* S165) incorporated diets before and after challenge with WSSV; to enhance the production of AMPs in the experimental animal i.e. *P.monodon* and to study the tissue-wise expression profile of AMP genes in response to immunostimulant administration and WSSV challenge

# 5.2. Materials and Methods

## *5.2.1. Experimental animals and rearing conditions*

Healthy adult *P. monodon* (20-25 g) were collected from a local shrimp farm in Vypeen, Kochi and maintained in aquarium tanks as mentioned in section 4.2.1.

### 5.2.2. *Test diets*

Four experimental feeds were prepared by incorporating two different yeast strains (*Candida haemulonii* S27 (CHY) and *Candida sake* S165 (CSY)) and β-glucans extracted from these yeasts (*Candida haemulonii* S27 glucan (CHG) and *Candida sake* S165 glucan (CSG)) to a standard shrimp diet (Higashi, India). Feed without supplementation of immunostimulant was used as the control feed (FC) (Table 5.1.).

#### 5.2.2.1. *Yeast strains*

*C. haemulonii* S27 and *C. sake* S165, isolated from coastal waters off Cochin and maintained in the Microbiology Laboratory of the Department of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, Kochi, India was used for the study. Selection of these isolates were based on the preliminary experiments with *F. indicus* and *P. monodon* that showed improved growth and survival when fed on a diet supplemented with marine yeasts/glucans (Sajeevan et al., 2003, 2006, 2009; Pillai, 2007; Shameeda, 2007).

Lawn culture of these yeast strains were prepared using malt extract agar (malt extract, 20g; mycological peptone, 5g; agar, 20g, 20ppt seawater, 1 liter; pH 6) and the cell biomass was harvested at exponential phase with sterile seawater (20ppt). Cells were separated by centrifugation at 7500 ×g for 10 min at 4 °C.

#### 5.2.2.2. *Preparation of Yeast incorporated experimental feed*

Experimental diets were prepared by incorporating 10% yeast biomass to the standard shrimp diet (Higashi, India). Briefly, 100g of the standard shrimp diet was powdered and mixed well in to a dough with 100 ml water and was steamed for 10 min in an autoclave. The test feeds were mixed with 10 g yeast biomass and pelletized using a laboratory model pelletizer having 1 mm diameter. The feeds were air dried for 2 hr and stored at -20 °C until used.

### *5.2.2.3. Glucan extraction*

Glucan used for the present work was extracted from two marine yeast strains viz. *C. haemulonii* S27 and *C. sake* S165. β- 1, 3 glucan was extracted from the cell wall of dried yeast biomass following the method of Williams et al. (1991).

***Procedure followed for extraction of β-glucan from yeast cell wall (as per Williams et al., 1991)***

### *5.2.2.4. Preparation of Glucan incorporated experimental feed*

Experimental diets were prepared by incorporating 0.2% glucan to a standard shrimp diet as described in section 5.2.2.2.

### *5.2.3. Feeding experiments and WSSV challenge*

Shrimps were randomly divided into five groups (n=15) and were fed on the respective diets for 14 days. Feeding experiments were done in triplicates. Group 1 (FC) shrimps fed standard shrimp diet without supplementation of any immunostimulant was used as the control. Group 2 (CHY) and Group 3 (CSY) were maintained on the feed containing 10% *C. haemulonii* S27 and *C. sake* S165 respectively, Group 4 (CHG) and Group 5 (CSG) were maintained on the diets containing 0.2% glucan extracted from *C. haemulonii* S27 and *C. sake* S165 respectively. Group 1 (standard diet), Group 2 (CHY) and Group 3 (CSY) shrimps were fed twice daily (8 AM and 7 PM) at a ration of 10–15% body weight. Whereas, Group 4 (CHG) and Group 5 (CSG) were fed 0.2% glucan diet once every seven days and the control diet on the rest of the days. Physico-chemical parameters of the rearing water was monitored regularly and maintained at optimal levels by water exchange. Six animals from each treatment group were sampled after 14 days of immunostimulant application. On the 15$^{th}$ day all the groups were challenged with WSSV by feeding WSSV infected frozen shrimp tissue at a rate of 1g/animal. Thereafter they were maintained on their respective diets for 48 h. After 48 h, six animals each from all the treatment groups were sampled for analysis. Only those in the intermoult stage were taken for sampling. Survival in each treatment group was recorded daily for a period of 7 days with dead animals removed promptly. Mortality by WSSV infection was confirmed by checking the characteristic white spots on the carapace of infected shrimps.

### 5.2.4. *Haemolymph and other tissues collection*

Haemolymph was collected from the rostral sinus using capillary tubes (RNase-free) rinsed using pre-cooled anticoagulant solution (RNase free, 10% sodium citrate, pH 7.0). Tissues including gill, muscle, heart, hepatopancreas and intestine were dissected out using RNase-free scissors and forceps. Haemolymph and the collected tissues were suspended in TRI reagent (Sigma) for total RNA isolation.

### 5.2.5. *Total RNA isolation and Reverse transcription*

Total RNA isolation and first strand cDNA synthesis were performed as described in section 2.2.3. to 2.2.6.

### 5.2.6. *Semi-quantitative RT-PCR analysis of target gene expression*

Expression of the target genes when supplemented with different immunostimulants pre- and post-challenge WSSV was determined by semi-quantitative RT-PCR analysis using 18S rRNA, β-actin and elongation factor (ELF) as the internal control (Marone et al., 2001). Haemocyte cDNA was diluted 5 times and amplified using Taq polymerase. PCR amplifications were performed for three control genes (18S rRNA, β-actin and ELF); five AMP genes (ALF, crustin-1, crustin-2, crustin-3, penaeidin-3 and penaeidin-5) and eight WSSV genes (DNA polymerase, endonuclease, immediate early gene, latency related gene, protein kinase, ribonucleotide reductase, thymidine kinase and VP28) in a 25 µl reaction volume as described in section 4.2.6.

From the four different immunostimulants screened, the best one was selected based on AMP gene expression, intensity of WSSV infection as envisaged from the WSSV gene expression and post-challenge survival data. The best performed immunostimulant group along with control group was subjected to detailed tissue-wise expression profile analysis. Tissue cDNA was amplified. PCR amplifications were performed for six AMP genes (ALF, crustin-1, crustin-2, crustin-3, penaeidin-3 and penaeidin-5) and two WSSV

genes (latency related gene and VP28) in a 25 µl reaction volume as described in section 4.2.6.

### *5.2.7. Statistical analysis of the post-challenge survival data*

Statistical significance of the post challenge survival data of the control group and immunostimulant administered groups were determined by One-way ANOVA and Duncan's multiple comparison of the means using the software SPSS 10.0. A probability ($p$) value of less than 0.05 was considered significant.

## 5.3. Results

### *5.3.1. Expression profile of control genes in the haemocytes of P. monodon in response to various immunostimulants pre- and post-challenge WSSV*

Expression profile of three control genes viz. 18SrRNA (Fig. 5.1), β-actin (Fig. 5.2) and ELF (Fig. 5.3) were analyzed prior to that of target AMP and WSSV genes. All experimental samples gave positive amplifications for the three control genes studied and the level of expression was found to be more or less stable. β-actin was found to vary the most among the three control genes analyzed followed by 18SrRNA and ELF.

### *5.3.2. Expression profile of AMP genes in the haemocytes of P. monodon in response to various immunostimulants pre- and post-challenge WSSV*

All AMP genes (ALF, crustin-1, crustin-2, crustin-3, penaeidin-3 and penaeidin-5) were found to be constitutively produced in the haemocytes of *P. monodon* and were found to be differentially expressed on administration of various immunostimulants. Also, the expression profile varied greatly for the various AMP genes studied (Fig.5.4 to 5.9)

***5.3.2.1. Expression profile of ALF***

Variation in the expression of ALF could be observed on administration of immunostimulants. ALF expression was found to up-regulate on administration of glucan incorporated diet pre-challenge WSSV. In the pre-challenge condition maximum up-regulation of the ALF gene was supported by CSG incorporated diet followed by CHG. much variation in expression level of ALF gene could not be observed on administration of marine yeasts (Fig. 5.4 A & C).

On challenge with WSSV; the ALF gene was found to up-regulate considerably in all experimental groups of shrimps including the control group. Noticeable up-regulation of the ALF gene could be noticed in all experimental diet fed groups post-challenge WSSV. CHY treated group supported maximum up-regulation of the ALF gene post-challenge WSSV (Fig. 5.4 B & C).

***5.3.2.2. Expression profile of crustin-1***

Not much variation in the expression profile of crustin-1 could be detected on administration of marine yeast and glucans as immunostimulants. The level of expression of crustin-1 gene remained more or less similar except for a slight down-regulation in case of CHY administered group (Fig. 5.5 A & C).

On WSSV challenge, crustin-1 was found to down-regulate considerably in the control group of shrimps. However, when compared to the control group, all experimental diet administered group supported up-regulation of the crustin-1 gene. CHY and CSG administered group was found to support maximum up-regulation of the crustin-1 gene post-challenge WSSV, which was followed by CSY and CHG (Fig. 5.5 B & C).

***5.3.2.3. Expression profile of crustin-2***

Not much variation in the expression profile of crustin-2 could be detected in the experimental group of shrimps pre-challenge WSSV (Fig. 5.6 A & C).

On WSSV challenge, crustin-2 followed the same pattern of expression as in case of crustin-1. Crustin-2 gene was found to be down-regulated in the control group of shrimps on challenge with WSSV. However, when compared to the control group, all experimental diet administered group supported up-regulation of the crustin-2 gene. CHY administered group was found to support maximum up-regulation of the crustin-2 gene post-challenge WSSV, which was followed by CSY, CHG and CSG (Fig. 5.6 B & C).

#### *5.3.2.4. Expression profile of crustin-3*

No variations could be detected in the expression profile of crustin-3 gene pre-challenge WSSV, except for a very slight up-regulation in the treated groups (Fig. 5.7 A & C).

Crustin-3 gene was found to be down-regulated in the control group of shrimps on WSSV challenge. No variations could be detected in the expression profile of crustin-3 gene on immunostimulant administration, post-challenge WSSV (Fig. 5.7 B & C).

#### *5.3.2.5. Expression profile of penaeidin-3*

Not much variation in the expression profile of penaeidin-3 could be detected on administration of immunostimulants pre-challenge WSSV except for a slight up-regulation in case of marine yeast administered group of shrimps (Fig. 5.8 A & C).

On WSSV challenge, down-regulation of the penaeidin-3 gene could be found in the control group of shrimps. However, significant variation in the expression of penaeidin-3 gene could be observed in the immunostimulant administered group of shrimps. Maximum up-regulation of the penaeidin-3 gene was supported by marine yeast administered group, followed by CSG and CHG administered groups (Fig. 5.8 B & C).

#### *5.3.2.6. Expression profile of penaeidin-5*

Not much variation in the expression profile of penaeidin-5 could be detected on administration of immunostimulants pre-challenge WSSV except

for a slight down-regulation in case of CHY and glucan administered group (Fig. 5.9 A & C).

On WSSV challenge, the expression of penaeidin-5 was found to be the same in case of control group of shrimps. However, when compared to the control group, the gene was found to be up-regulated in all the immunostimulant administered groups. Marine yeasts supported maximum up-regulation of the penaedin-5 gene post-challenge WSSV (Fig. 5.9 B & C).

### *5.3.3. Expression of WSSV genes in haemocytes of P. monodon in response to the administration of various immunostimulants*

All WSSV gene transcripts could be detected in the haemocytes of the control group of shrimps and all the genes followed more or less similar pattern of expression for the various experimental groups analyzed. WSSV infection could be confirmed by the expression of DNA polymerase (Fig. 5.10), endonuclease (Fig. 5.11), immediate early gene (Fig. 5.12), latency related gene (Fig. 5.13), protein kinase (Fig. 5.14), ribonucleotide reductase (Fig. 5.15), thymidine kinase (Fig. 5.16) and VP28 (Fig. 5.17) genes of WSSV in control group of shrimps. Glucan diet fed animals viz. CHG and CSG, also exhibited the presence of WSSV gene transcripts confirming infection in these groups of animals also. However, CHG was found to support low level of WSSV gene expression when compared to the CSG group. Interestingly, the marine yeast fed group was with minimum WSSV gene transcripts. CHY did not show the presence of any of the WSSV genes studied. However, CSY exhibited the presence of protein kinase and VP28 gene transcripts of WSSV.

Since CHY administered shrimps were found to perform best in terms of AMP gene expression and WSSV gene expression, a detailed tissue-wise expression profile was performed for the above group of shrimps.

### *5.3.4. Expression profile of AMP genes in various tissues of P. monodon in response to the administration of Candida haemulonii yeast pre- and post-challenge WSSV*

mRNAs of AMP genes could be detected in all of the target tissues at varying levels in the animals pre- and post- challenge WSSV. The expression profile of each AMP was found to vary for the various tissue targets (Fig. 5.18 to 5.23).

#### *5.3.4.1. Expression profile of ALF*

ALF gene transcripts could be detected at high levels in the gills, intestine and hepatopancreas of the control group of shrimps prior to WSSV challenge. In case of the control group of shrimps, gills and intestine supported maximum expression of the ALF gene followed by hepatopancreas. The ALF gene was found to be down-regulated in the gills, intestine and hepatopancreas on CHY treatment. However, an up-regulation of the gene could be noticed in the muscle and heart of the experimental group of shrimps (Fig. 5. 18 A & C).

ALF gene was found to be up-regulated on WSSV challenge even in the control group. Up-regulation of ALF could be noticed in all the tissues of control shrimps except muscle. In CHY treated group of shrimps, a noticeable up-regulation of the gene could be seen in case of gills, intestine and muscle (Fig. 5. 18 B & D).

#### *5.3.4.2. Expression profile of crustin-1*

mRNA of crustin-1 gene could be detected in all of the target tissues in the both control and CHY treated groups of animals prior to WSSV challenge. Gill, muscle, and intestine was found to support maximum expression of the crustin-1 gene in the control shrimps whereas in case of CHY treated group of shrimps, gill, muscle and heart supported maximum expression followed by intestine and hepatopancreas (Fig. 5. 19 A & C).

On challenge with WSSV, a complete down-regulation of the crustin-1 gene could be observed in the various target tissues of both the control group and CHY treated group of shrimps. Maximum level of expression was found in the gills. No transcripts of crustin-1 could be detected in muscle, hepatopancreas or heart of both the control group and CHY treated group of shrimps (Fig. 5. 19 B & D).

***5.3.4.3. Expression profile of crustin-2***

Crustin-2 was found to be expressed at very high levels in all the target tissues of the control group of shrimps pre-challenge WSSV. However, the gene was found to be completely down-regulated in the CHY treated group prior to WSSV challenge. Gill was the only tissue that was found to express the crustin-2 gene in the CHY treated group, that too at very low level (Fig. 5.20 A & C)

On challenge with WSSV, a general down-regulation of the crustin-2 gene could be noticed in all the target tissues of the control group. However, in case of CHY treated group of shrimps, the crustin-2 gene was found to be up-regulated considerably in all the tissues except in case of hepatopancreas. Maximum expression was found in the gill and intestine (Fig. 5. 20 B & D).

***5.3.4.4. Expression profile of crustin-3***

Crustin-3 was found to be expressed in all the target tissues of the control group of shrimps pre-challenge WSSV. Maximum expression of the gene was found in gill and intestine. However, the gene was found to be completely down-regulated on administration of CHY (Fig. 5. 21 A & C)

On challenge with WSSV, down-regulation of the crustin-3 gene could be noticed in various target tissues. Gill was the only tissue that was found to express the crustin-3 gene, that too at very low level. However, when compared to the control of shrimps, the CHY treated group up-regulated the crustin-3 gene, especially in gill and intestine (Fig. 5. 21 B & D).

***5.3.4.5. Expression profile of penaeidin-3***

Penaeidin-3 was found to be expressed at very high levels in all the target tissues, except gills of the control group of shrimps prior to WSSV challenge. However, the gene was found to be completely down-regulated in the CHY treated group. Intestine was the only tissue that was found to express the penaeidin-3 gene, that too at very low level (Fig. 5. 22 A & C)

On challenge with WSSV, a general down-regulation of the penaeidin-3 genes could be noticed in various target tissues. Penaedin-3 transcripts could be found only in the gills and intestine of the WSSV challenged shrimps, intestine supporting maximum expression of the gene. However, in the CHY treated group of shrimps, the gene was found to be up-regulated to high levels. Transcripts of penaeidin-3 could be detected in all the tissues with gill and intestine supporting maximum expression followed by muscle, heart and hepatopancreas (Fig. 5. 22 B & D).

***5.3.4.6. Expression profile of penaeidin-5***

Very low level of penaeidin-5 expression could be observed in the various target tissues like, gill, muscle and heart of the control group of shrimps. And no transcripts were detected in the hepatopancreas and intestine prior to WSSV challenge. The penaeidin-5 gene was found to be completely down-regulated in all the tissues on CHY treatment (Fig. 5. 23 A & C).

On challenge with WSSV, up-regulation of the penaedin-5 gene could be noticed in the various target tissues of the control group of shrimps. Maximum expression was supported by the gills and intestine. CHY treated group also supported maximum up-regulation of the penaeidin-5 gene, and maximum expression was found in the gill and muscle followed by intestine (Fig. 5. 23 B & D).

### *5.3.5. Expression of WSSV genes in various tissues of P. monodon in response to the administration of Candida haemulonii yeast*

In the control group of shrimps, both the latency related gene and VP 28 gene analyzed gave positive amplifications for in all the target tissues and followed more or less similar pattern of expression for the various target tissues analyzed. Muscle was found to support minimum expression of the WSSV genes. Interestingly, various target tissues of the CHY treated group of shrimps did not give amplification for any of the WSSV gene studied (Fig. 5. 24 to 5.25).

### *5.3.6. Post-challenge survival*

Marine yeast incorporated diets showed significantly high survival compared to β-glucan incorporated diet fed shrimps and the control group. *C. haemulonii* S 27 (CHY) fed group showed maximum survival (93%) followed by *C. sake* S165 (CSY) (75%), *C. haemulonii* glucan (CHG) (50%) and *C. sake* glucan (CSG) (38%) (Fig. 5.26).

## 5.4. Discussion

The prophylactic 'immunostimulation' in crustaceans must act on the innate immune system and therefore presumes that it can be educated or boosted to improve surveillance of and reactivity towards potential non-self threats. This, at first, might seem incompatible with the very nature of non-specific immune responses, as they are in essence the 'rapid reaction force' in all animals. On the other hand, pathogens may have damaging effects precisely because the evolutionary 'arms race' between host and parasite has led to the development of pathogen traits that enable them to avoid or disrupt the various defense responses mounted by the host. In this case, it is conceivable that, depending upon which part(s) of the immune system the pathogen circumvents, interventional strategies might be used to compensate. Clearly, non-specific immune stimulation, if it is to be effective,

must firstly rest on a sound functional basis to target those aspects of the innate system that pathogens might evade. It then must induce or enhance this response to effect pathogen destruction or elimination without detriment to the host itself (Smith et al., 2003).

Many of the compounds claimed to have immunostimulating or potentiating effects are known from invivo or invitro experiments to have direct effects on various aspects of the crustacean immune system. Glucan, LPS, bacteria and other non-self agents are known to invoke various invivo responses, such as change in haemocyte counts (Smith et al., 1984; Holmblad and Soderhall, 1999; Lorenzon et al., 1999) and induction of encapsulation reactions (Smith and Ratcliffe, 1980). They also are known to induce prophenoloxidase activation and melanisation reactions (Smith and Soderhall, 1983; Soderhall et al., 1986) while invitro glucans have been shown to initiate cell degranulation and to enhance phagocytosis (Smith and Soderhall, 1983; Soderhall et al., 1986). Research on a number of crustaceans has further demonstrated that phenoloxidase activation by glucans or other non-self molecules generates a range of immunoactive agents and activities, including peroxinectin and reactive oxygen species (Holmblad and Soderhall, 1999). However, the molecular mechanisms by which immunostimulants affect the expression of immune genes still remains less understood.

Yeasts and β-glucans are widely accepted as immunostimulants in the shrimp industry. Yeast is generally considered as a good source of protein, nucleic acid, vitamins and polysaccharides. Yeast supplement as immunostimulant is believed to protect the cultured organism against the negative effects of stress on the immune system (Burgents et al., 2004). Previous works by Scholz et al., 1999, showed that other diets, with different yeast additives, which were recorded as enhancing survival, were not found to be significantly better than the control diet. Curiously, these authors

concluded that, although the yeast diets did not appear to have any immunostimulatory effects, they improved survival. It was also shown that the proPO activity expressed by *L. vannamei* fed on different yeast supplemented diets was highly variable between individuals reared on the same diet (Scholz et al., 1999).

The immunostimulatory effects of β-glucan have been well-studied (Sakai, 1999). β-glucans have successfully been used to enhance resistance of fish and crustaceans against bacterial or viral infections (Olivier et al., 1986; Engstad et al., 1992; Matsuyama et al., 1992; Itami et al., 1994; Sung et al., 1994; Su et al., 1995; Chang et al., 2000, 2003). The effects of several types of glucan; e.g. yeast glucan, peptide-glucan, β-1,3-glucan, have been investigated in fish. Yeast glucan is the most extensively studied of these glucans (Sakai, 1999). Yeast glucan has been applied by immersion and oral administration methods. Yeast cell wall glucans have also been shown to enhance the resistance of mammals against bacterial, fungal, viral and protozoan pathogens (Di Luzio, 1985). Glucans are reported to enhance disease resistance by stimulating nonspecific components of the immune system or by improving processing and presentation of antigens during specific adaptive immune responses. For example, the protective effects of β-glucan in crustaceans have been associated with activation of the prophenoloxidase system inducing antimicrobial activity in plasma and enhancing phagocytosis, cell adhesion and superoxide production in haemocytes (Itami et al., 1994; Song and Hsieh, 1994; Chang et al., 2000). Dietary administration of β-glucan increases the resistance of *M. japonicus* against vibriosis (Itami et al., 1994). By immersion or injection of a different glucan, β-1,3-glucan extracted from yeast cell wall, Sung et al. (1994) and Song et al. (1997) demonstrated an enhanced resistance of *P. monodon* to vibriosis and WSSV infection.

For the present study two marine yeasts (*C. haemulonii* S27 and *C. sake* S165) and the β-glucans extracted from the cell wall of these yeasts were used. The work was carried out to find out the role of immunostimulants on AMP gene expression in adult shrimps, since AMPs play an important role in the shrimp defense.

The efficacy of marine yeast *C. sake* S165 (1, 10 and 20%) as a feed supplement and immunostimulant to Indian white shrimp *F. indicus* has been studied by Sajeevan and co-workers (2006). *C. sake* at 10% level in the diet was found to support significantly high growth and maximum immune response in the animals. The immune enhancing capability of the marine yeast *C. haemulonii* S27 has been studied by Pillai (2007), which showed increased survival rate of shrimps on challenge with WSSV when fed with S27 incorporated diet. Also, previous studies by Shameeda (2007) has reported that *C. haemulonii* S27 incorporated feeds supported better performance in terms of various growth parameters i.e. weight gain (WG), food conversion ratio (FCR), feed efficiency ratio (FE) and specific growth rate (SGR) in shrimps compared to the control feed.

Shrimp feed containing 0.2% glucan when administered once every seven days have also proved to be a good source of immuostimulant in *F. indicus* under WSSV challenged conditions (Sajeevan et al., 2009). Haemocytes, being the site of AMP production and storage, was the perfect tissues to study the expression profile of AMP genes. Since WSSV infection is believed to occur mainly via ingestion of WSSV-infected flesh under natural and culturing conditions, in the present study also WSSV challenge was performed orally so as to mimic the natural mode of WSSV infection. Semi-quantitative RT-PCR was employed to examine changes in AMP gene expression in the haemocytes of the black tiger shrimp *P. monodon* challenged with WSSV. Gene expression analysis using semi-quantitative RT-PCR is an easiest sensitive and reproducible method of detecting and

quantifying an mRNA transcript. The method evaluates the product accumulation during the exponential phase of the reaction, has a large dynamic range and a high throughput capacity (Marone et al., 2001).

Generally the immunological parameters were at peak 48 hrs post-challenge with WSSV via diet (Sajeevan et al., 2006; Pillai, 2007). Hence in the present work, sampling was done at $48^{th}$ hr to assess the variation in the expression of AMPs in response to varying stimuli. Chang et al., (2000) evaluated the effectiveness of β-1,3-glucan derived from *Schizophyllum commune* in enhancing shrimp survival as well as phagocytosis and superoxide anion production in brooder *P. monodon* and showed that immunostimulatory enhancement peaked at day 24 after starting the dietary exposure and subsequently decreased to the pre-feeding level at the end of 40 days feeding trial. Itami et al (1994) reported that the dietary administration of Schizophyllan, a water soluble β-1,3-glucan with some β-1,6-glucoside side chains derived from the fungus *S. commune*, increased the resistance of *P. japonicus* against vibriosis. Using β-1,3-1,6 glucan, extracted from the yeast cell wall, Sung et al. (1994) and Song et al. (1997) demonstrated enhanced resistance of *P. monodon* to vibriosis and WSSV infection. But no works have been carried till date out analyzing the expression pattern of important AMP families of shrimps in response to immunostimulants and WSSV challenge. Immune genes differentially expressed during WSSV infection are considered of special interest as they could represent a potential target for the development of antiviral strategies.

Expression profile of three control genes viz. 18S rRNA, β-actin and ELF were analyzed in the present study since these are routinely used as internal controls in a variety of experimental conditions and are widely used for semi-quantitative RT-PCR studies (Suzuki et al., 2000; Marone et al., 2001). AMPs were found to be differentially expressed by various immunostimulants used in the study. ALFs, first purified from the

ameobocytes of two marine chelicerate arthropods (Tanaka et al., 1982), exhibits a potent and broad spectrum of antimicrobial activity against a large number of both Gram-positive and Gram-negative bacteria, including several opportunistic/pathogenic *Vibrio* species, fungi and human enveloped virus (Somboonwiwat et al., 2005; Carriel-Gomes et al., 2007). ALF was found to be up-regulated on administration of glucans (Fig. 5.4). However, application of yeasts did not show any visible variation in the pattern of expression of ALF gene. This indicates some degree of ALF specificity in the immune response of ALFs to yeast cells. It has been reported in several shrimps that ALF transcription is induced upon bacterial challenge (Supungul et al., 2004; Liu et al., 2005; Nagoshi et al., 2006) and in the crayfish (*P. leniusculus*) ALF was found to get induced upon WSSV infection (Liu et al., 2006).

Crustins are generally defined as multi-domain cationic antibacterial polypeptides (7-14 kDa) containing one whey acidic protein (WAP) domain at the C-terminus (Smith et al., 2008). In the present study differential expression of three isoforms of crustins viz. crustin-1, -2 and -3 were analyzed (Fig. 5.5 to 5.7). However, not much variation could be detected in the expression profile of crustins on administration of marine yeast and glucans as immunostimulants, except for some very slight variations. Antimicrobial studies have revealed that all crustin groups are mainly active against gram-positive bacteria. Susceptible bacteria include the gram-positive strains of the genera *Micrococcus*, *Aerococcus*, *Planococcus*, *Staphylococcus*, *Streptococcus*, *Corynebacterium* and *Bacillus* (Relf et al., 1999; Zhang et al., 2007; Supungul et al., 2008; Imjongjirak et al., 2009).

Penaeidins are unquestionably the well-characterized family of AMPs described in crustaceans so far and have been the subject of many review articles (Bachere et al., 2000a, 2004; Destoumieux et al., 2000a; Cuthbertson et al., 2008; Tassanakajon et al., 2010). In the present study penaeidin belonging

to subgroups 3 and 5 present in *P. monodon* were analyzed. Much variation could not be detected in the expression level of penaeidins on administration of these immunostimulants except for some slight variations (Fig. 5.8 to 5.9).

An entirely different expression pattern of the immune genes could be noted post- WSSV challenge. The expression of the gene varied greatly in the control and the immunostimulant fed groups. A general down-regulation of all the target genes could be noticed in the control group of organisms except in case of ALF (Fig. 5.4 to 5.9). This is in agreement with the previous works where a general down-regulation of the immune genes has been reported with bacterial or viral infection (Burgents et al., 2004; Vargas-Albores et al., 2004). However, expression profile of AMPs on administration of immunostimulants post-challenge WSSV was worth noticeable (Fig. 5.4 to 5.9).

ALF was the only AMP gene that was found to be up-regulated post-challenge WSSV even in the control group of shrimps. Expression pattern of ALF post-challenge WSSV clearly indicates its possible antiviral activity. This is in agreement with previous reports by Liu and co-workers (2006), where an up-regulation of ALF gene was noticed in *P. leniusculus* on WSSV challenge. ALF have also shown to be up-regulated on *Vibrio fluvialis* infection (Beale et al., 2008). Also, immunostimulant administrations, especially marine yeasts, were found to up-regulate the gene on WSSV infection, when compared to that of the control group of organisms.

Crustin genes were found to be down-regulated in the control group on WSSV challenge. Crustin-1 and -2 were found to up-regulated to high levels in case of immunostimulant fed groups post-challenge WSSV. Maximum up-regulation was supported by CHY administered group of shrimps. Whereas, only very slight variations could be detected for crustin-3 gene post-challenge WSSV. According to Smith et al. (2008) crustin-1 and -2 belong to type II crustins and crustin-3 belongs to type III crustins. The

variation in expression observed might be because of the fact that these are different crustins with different signature, and they act differently. Also, previous work in *L. vannamei* has shown that expression of crustin does not vary significantly with the administration of β-glucan incorporated diet (Wang et al., 2008). Previous study on administration of β-glucans extracted from sources other than yeast (Scizophyllan) has also reported a down regulation of the AMP genes (Wang et al., 2008).

Expression of penaeidins was worth noticeable when compared to that of crustins, where great variation in the expression pattern was noticed for the marine yeast administered groups. Both penaeidin-3 and -5 were found to be up-regulated considerably in the marine yeast fed groups.

When the expression of all the target AMP genes and WSSV genes were taken into account, marine yeasts proved to be better immunostimulant compared to glucans. Marine yeasts are a good source of vitamins, nucleotides and polysaccharides which act as immunostimulants; besides having proteins, lipids and β-1, 3 glucans have already been proved to be immunostimulants (Chang et al., 1996; Burrells et al., 2001a, 2001b; Sakai et al., 2001; Chang et al., 2003; Burgents et al., 2004). Also, marine yeasts are rich in minerals and these minerals might be imparting immunostimulation. Joseph and Philip (2007) has shown that cadmium and zinc at low concentration act as immunostimulants in *P. monodon*.

Besides, β-1,3-glucans, nucleotides are also proved to be immunostimulants (Sakai et al., 2001). The extraction process of the commonly used β-1,3-glucans is tedious, time consuming and cause additional expenditure. This results in the loss of valuable nutrients from yeast biomass as effluent to be treated and disposed. Comparison of expression levels of *P. monodon* AMP revealed that the crustin expression was highest in unchallenged *P. monodon* followed by penaeidins. This is in agreement with the results of Supungul et al., (2004). Also, Administration of

β-glucan as immunostimulant during the different larval stages of *P. monodon* has been shown to enhance the resistance to vibrios and WSSV (Su et al., 1995; Chang et al., 1996; Liao et al., 1996; Chang et al., 1999, 2003).

In the present study, AMP genes that were most differentially expressed during immuostimulant administration and WSSV challenge were ALF and penaeidins. According to previous works, the genes differentially expressed in the WSSV-infected shrimps included penaeidin-2 (Rojtinnakorn et al., 2002), penaeidin-3 isoforms (O'Leary and Gross, 2006), crustin (Bartlett et al., 2002), penaeidin-3 (Garcia et al., 2009), a C-type lectin (Weis et al., 1998), a protease inhibitor and a chitin-binding domain containing protein (Wang et al., 2007b); these molecules are all directly involved in the immune response of invertebrates.

Analysis of the WSSV gene expression profile also proved marine yeast to be a better immunostimulant than β-glucan (Fig. 5. 10 to 5.17). When control group and glucan administered group gave positive amplifications for all the eight WSSV genes studied, none of the WSSV genes were found to amplify in the CHY administered group of shrimps. This shows that marine yeast administered groups were less infected with WSSV when compared to that of other groups. The above observation is reflected in the post-challenge survival also, where maximum survival rate was supported by the marine yeast fed group, followed by glucan fed groups of shrimps (Fig. 5.26).

Since *C. haemulonii* was found to be the best immunostimulant among the four, tissue-wise analysis of AMP expression was carried out for *C. haemulonii* yeast treated group of animals. Tissue-wise expression displayed haemocytes with maximum transcripts followed by gill, intestine and other tissues, where the expression was comparatively less. Results were in agreement concordant to those in other arthropods where haemocytes are the major sites of AMP synthesis (Iwanaga and Kawabata, 1998).

It has been previously shown that AMPs are constitutively produced and stored in granular haemocytes of shrimps that have not been experimentally infected, indicating haemocytes as the main site of production of the peptides (Destoumieux et al., 2000b). In shrimp tissues, the distribution of penaeidin transcripts is restricted to haemocytes either circulating in blood vessels irrigating tissues such as the brain, hepatopancreas or gills, or infiltrating tissues such as sub-cuticular epithelia or midgut caecum. AMPs are solely present in large-granule haemocytes and small granule haemocytes (also called semigranular cells), and are absent from the hyaline haemocyte population, devoid of granules (Munoz et al., 2002).

mRNA expression of AMPs was found to vary with the tissue (Fig. 5.18 to 5.23). ALF was expressed in high levels in the gills and intestine, followed by hepatopancreas, muscle and heart. Earlier works have also reported that gills and intestine supported maximum expression of ALF gene (Supungul et al., 2004; de la Vega et al., 2008; Beale et al., 2008). Contrasting observations have also been reported by earlier workers (de la Vega et al., 2008) where highest expression was noticed in the heart followed by gills and intestine. Tissue expression profile of crustins in unchallenged shrimps revealed highest expression in the gill followed by intestine, muscle and hepatopancreas. Variation in the expression of the AMPs in tissues might be due to the differential migration of haemocytes to the site of infection for fighting the pathogens. This in turn depends on the tissue specificity of the pathogen for proliferation and establishment. Secondary expression sites such as intestines or gonads have been reported for AMPs in insects (Manetti et al., 1998). Increased expression of AMPs in shrimp intestine also suggests intestine to be an expression site for AMPs. Previous reports in *L. vannamei* are in agreement with the present result where mRNA transcripts could be observed in all tissues including hepatopancreas (Wang

et al., 2007a). However, earlier works in *P. monodon* revealed the absence of crustin transcripts in hepatopancreas (Supungul et al., 2004). In *M. japonicus*, crustin mRNA was detected in haemolymph and not in any other tissue (Rattanachai et al., 2004).

Shrimps possess an open circulatory system that allows haemocytes to infiltrate and adhere to many tissues. In aquatic animals such as shrimps, gills are the major sites for clearance of bacteria (van de Braak et al., 2002). All gene expressions are from the infiltrating haemocytes and the relative expression levels of these genes would represent the amount of haemocytes infiltrating or fixed in tissues (Kang et al., 2004; Liu et al., 2005). Another possible reason is that fixed or infiltrating haemocytes tend to express differentially from circulatory haemocytes because the former is more differentiated than the latter, which affects their gene expressions (Wang et al., 2007a).

With the wide spread occurrence of severe epidemic diseases in shrimp aquaculture, studies on shrimp immune-related genes and their expression receive increasing attention. Based on the present results, we suggest that quantification of AMP gene expression could be used as a potent tool to gauge the state of immune response in shrimps, at least in terms of immunostimulant treatment and WSSV infection. The suggestion is based on the notion that up-regulation of AMP genes is closely related to the increased immune response against WSSV that have been linked to the pre disposition of the shrimp to a state of high immune responsiveness.

Expressions of WSSV related genes showed that marine yeast, *C. haemulonii* treated groups were not infected by WSSV and marine yeast, *C. sake* treated groups less infected with WSSV at the time of sampling (post-challenge 48 hr). This is well supported with the up-regulation pattern of AMP genes noted for these treatment groups. This observation is a good supporting evidence for the better performance of yeast fed group

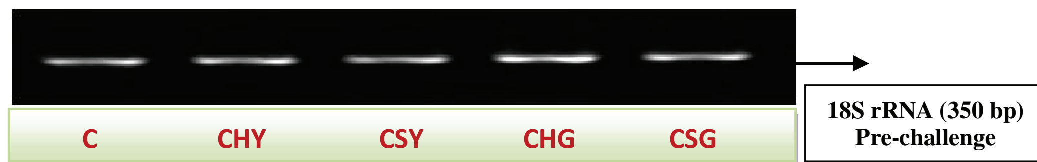
manifested in terms of greater protection and survival on challenge with WSSV (Fig. 5.26).

This study shows that shrimp defense system is equipped enough to evade WSSV infection when the animals were maintained on marine yeast diet, whereas the glucan fed group succumbed to WSSV infection. Factors responsible for this additional protection need to be investigated. This study confirms that marine yeast and β-1,3-glucan supplemented diet can delay the process of WSSV infection and confer greater protection to the animals. Particularly, the protection conferred by marine yeast, *C. haemulonii* S27 was highly promising imparting greater hope to the aquaculture community to overcome the prevailing disease problems in aquaculture.

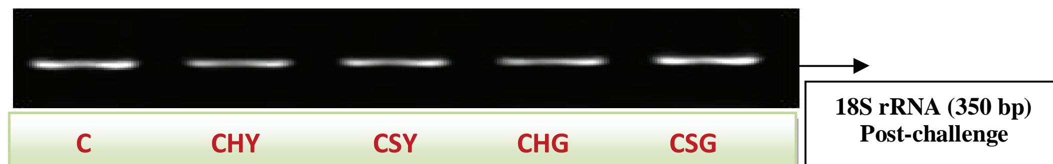
**Table 5.1. Experimental feeds used in the study**

| FEED CODE | FEED COMPOSITION |
|---|---|
| **FC** | Control feed |
| **CHY** | *Candida haemulonii* S27 Yeast (10 %) incorporated diet |
| **CSY** | *Candida sake* S 165Yeast (10 %) incorporated diet |
| **CHG** | *Candida haemulonii* S27 glucan (0.2 %) incorporated diet |
| **CSG** | *Candida sake* S165 glucan (0.2 %) incorporated diet |

(A)




(B)




(C)

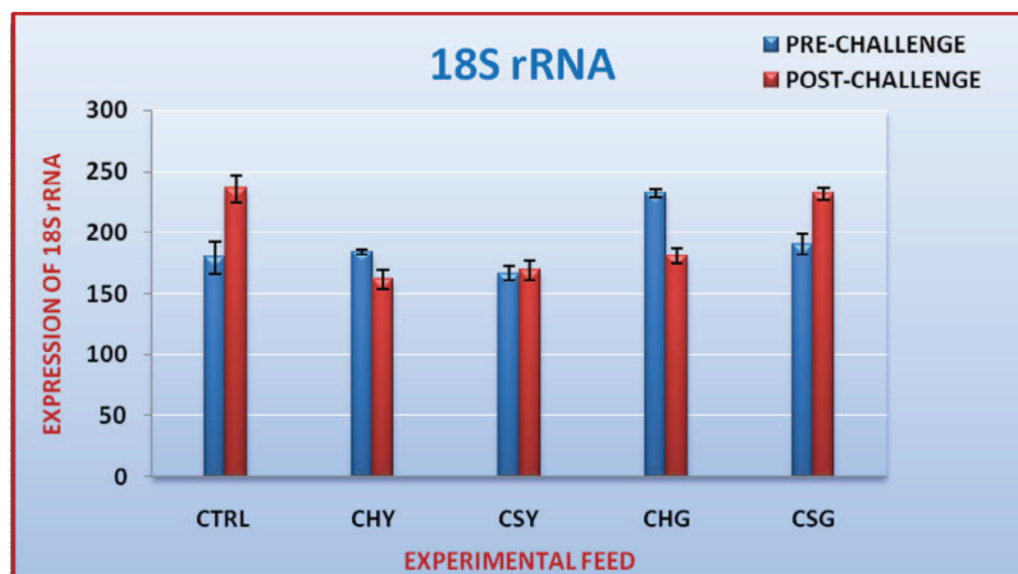



**Fig. 5.1. Expression profile of 18SrRNA (control gene) in the haemocytes of giant tiger shrimp, *P. monodon* in response to the administration of marine whole yeast cell and β-1, 3 glucan as immunostimulants and WSSV challenge**

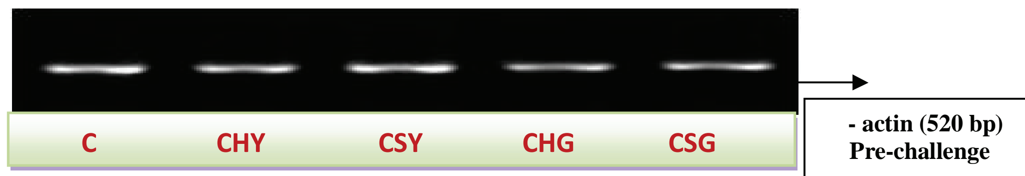
**(A) Agarose gel electrophoretogram - Pre-challenge**

**(B) Agarose gel electrophoretogram - Post-challenge WSSV**

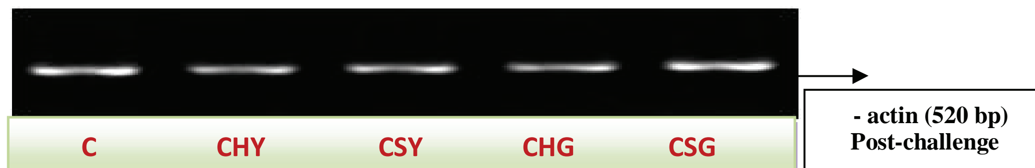
**(C) Graphical representation of the expression levels of 18S rRNA gene (x-axis = immunostimulants used, y-axis = Expression levels of the 18S rRNA gene)**

*(CTRL – Control Feed, CHY – Candida haemulonii yeast, CSY – Candida sake yeast, CHG – Candida haemulonii glucan, CSG – Candida sake glucan)*

(A)




(B)




(C)

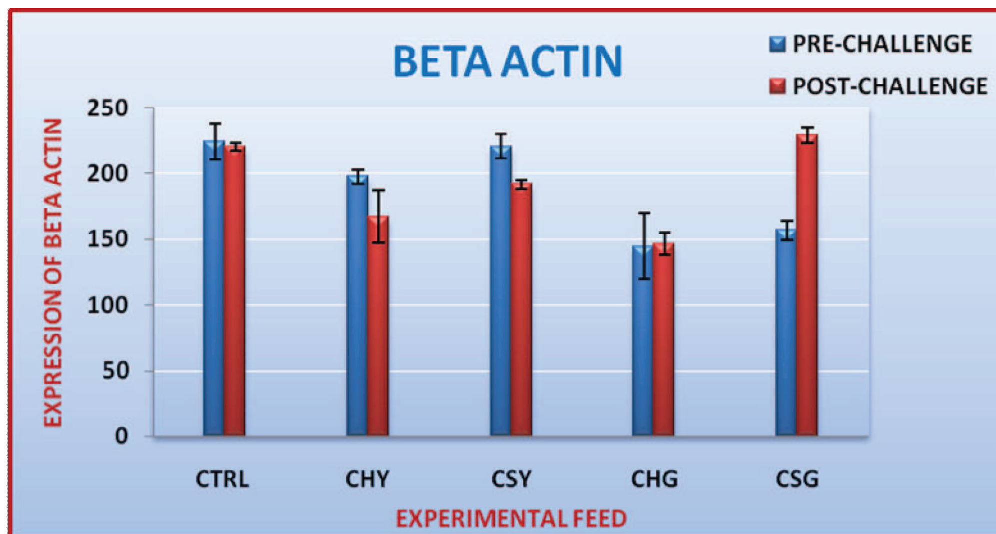



**Fig. 5.2. Expression profile of β-actin (control gene) in the haemocytes of giant tiger shrimp, *P. monodon* in response to the administration of marine whole yeast cell and β-1, 3 glucan as immunostimulants and WSSV challenge**

**(A) Agarose gel electrophoretogram - Pre-challenge**

**(B) Agarose gel electrophoretogram - Post-challenge WSSV**

**(C) Graphical representation of the expression levels of β-actin gene (x-axis = immunostimulants used, y-axis = Expression levels of the β-actin gene)**

*(CTRL – Control Feed, CHY – Candida haemulonii yeast, CSY – Candida sake yeast, CHG – Candida haemulonii glucan, CSG – Candida sake glucan)*

**(A)**

**(B)**

**(C)**

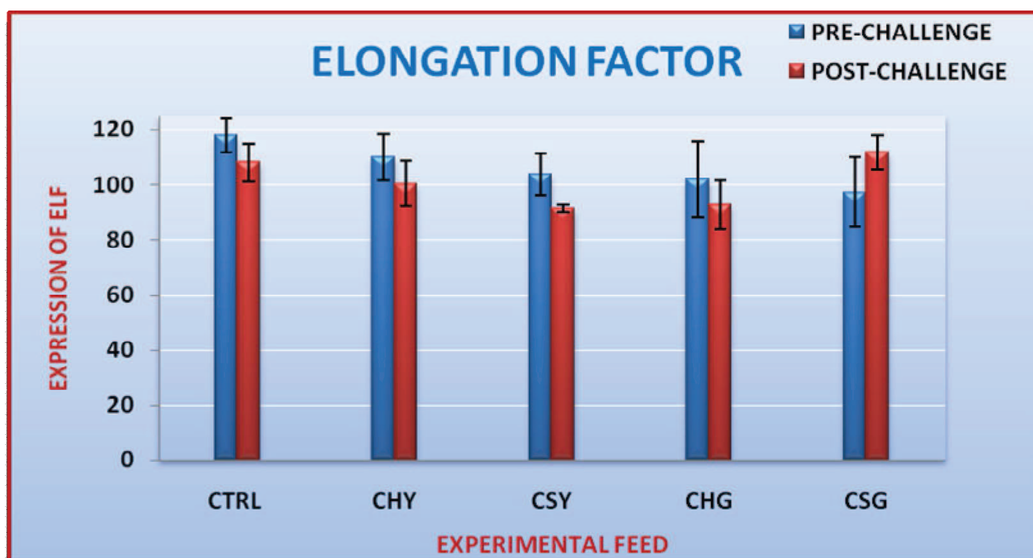



**Fig. 5.3. Expression profile of elongation factor (control gene) in the haemocytes of giant tiger shrimp, *P. monodon* in response to the administration of marine whole yeast cell and β-1, 3 glucan as immunostimulants and WSSV challenge**

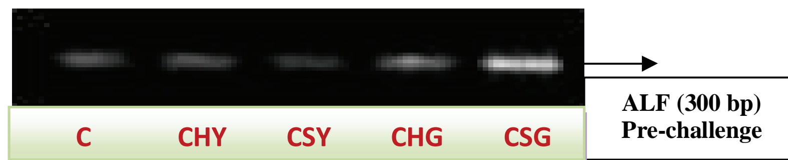
**(A) Agarose gel electrophoretogram - Pre-challenge**

**(B) Agarose gel electrophoretogram - Post-challenge WSSV**

**(C) Graphical representation of the expression levels of elongation factor gene (x-axis = immunostimulants used, y-axis = Expression levels of the elongation factor gene)**

***(CTRL – Control Feed, CHY – Candida haemulonii yeast, CSY – Candida sake yeast, CHG – Candida haemulonii glucan, CSG – Candida sake glucan)***

(A)




(B)

(C)

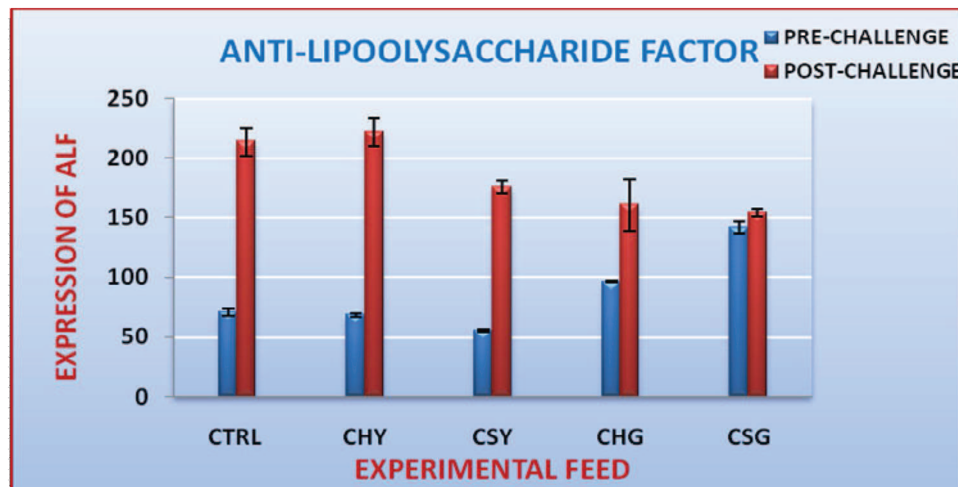



**Fig. 5.4. Expression profile of anti-lipopolysaccharide factor (AMP gene) in the haemocytes of giant tiger shrimp, *P. monodon* in response to the administration of marine whole yeast cell and β-1, 3 glucan as immunostimulants and WSSV challenge**

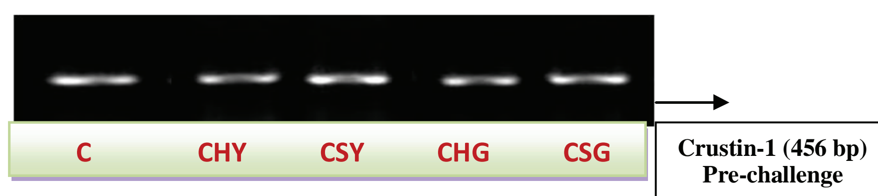
**(A) Agarose gel electrophoretogram - Pre-challenge**

**(B) Agarose gel electrophoretogram - Post-challenge WSSV**

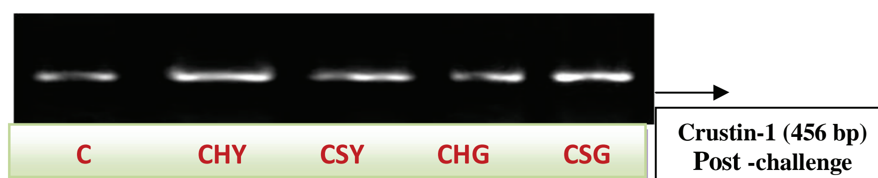
**(C) Graphical representation of the expression levels of anti-lipopolysaccharide factor gene (x-axis = immunostimulants used, y-axis = Expression levels of the anti-lipopolysaccharide factor gene)**

*(CTRL – Control Feed, CHY – Candida haemulonii yeast, CSY – Candida sake yeast, CHG – Candida haemulonii glucan, CSG – Candida sake glucan)*

(A)




(B)




(C)

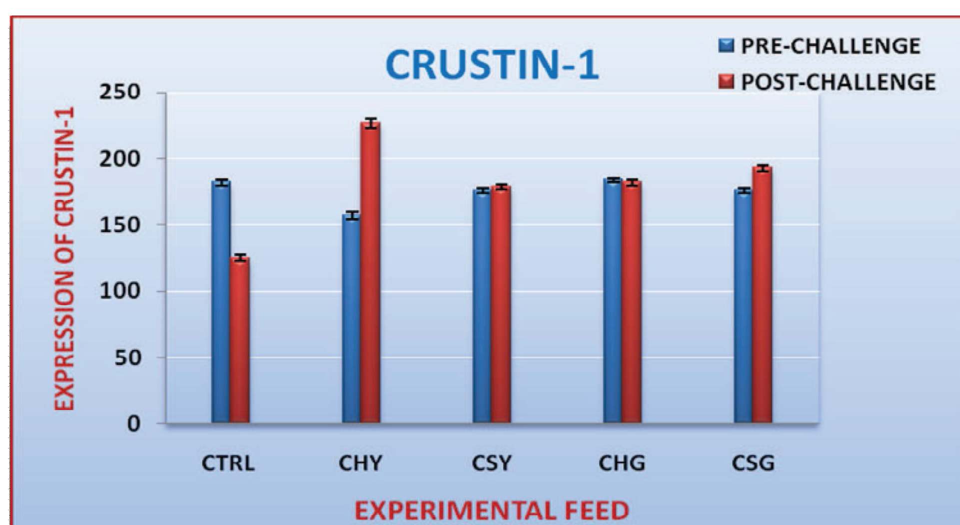



**Fig. 5.5. Expression profile of crustin-1 (AMP gene) in the haemocytes of giant tiger shrimp, *P. monodon* in response to the administration of marine whole yeast cell and β-1, 3 glucan as immunostimulants and WSSV challenge**

**(A) Agarose gel electrophoretogram - Pre-challenge**

**(B) Agarose gel electrophoretogram - Post-challenge WSSV**

**(C) Graphical representation of the expression levels of crustin-1 gene (x-axis = immunostimulants used, y-axis = Expression levels of the crustin-1 gene)**

***(CTRL – Control Feed, CHY – Candida haemulonii yeast, CSY – Candida sake yeast, CHG – Candida haemulonii glucan, CSG – Candida sake glucan)***

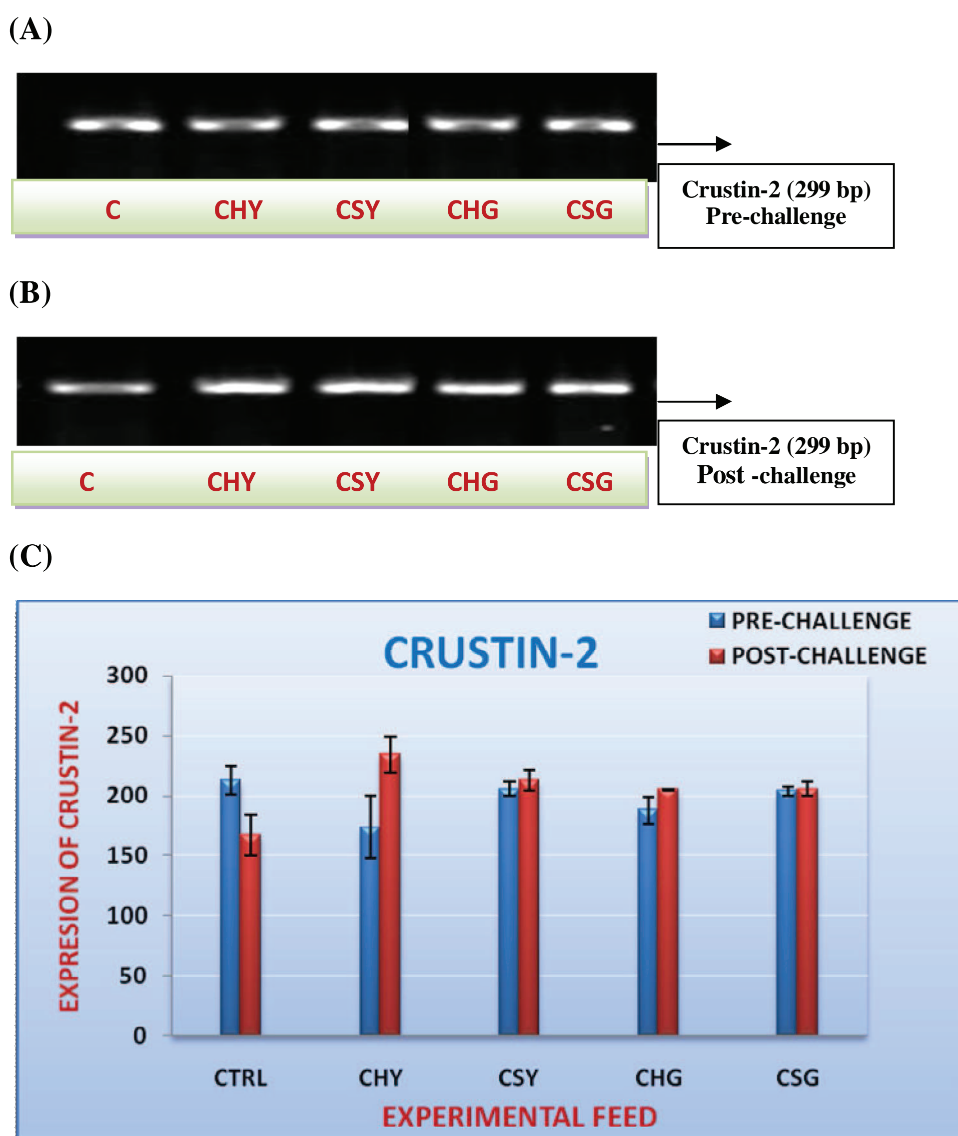



**Fig. 5.6. Expression profile of crustin-2 (AMP gene) in the haemocytes of giant tiger shrimp, *P. monodon* in response to the administration of marine whole yeast cell and β-1, 3 glucan as immunostimulants and WSSV challenge**

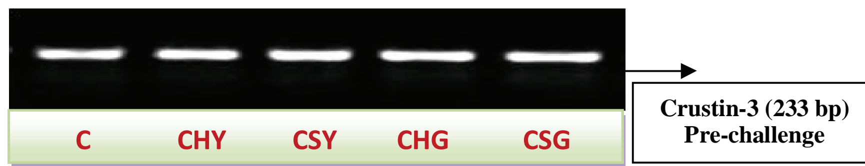
**(A) Agarose gel electrophoretogram - Pre-challenge**

**(B) Agarose gel electrophoretogram - Post-challenge WSSV**

**(C) Graphical representation of the expression levels of crustin-2 gene (x-axis = immunostimulants used, y-axis = Expression levels of the crustin-2 gene)**

*(CTRL – Control Feed, CHY – Candida haemulonii yeast, CSY – Candida sake yeast, CHG – Candida haemulonii glucan, CSG – Candida sake glucan)*

(A)




(B)

(C)

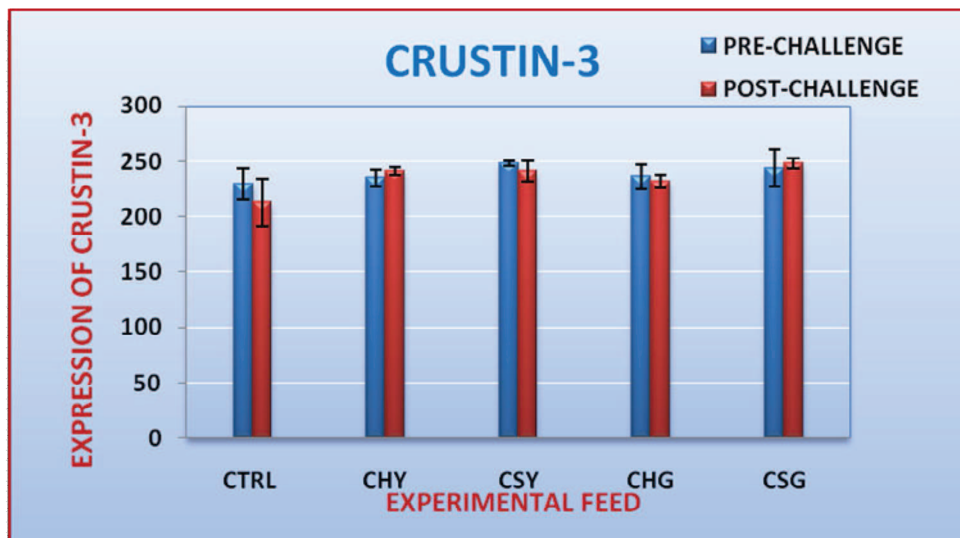



**Fig. 5.7. Expression profile of crustin-3 (AMP gene) in the haemocytes of giant tiger shrimp, *P. monodon* in response to the administration of marine whole yeast cell and β-1, 3 glucan as immunostimulants and WSSV challenge**

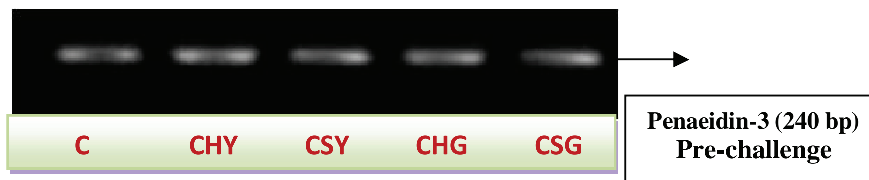
**(A) Agarose gel electrophoretogram - Pre-challenge**

**(B) Agarose gel electrophoretogram - Post-challenge WSSV**

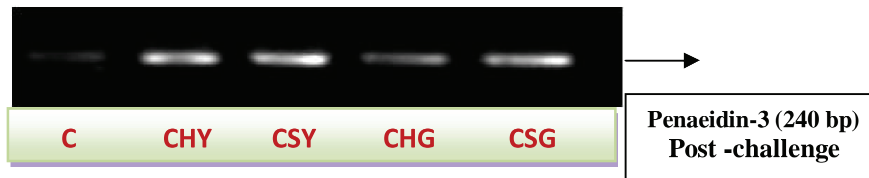
**(C) Graphical representation of the expression levels of crustin-3 gene (x-axis = immunostimulants used, y-axis = Expression levels of the crustin-3 gene)**

*(CTRL – Control Feed, CHY – Candida haemulonii yeast, CSY – Candida sake yeast, CHG – Candida haemulonii glucan, CSG – Candida sake glucan)*

(A)




(B)




(C)

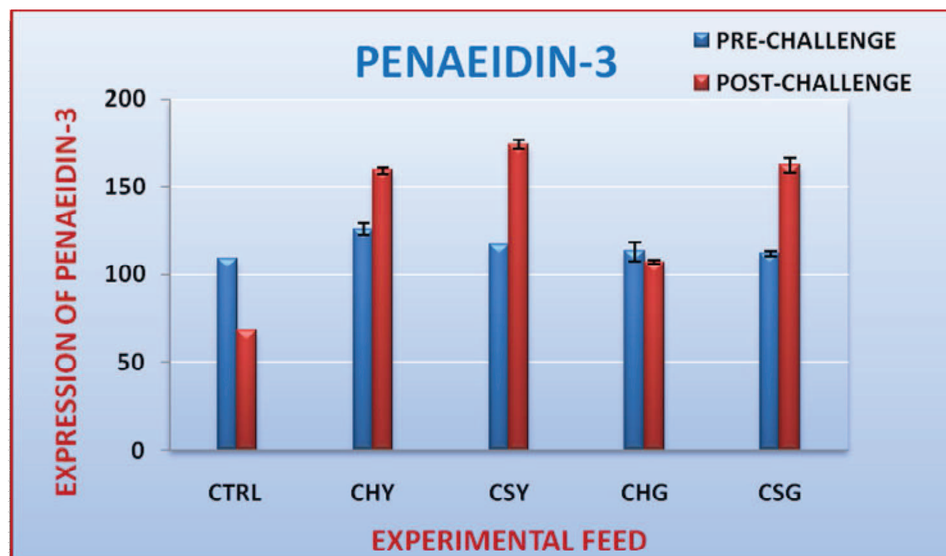



**Fig. 5.8. Expression profile of penaeidin-3 (AMP gene) in the haemocytes of giant tiger shrimp, *P.monodon* in response to the administration of marine whole yeast cell and β-1, 3 glucan as immunostimulants and WSSV challenge**

**(A) Agarose gel electrophoretogram - Pre-challenge**

**(B) Agarose gel electrophoretogram - Post-challenge WSSV**

**(C) Graphical representation of the expression levels of penaeidin-3 gene (x-axis = immunostimulants used, y-axis = Expression levels of the penaeidin-3 gene)**

*(CTRL – Control Feed, CHY – Candida haemulonii yeast, CSY – Candida sake yeast, CHG – Candida haemulonii glucan, CSG – Candida sake glucan)*

(A)

(B)

(C)

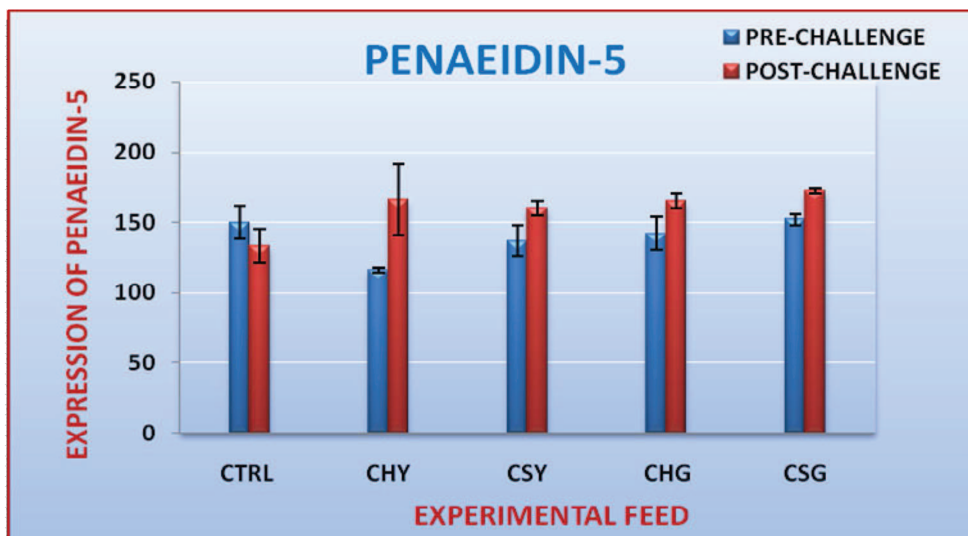



**Fig.5. 9. Expression profile of penaeidin-5 (AMP gene) in the haemocytes of giant tiger shrimp, *P. monodon* in response to the administration of marine whole yeast cell and β-1, 3 glucan as immunostimulants and WSSV challenge**

**(A) Agarose gel electrophoretogram - Pre-challenge**

**(B) Agarose gel electrophoretogram - Post-challenge WSSV**

**(C) Graphical representation of the expression levels of penaeidin-5 gene (x-axis = immunostimulants used, y-axis = Expression levels of the penaeidin-5 gene)**

*(CTRL – Control Feed, CHY – Candida haemulonii yeast, CSY – Candida sake yeast, CHG – Candida haemulonii glucan, CSG – Candida sake glucan)*

(A)

(B)

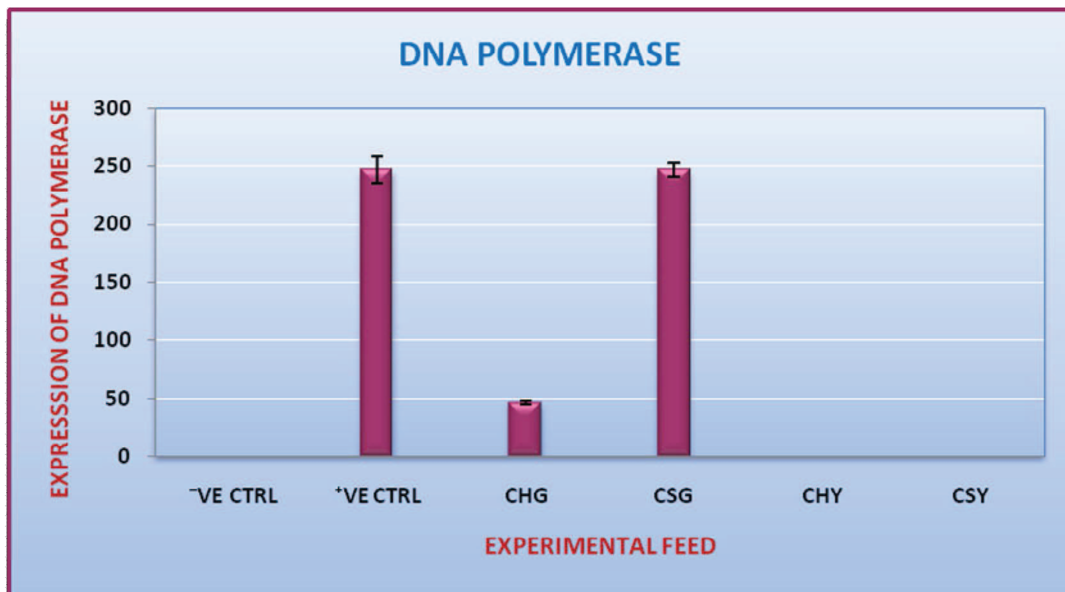



**Fig. 5.10. Expression profile of DNA polymerase (WSSV gene) in the haemocytes of giant tiger shrimp, *P. monodon* in response to the administration of marine whole yeast cell and β-1, 3 glucan as immunostimulants and WSSV challenge**

**(A) Agarose gel electrophoretogram**

**(B) Graphical representation of the expression levels of DNA polymerase gene (x-axis = immunostimulants used, y-axis = Expression levels of the DNA polymerase gene)**

*(-ve control – Unchallenged shrimp, +ve control - WSSV Challenged shrimp, CHY – Candida haemulonii yeast, CSY – Candida sake yeast, CHG – Candida haemulonii glucan, CSG – Candida sake glucan)*

(A)

(B)

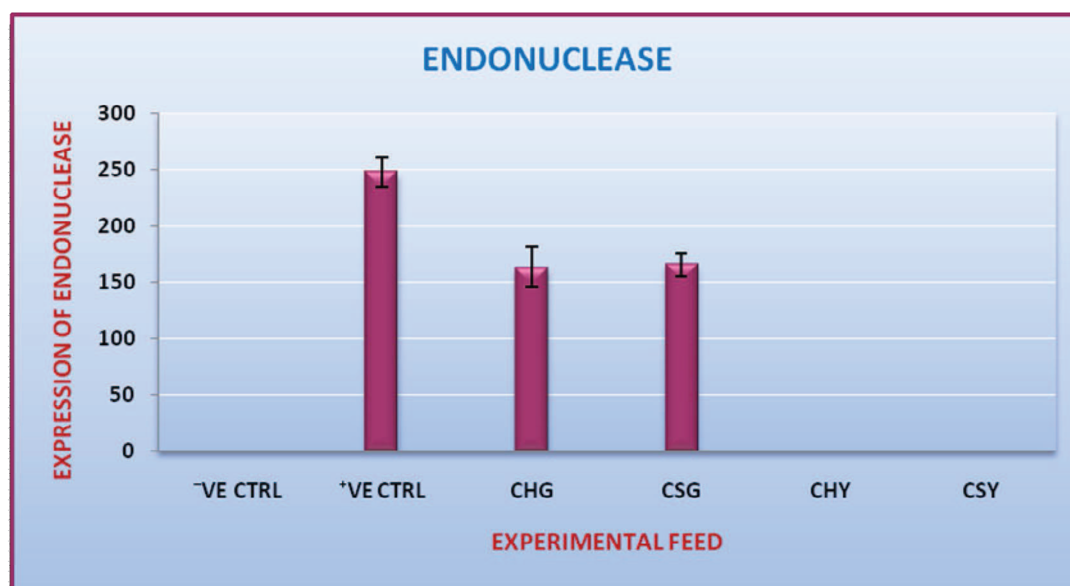


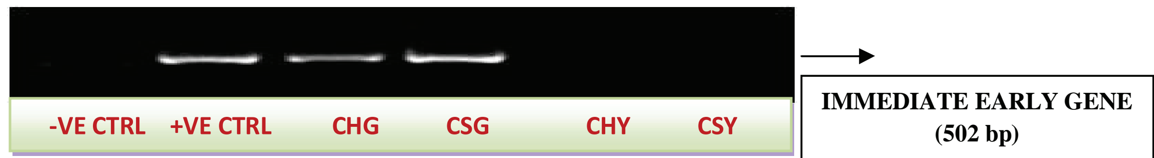

**Fig. 5.11. Expression profile of endonuclease (WSSV gene) in the haemocytes of giant tiger shrimp, *P. monodon* in response to the administration of marine whole yeast cell and β-1, 3 glucan as immunostimulants and WSSV challenge**

**(A) Agarose gel electrophoretogram**

**(B) Graphical representation of the expression levels of endonuclease gene (x-axis = immunostimulants used, y-axis = Expression levels of the endonuclease gene)**

*(-ve control – Unchallenged shrimp, +ve control - WSSV Challenged shrimp, CHY – Candida haemulonii yeast, CSY – Candida sake yeast, CHG – Candida haemulonii glucan, CSG – Candida sake glucan)*

(A)




(B)

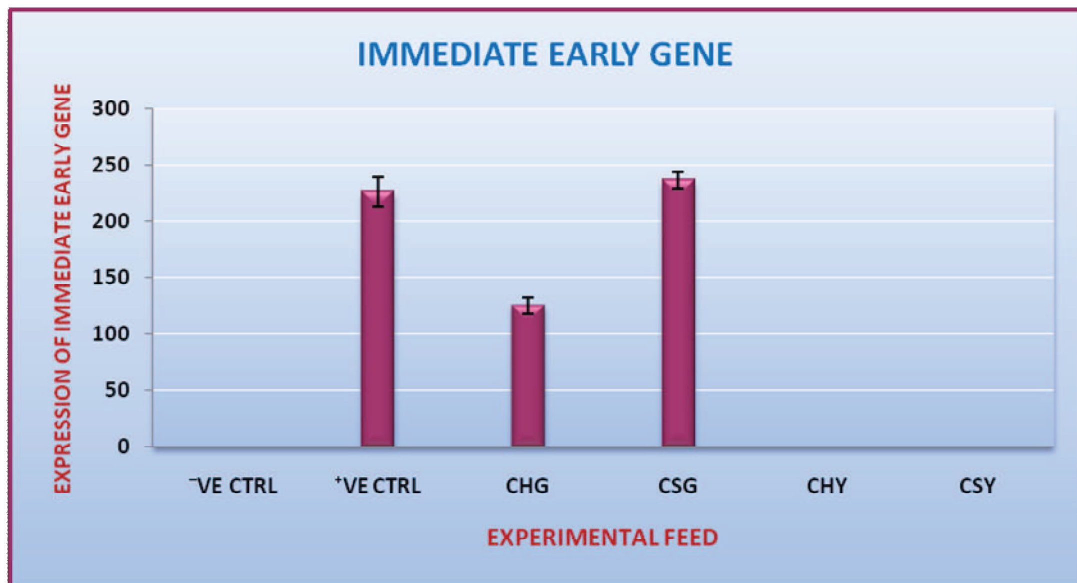


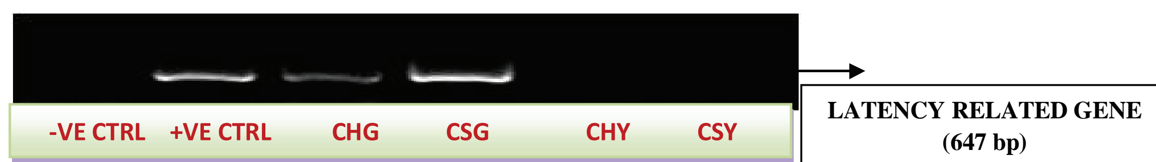

**Fig.5.12. Expression profile of immediate early gene (WSSV gene) in the haemocytes of giant tiger shrimp, *P. monodon* in response to the administration of marine whole yeast cell and β-1, 3 glucan as immunostimulants and WSSV challenge**

**(A) Agarose gel electrophoretogram**

**(B) Graphical representation of the expression levels of immediate early gene (x-axis = immunostimulants used, y-axis = Expression levels of the immediate early gene)**

*(-ve control – Unchallenged shrimp, +ve control - WSSV Challenged shrimp, CHY – Candida haemulonii yeast, CSY – Candida sake yeast, CHG – Candida haemulonii glucan, CSG – Candida sake glucan)*

(A)




(B)

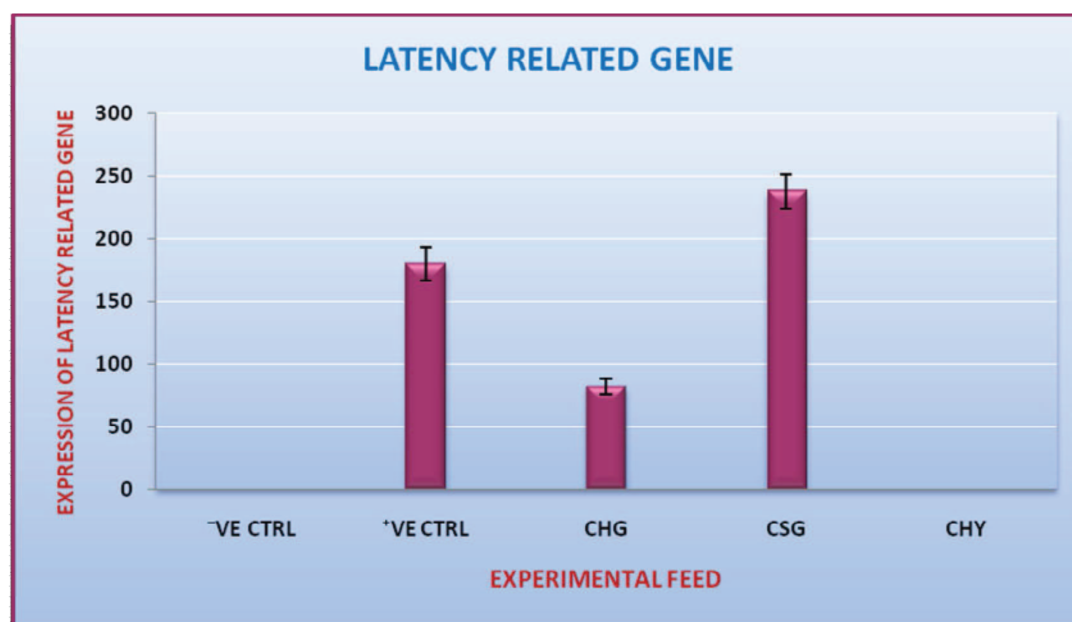



**Fig. 5.13. Expression profile of latency related gene (WSSV gene) in the haemocytes of giant tiger shrimp, *P. monodon* in response to the administration of marine whole yeast cell and β-1, 3 glucan as immunostimulants and WSSV challenge**

**(A) Agarose gel electrophoretogram**

**(B) Graphical representation of the expression levels of latency related gene gene (x-axis = immunostimulants used, y-axis = Expression levels of the latency related gene)**

*(-ve control – Unchallenged shrimp, +ve control - WSSV Challenged shrimp, CHY – Candida haemulonii yeast, CSY – Candida sake yeast, CHG – Candida haemulonii glucan, CSG – Candida sake glucan)*

(A)

(B)

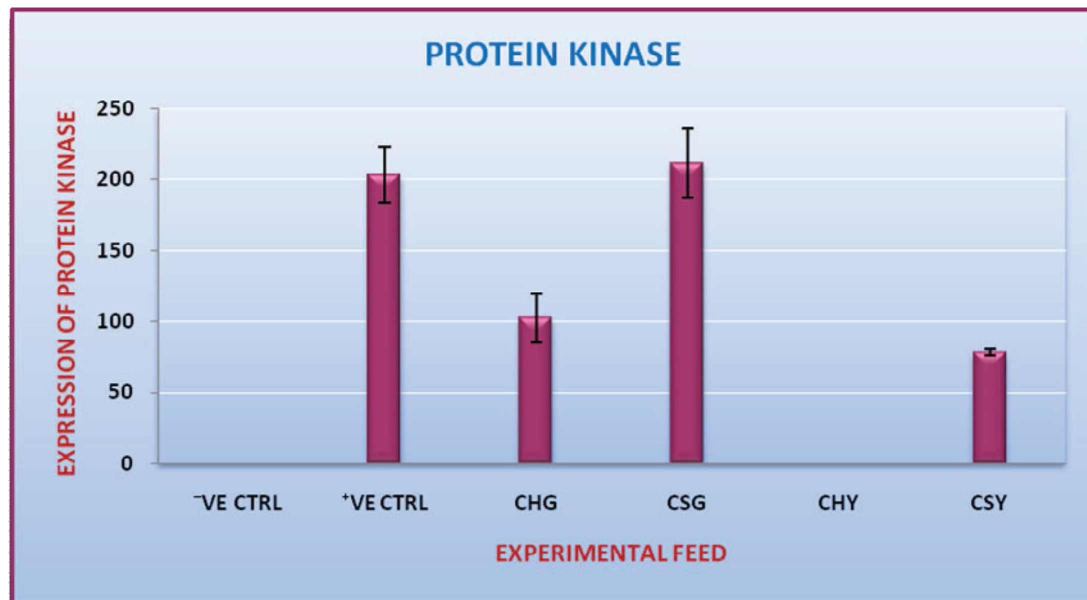


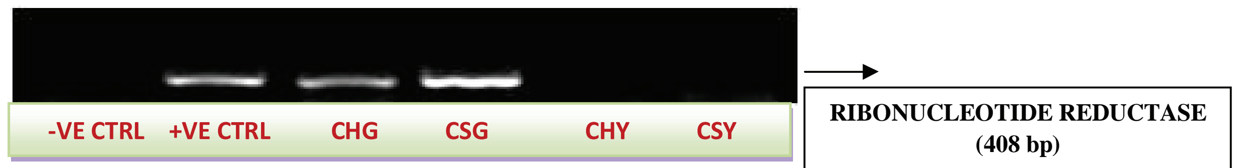

**Fig. 5.14. Expression profile of protein kinase (WSSV gene) in the haemocytes of giant tiger shrimp, *P. monodon* in response to the administration of marine whole yeast cell and β-1, 3 glucan as immunostimulants and WSSV challenge**

**(A) Agarose gel electrophoretogram**

**(B) Graphical representation of the expression levels of protein kinase gene (x-axis = immunostimulants used, y-axis = Expression levels of the protein kinase gene)**

*(-ve control – Unchallenged shrimp, +ve control - WSSV Challenged shrimp, CHY – Candida haemulonii yeast, CSY – Candida sake yeast, CHG – Candida haemulonii glucan, CSG – Candida sake glucan)*

(A)




(B)

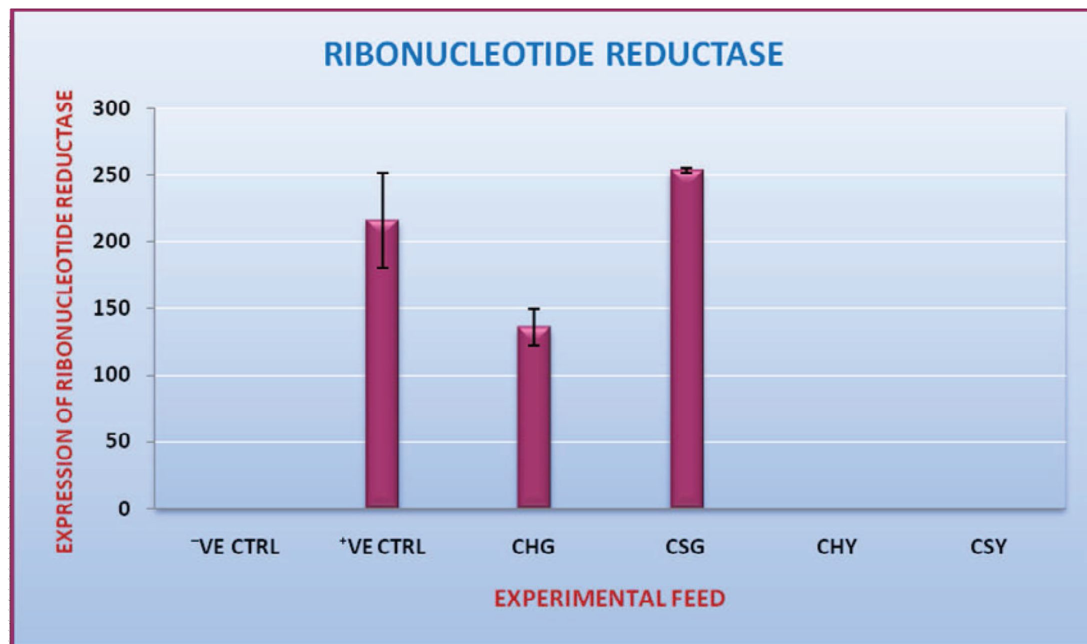


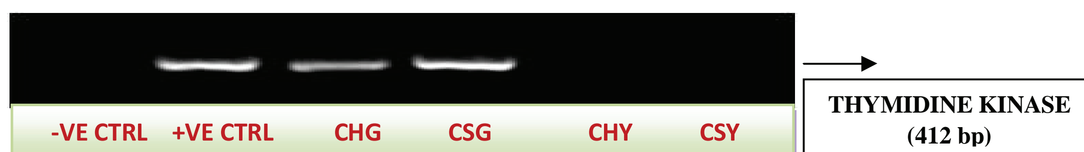

**Fig.5.15. Expression profile of ribonucleotide reductase (WSSV gene) in the haemocytes of giant tiger shrimp, *P. monodon* in response to the administration of marine whole yeast cell and β-1, 3 glucan as immunostimulants and WSSV challenge**

**(A) Agarose gel electrophoretogram**

**(B) Graphical representation of the expression levels of ribonucleotide reductase gene (x-axis = immunostimulants used, y-axis = Expression levels of the ribonucleotide reductase gene)**

*(-ve control – Unchallenged shrimp, +ve control - WSSV Challenged shrimp, CHY – Candida haemulonii yeast, CSY – Candida sake yeast, CHG – Candida haemulonii glucan, CSG – Candida sake glucan)*

(A)




(B)

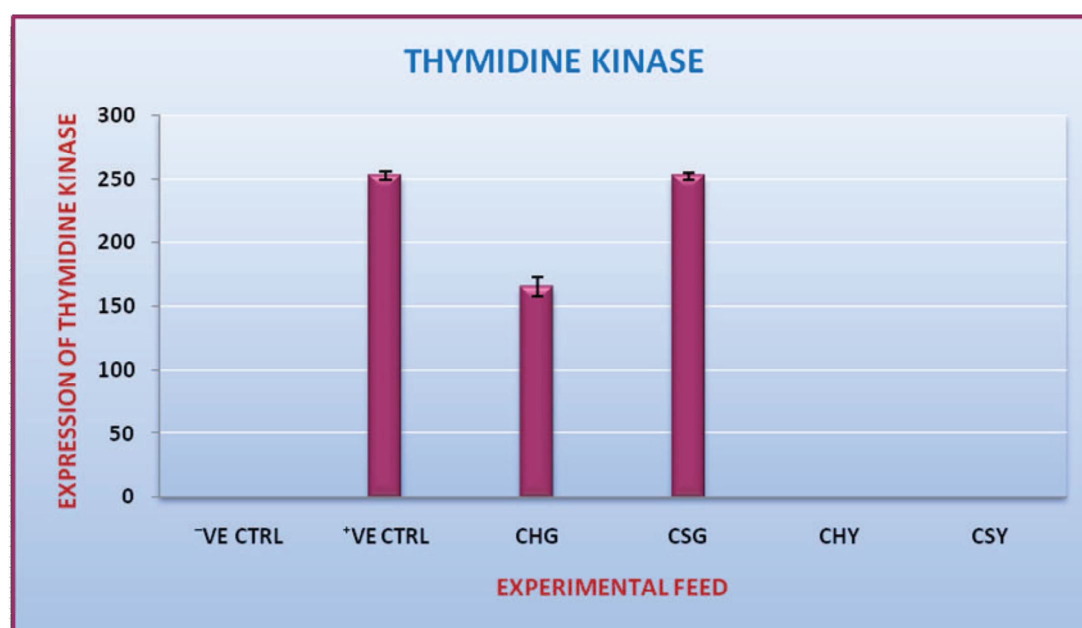



**Fig. 5.16. Expression profile of thymidine kinase (WSSV gene) in the haemocytes of giant tiger shrimp, *P. monodon* in response to the administration of marine whole yeast cell and β-1, 3 glucan as immunostimulants and WSSV challenge**

**(A) Agarose gel electrophoretogram**

**(B) Graphical representation of the expression levels of thymidine kinase gene (x-axis = immunostimulants used, y-axis = Expression levels of the thymidine kinase gene)**

*(-ve control – Unchallenged shrimp, +ve control - WSSV Challenged shrimp, CHY – Candida haemulonii yeast, CSY – Candida sake yeast, CHG – Candida haemulonii glucan, CSG – Candida sake glucan)*

(A)

(B)

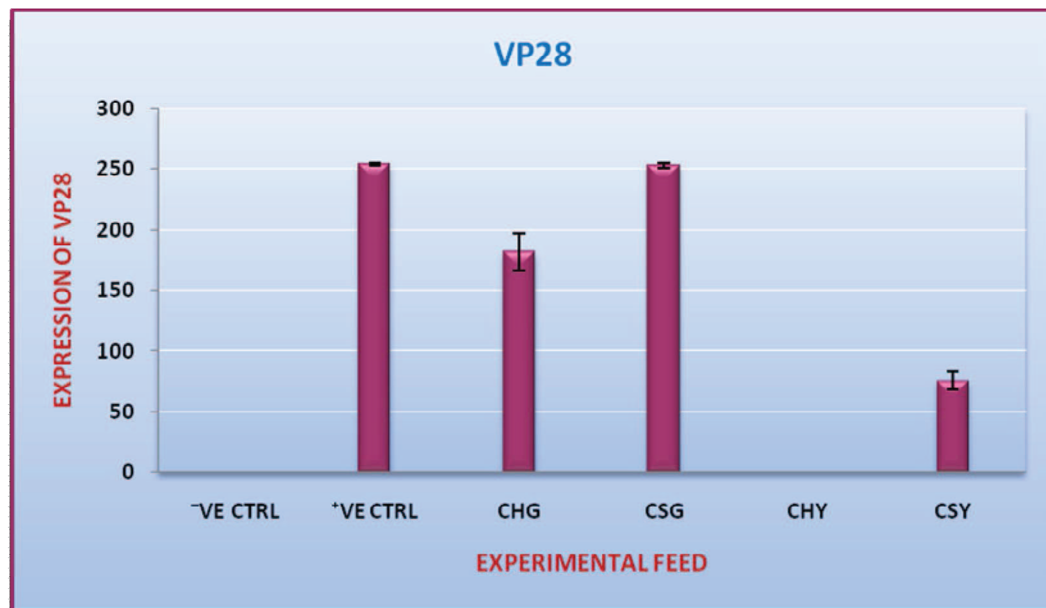



**Fig.5.17. Expression profile of VP28 (WSSV gene) in the haemocytes of giant tiger shrimp, *P. monodon* in response to the administration of marine whole yeast cell and β-1, 3 glucan as immunostimulants and WSSV challenge**

**(A) Agarose gel electrophoretogram**

**(B) Graphical representation of the expression levels of VP28 gene (x-axis = immunostimulants used, y-axis = Expression levels of the VP28 gene)**

*(-ve control – Unchallenged shrimp, +ve control - WSSV Challenged shrimp, CHY – Candida haemulonii yeast, CSY – Candida sake yeast, CHG – Candida haemulonii glucan, CSG – Candida sake glucan)*

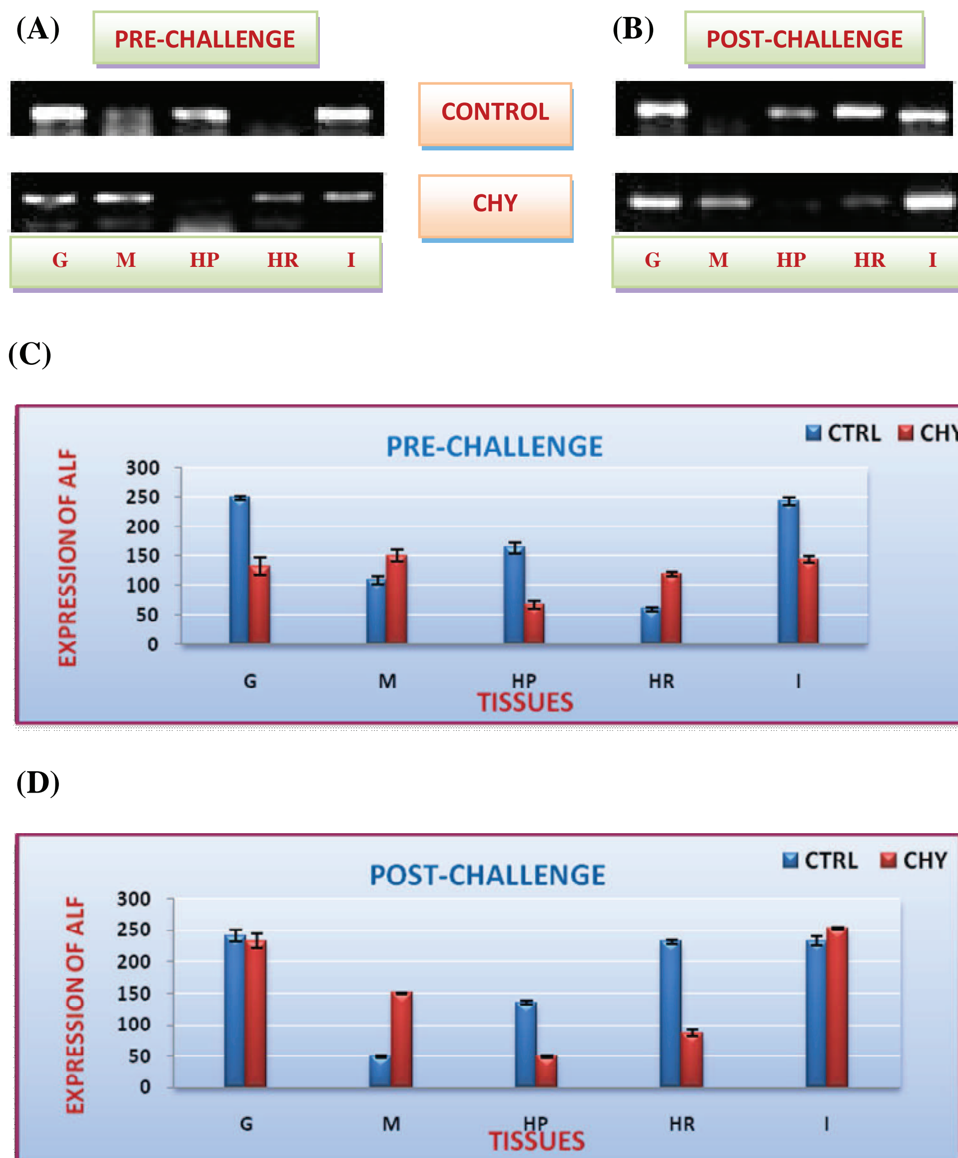



**Fig. 5.18. Tissue-wise expression profile of ALF (AMP gene) in various tissues of giant tiger shrimp, *P. monodon* in response to the administration of marine yeast *C. haemulonii* (CHY) as immunostimulant and WSSV challenge**

**(A) Agarose gel electrophoretogram of control and CHY treated groups pre-challenge WSSV**

**(B) Agarose gel electrophoretogram of control and CHY treated groups Post-challenge WSSV**

**(C) & (D) Graphical representation of expression level of ALF gene pre- and post-challenge WSSV (x-axis = tissues analyzed, y-axis = expression level of ALF gene)**

*(G-Gill, M-Muscle, HP-Hepatopancreas, HR-Heart, I-Intestine)*

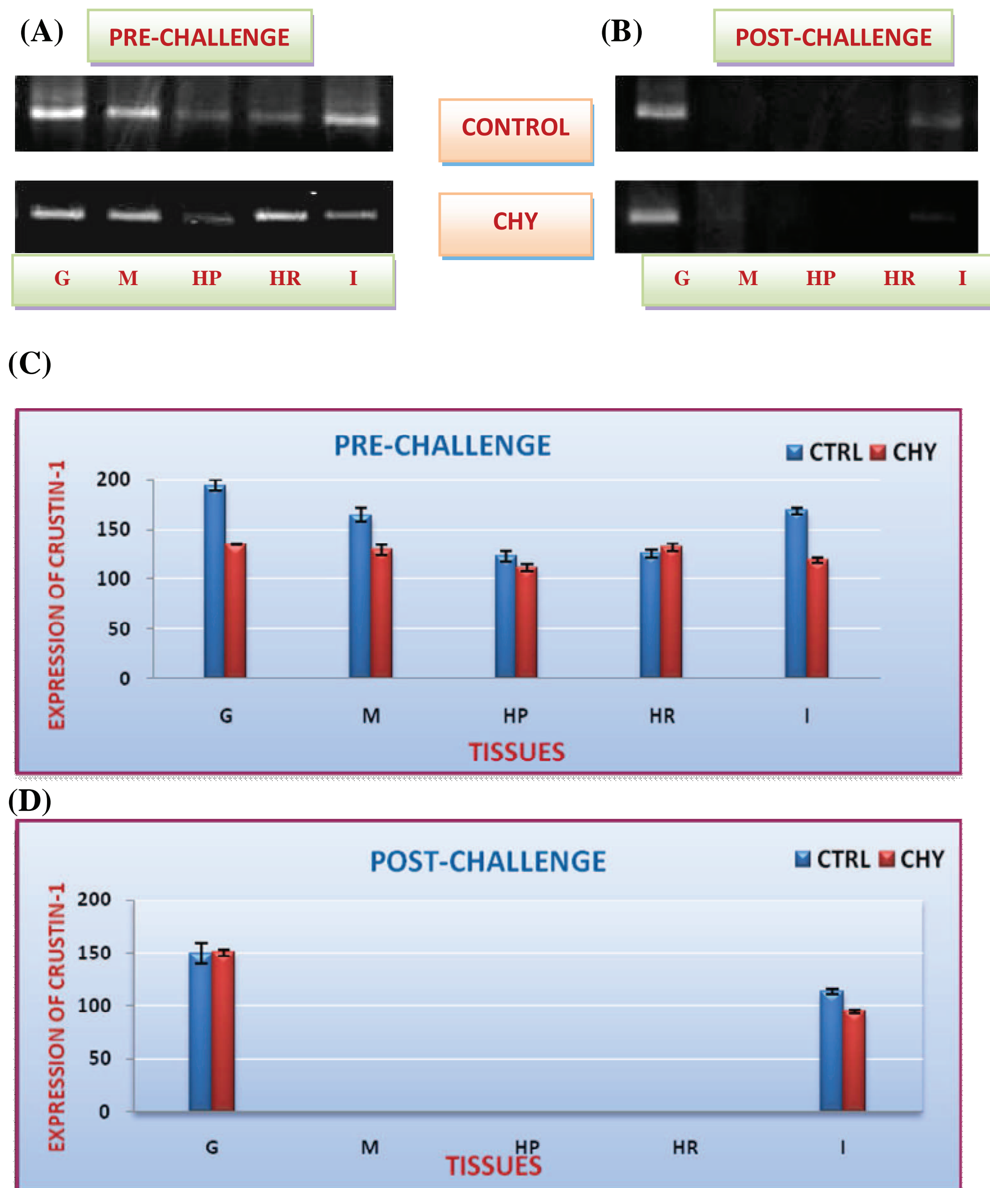



**Fig. 5.19. Tissue-wise expression profile of crustin-1 (AMP gene) in various tissues of giant tiger shrimp, *P. monodon* in response to the administration of marine yeast *C. haemulonii* (CHY) as immunostimulant and WSSV challenge**

(A) Agarose gel electrophoretogram of control and CHY treated groups pre-challenge WSSV

(B) Agarose gel electrophoretogram of control and CHY treated groups Post-challenge WSSV

(C) & (D) Graphical representation of expression level of ALF gene pre- and post-challenge WSSV (x-axis = tissues analyzed, y-axis = expression level of ALF gene)

*(G-Gill, M-Muscle, HP-Hepatopancreas, HR-Heart, I-Intestine)*

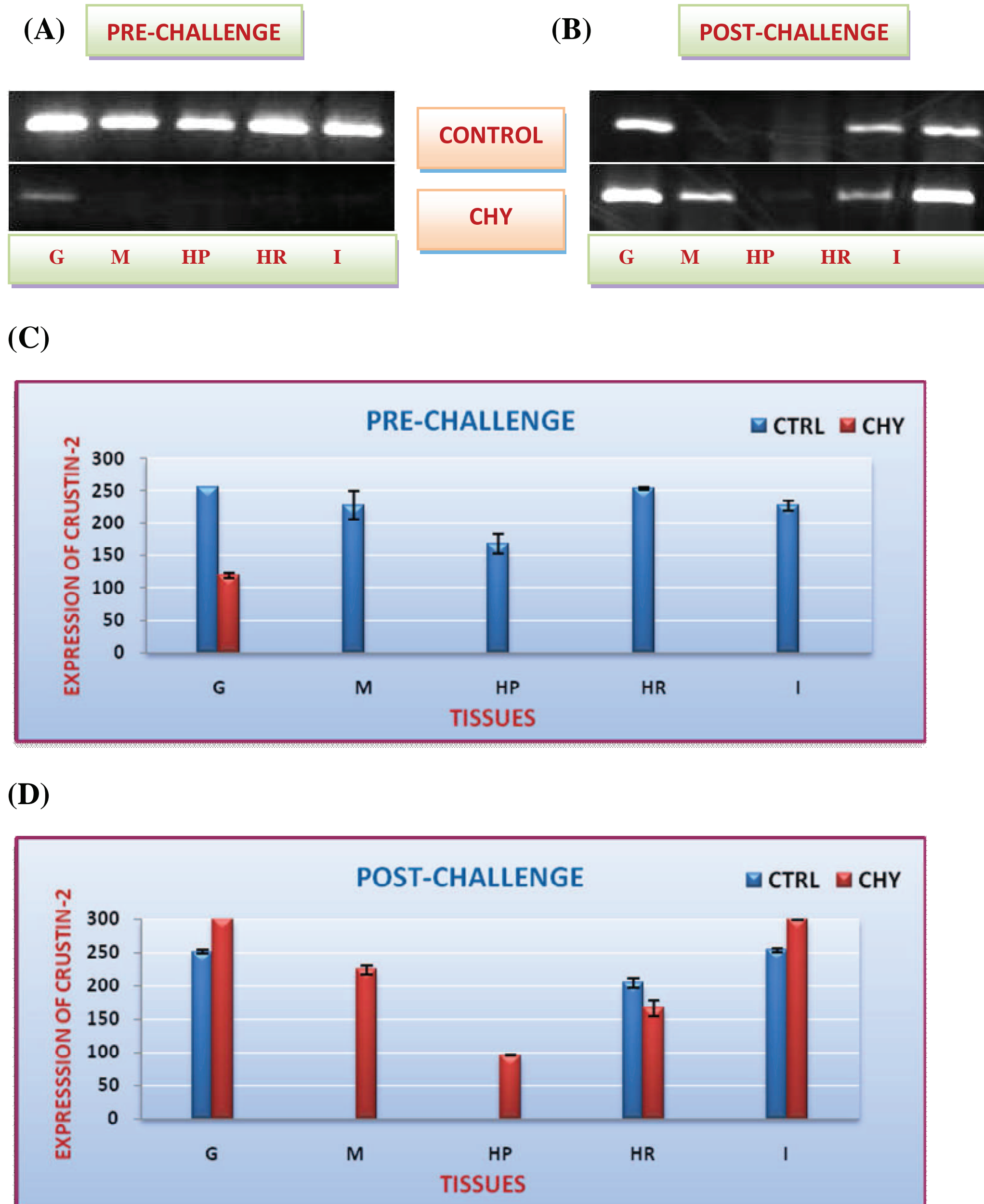



**Fig. 5.20. Tissue-wise expression profile of crustin-2 (AMP gene) in various tissues of giant tiger shrimp, *P. monodon* in response to the administration of marine yeast *C. haemulonii* (CHY) as immunostimulant and WSSV challenge**

**(A) Agarose gel electrophoretogram of control and CHY treated groups pre-challenge WSSV**

**(B) Agarose gel electrophoretogram of control and CHY treated groups Post-challenge WSSV**

**(C) & (D) Graphical representation of expression level of ALF gene pre- and post-challenge WSSV (x-axis = tissues analyzed, y-axis = expression level of ALF gene)**

*(G-Gill, M-Muscle, HP-Hepatopancreas, HR-Heart, I-Intestine)*

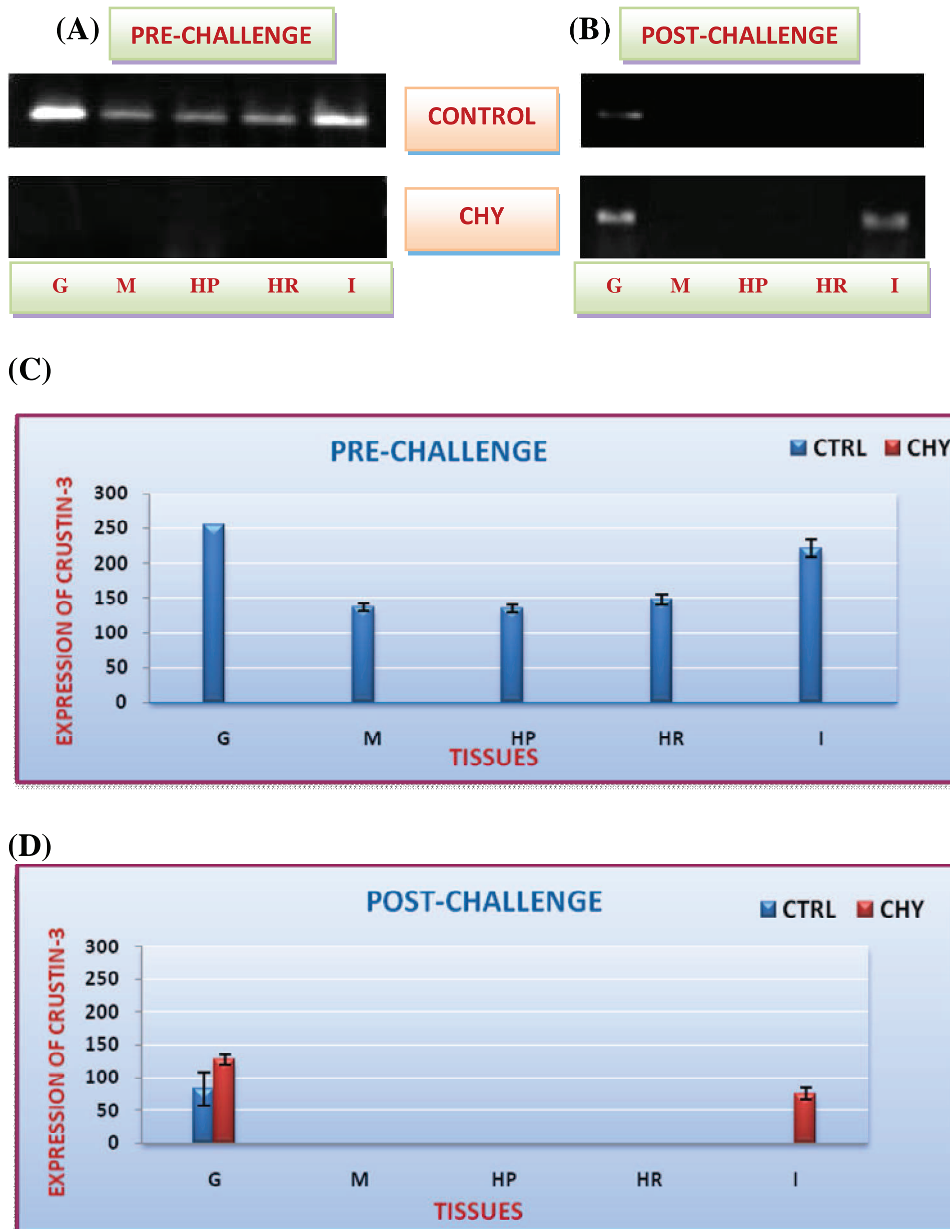



**Fig. 5.21. Tissue-wise expression profile of crustin-3 (AMP gene) in various tissues of giant tiger shrimp, *P. monodon* in response to the administration of marine yeast *C. haemulonii* (CHY) as immunostimulant and WSSV challenge**

**(A) Agarose gel electrophoretogram of control and CHY treated groups pre-challenge WSSV**

**(B) Agarose gel electrophoretogram of control and CHY treated groups Post-challenge WSSV**

**(C) & (D) Graphical representation of expression level of ALF gene pre- and post-challenge WSSV (x-axis = tissues analyzed, y-axis = expression level of ALF gene)**

***(G-Gill, M-Muscle, HP-Hepatopancreas, HR-Heart, I-Intestine)***

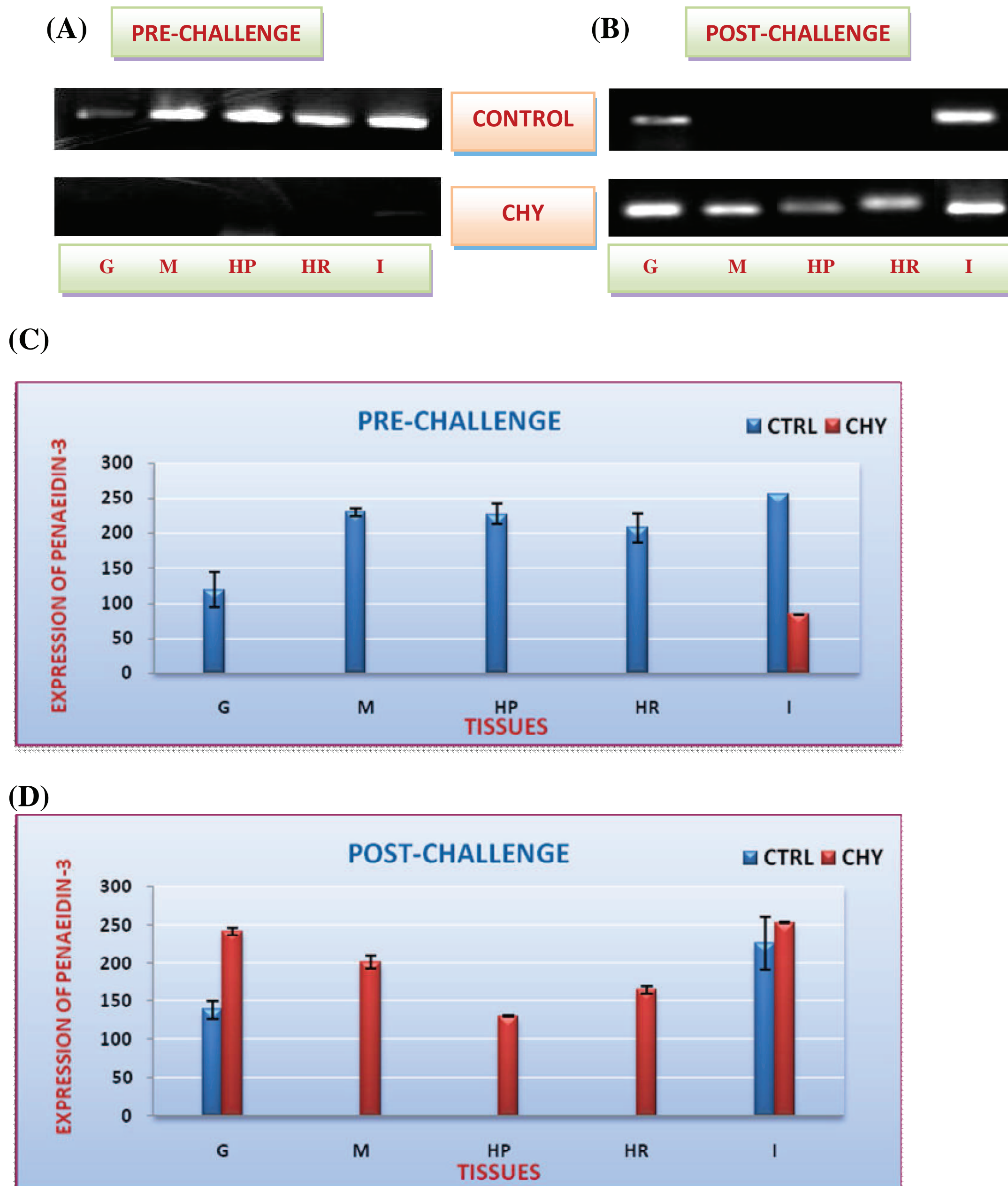



**Fig. 5.22. Tissue-wise expression profile of penaeidin-3 (AMP gene) in various tissues of giant tiger shrimp, *P. monodon* in response to the administration of marine yeast *C. haemulonii* (CHY) as immunostimulant and WSSV challenge**

**(A) Agarose gel electrophoretogram of control and CHY treated groups pre-challenge WSSV**

**(B) Agarose gel electrophoretogram of control and CHY treated groups Post-challenge WSSV**

**(C) & (D) Graphical representation of expression level of ALF gene pre- and post-challenge WSSV (x-axis = tissues analyzed, y-axis = expression level of ALF gene)**

***(G-Gill, M-Muscle, HP-Hepatopancreas, HR-Heart, I-Intestine)***

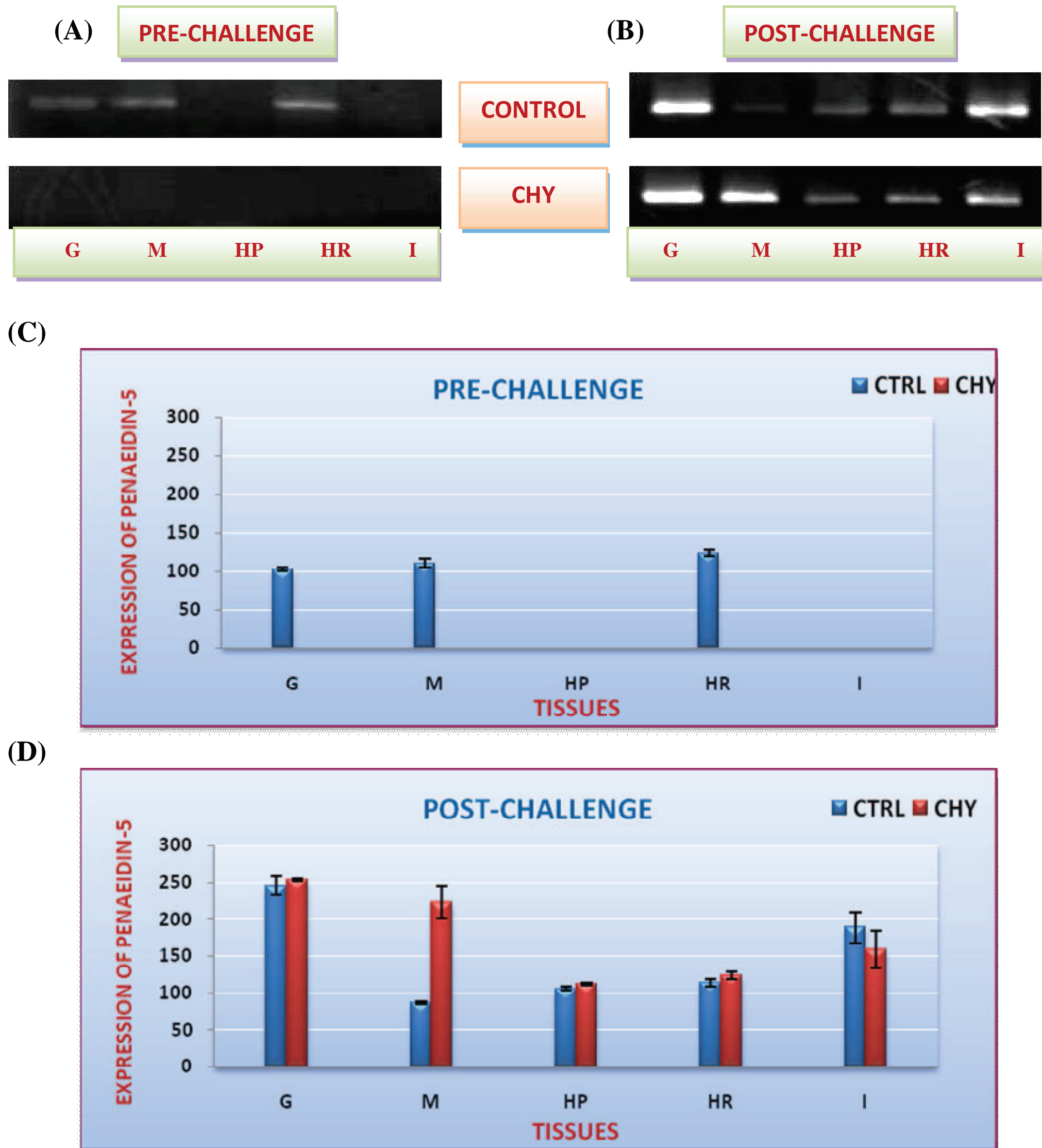



**Fig. 5.23. Tissue-wise expression profile of penaeidin-5 (AMP gene) in various tissues of giant tiger shrimp, *P. monodon* in response to the administration of marine yeast *C. haemulonii* (CHY) as immunostimulant and WSSV challenge**

(A) Agarose gel electrophoretogram of control and CHY treated groups pre-challenge WSSV

(B) Agarose gel electrophoretogram of control and CHY treated groups Post-challenge WSSV

(C) & (D) Graphical representation of expression level of ALF gene pre- and post-challenge WSSV (x-axis = tissues analyzed, y-axis = expression level of ALF gene)

*(G-Gill, M-Muscle, HP-Hepatopancreas, HR-Heart, I-Intestine)*

(A)

(B)

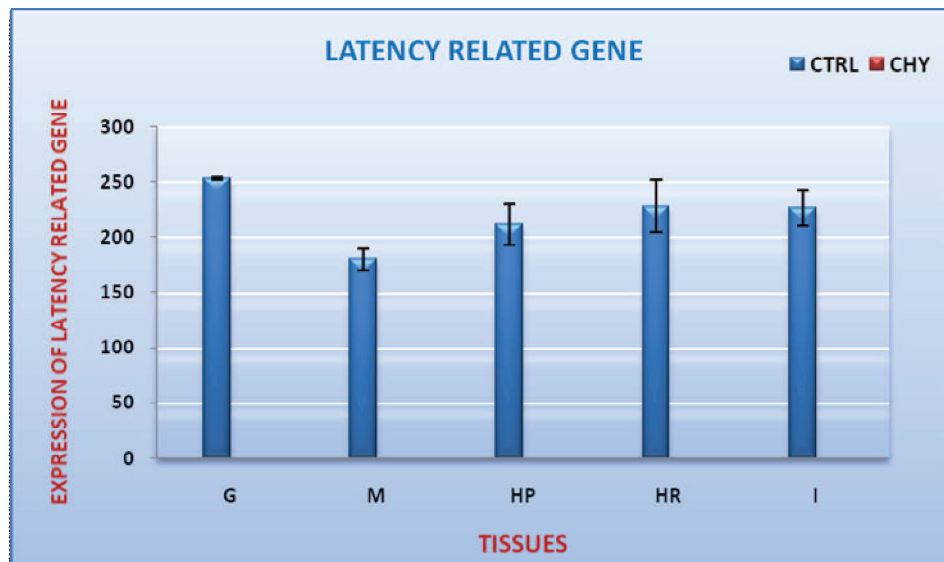


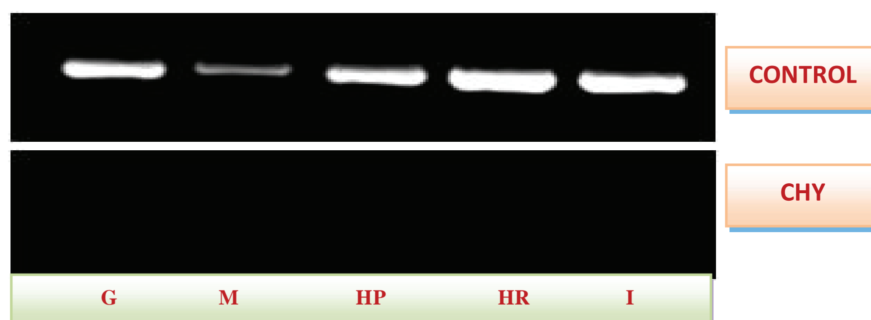

**Fig. 5.24. Tissue-wise expression profile of latency related gene (WSSV gene) in various tissues of giant tiger shrimp, *P. monodon* in response to the administration of marine yeast *C. haemulonii* (CHY) as immunostimulant and WSSV challenge**

**(A) Agarose gel electrophoretogram of control and CHY treated groups**

**(B) Graphical representation of expression level of latency related gene (x-axis = tissues analyzed, y-axis = expression level of latency related gene)**

***(G-Gill, M-Muscle, HP-Hepatopancreas, HR-Heart, I-Intestine)***

(A)




(B)

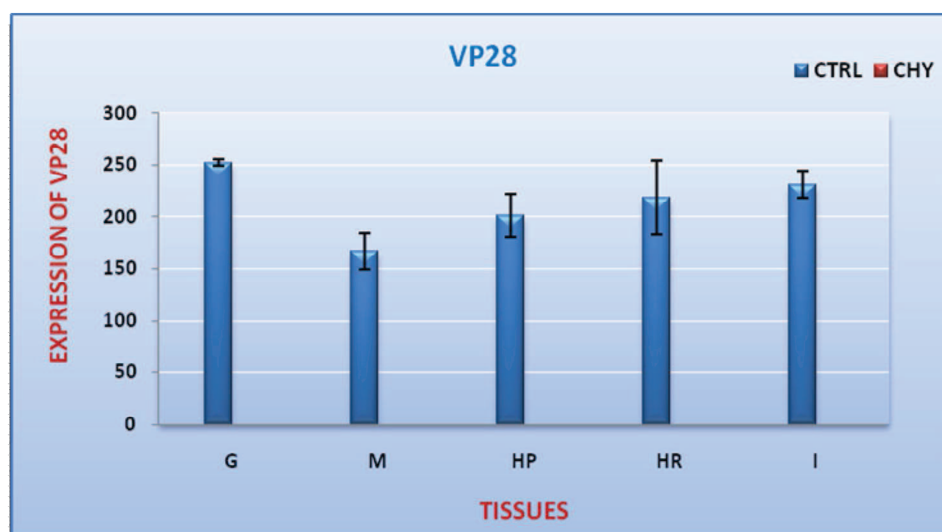



**Fig. 5.25. Tissue-wise expression profile of VP28 (WSSV gene) in various tissues of giant tiger shrimp, *P. monodon* in response to the administration of marine yeast *C. haemulonii* (CHY) as immunostimulant and WSSV challenge**

**(A) Agarose gel electrophoretogram of control and CHY treated groups**

**(B) Graphical representation of expression level of VP28 gene (x-axis = tissues analyzed, y-axis = expression level of VP28 gene)**

***(G-Gill, M-Muscle, HP-Hepatopancreas, HR-Heart, I-Intestine)***

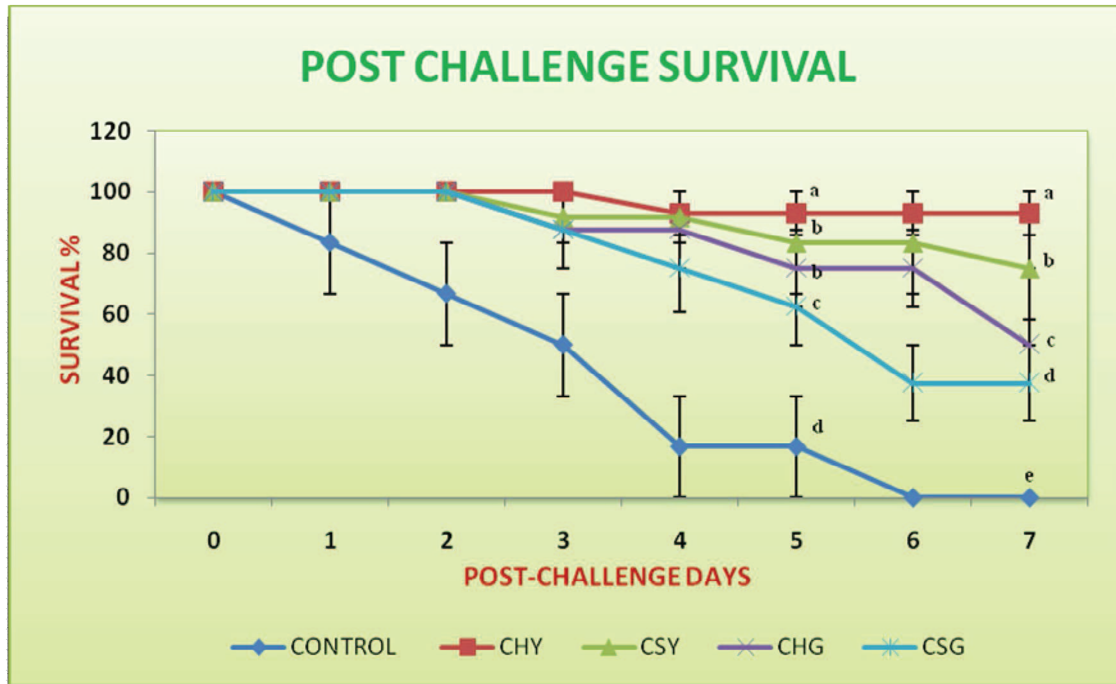



**Fig. 5.26. Post challenge survival of *P. monodon* fed with different immunostimulant incorporated diets and challenged with WSSV**

*(CHY – Candida haemulonii yeast fed group, CSY – Candida sake yeast fed group, CHG – Candida haemulonii glucan fed group, CSG – Candida sake glucan fed group)*

# CHAPTER-6

## *Expression Profile of Antimicrobial Peptide Genes in Response to the Administration of Probiotic Bacteria*

## 6.1. Introduction

Prophylactic approach is gaining more importance in aquaculture, because of the ever increasing incidence of viral pathogens and the ban of many antibiotics in aquaculture. Antibiotic therapy kills beneficial microbes besides pathogens affecting aquatic ecosystem health and emergence of resistant microbial strains leaving deadly diseases untreatable in animals. Several alternative strategies for the prevention and control of diseases have been proposed and have already been applied successfully in aquaculture, such as the use of vaccines, immunostimulants and probiotics (Ahilan, 2003). Only in the late 1980's did the first publication on biological control in aquaculture emerged and since then the research effort has continually increased. In the last two decades, there has been growing interest in bio-control of microbial pathogens in aquaculture using antagonistic microorganisms (Westerdahl et al., 1991; Maeda, 1994). The concept of microfloral manipulation was first appreciated by Metchnikoff in 1907, who viewed the consumption of yoghurt by Bulgarian peasants as conferring long span of life. Elie Metchnikoff's work at the beginning of 20$^{th}$ century is regarded as the first work on probiotics (Fuller, 1992). He described them as "microbes ingested with the aim of promoting good health". Fuller (1989) defined probiotics as 'A live microbial feed supplement, which beneficially affects the host animal by improving its microbial intestinal balance'. The new definition of probiotics is that it is 'a mono- or mixed culture of live microorganisms which, applied to animal or man, affect beneficially the host by improving the properties of the indigenous micro flora'.

Commonly, probiotics are considered as the counter part of antibiotics. While antibiotics are chemical compounds produced by living

organisms, probiotics are defined as "live organisms, which contribute to intestinal microbial balance". The beneficial effects of probiotics have been described as early as 1907. But it is only in the last thirty years that probiotics are being commercially used as feed additive (Cheeke, 1987). The term probiotics was first coined by Parker (1974). It originated from two Greek words, 'pro' and 'bios', which means 'for life'. Probiotics generally include bacteria, cyanobacteria, fungi etc. and they may be called as "normal microbiota" or "effective microbiota". "Probiotics", "probiont", "beneficial bacteria" or "friendly bacteria", are the terms synonymously used for probiotic bacteria (Rao, 2002). Most probiotics proposed as biological control agents in aquaculture belong to the *Lactobacillus* (Sugitha et al., 1996; Byun et al., 1997; Harzevili et al., 1998), *Bacillus* (Sugitha et al., 1998), *Micrococcus* (Irianto and Austin, 2002), *Pseudomonas* (Gram et al., 1999; Smith and Davey., 1993), *Vibrio* (Nair et al., 1985; Austin et al., 1995; Bergh 1995; Direkbusarakom et al., 1998) and *Aeromonas* (Gibson et al., 1998).

### 6.1.1. Role of probiotics in aquaculture

Now the use of bacterial amendments has been recommended for use in aquaculture ponds to obtain several benefits such as to improve FCR, to enhance immunity and resistance to diseases, to improve nutrient absorption, to change bacterial composition in guts excluding undesirable forms, to reduce mortality in shrimps, to enhance the production and yield and to eliminate the use of antibiotics (Mishra et al., 2001; Green and Green, 2003; Haung, 2003; Ahilan, 2003; Jameson, 2003).

### 6.1.2. Modes of action of probiotics

Several modes of actions have been proposed for probiotics such as the suppression of bacterial pathogens by production of antibacterial compounds like antibiotics, bacteriocin, siderophores, lysozymes, proteases, hydrogen peroxide, organic acids etc. (Fuller, 1992); Competitive exclusion by which the probiotics antagonizes the potential pathogen by the

production of inhibitory compounds or by competition for nutrients, space or oxygen (Fuller, 1989); Adherence and colonization of the probiotic microbes thereby preventing the pathogens from inhabiting the gastro-intestinal tract; production of essential nutrients to enhance the nutrition of the cultured animals (Ahilan, 2003); reduction in the potential availability of oxygen to pathogens; stimulation of humoral and/or cellular immune response (Fuller, 1989); alteration of microbial metabolism by the increase or decrease of the relevant enzyme level (Fuller, 1989); facilitation of improved lactose utilization by lactic acid bacteria and detoxification of the metabolites produced by intestinal pathogens.

Probiotics are a welcome addition to the armament of disease prophylaxis in aquaculture although the technology and science behind it is still very much in a developmental phase. In recent years there has been an increasing interest to understand the relevance of microbial communities in aquaculture systems and their importance in pond productivity (Moriarty, 1997). Reports on the potential of probiotics in shrimp aquaculture are, however, on the increase. Although several of publications about the probiotics and its role in aquaculture emerged (Moriarty, 1996; Mishra et al., 2001; Ahilan, 2003; Haung, 2003; Nayak et al., 2003; Antony and Philip, 2008), the approach is generally empirical and the arguments with regard to the mode of action are often circumstantial and have not been studied systematically. The mode of action of probiotics is likely to be multi-factorial and from existing evidence, appears to be strain specific (Touhy et al., 2003; Antony and Philip, 2008). Variation in biochemical parameters (prophenol oxidase activity and total haemocyte count) seems to be the usual criteria for studying the effects of these probiotics in the experimental organism (Nappi and Vass, 1993; Rodriguez and Le Moullac, 2000).

In some countries it is reported that probiotic use has significantly reduced antibiotic use in shrimp hatcheries. Probiotics have been used to

suppress the growth of pathogenic *Vibrio* spp. in many shrimp hatcheries by introducing (inoculating) non-pathogenic strains or species. This procedure appears effective and economical and demonstrates a clear need for further research into identifying potential probiotic strains of micro-organisms and evaluating their efficacy under field/farm conditions (Subasinghe et al., 1998). However, the exact mode of action of the probionts, especially at the molecular level still remains enigmatic. Further research and field trials are clearly essential to determine the precise mechanisms of the action of these products and to evaluate their efficacy in commercial shrimp production (Flegel, 1996; Subasinghe et al., 1998). It seems likely that the use of probiotics will gradually increase and if validated through rigorous scientific investigation and used widely, may prove to be a boon for the aquaculture industry.

Eukaryotic antimicrobial proteins or peptides (AMPs) are promising molecular tools that have been a subject of intense research in the last few decades, regarding their biosynthesis, activity against microorganisms, mechanism of action and potential clinical application (Lofgren et al., 2008). Although, the use of antimicrobial peptides as a therapeutic tool has been among the most promising avenues investigated, to date, for addressing antibiotic resistance (Sheynis et al., 2003), there are hardly any studies on the effect of the probiotic bacteria on AMP gene expression.

Therefore, the present work was carried out to analyze the expression profile of AMP genes in *P. monodon* fed probiotic bacteria incorporated diets pre- and post-challenge WSSV; to enhance the production of AMPs within the body of the experimental animal; to study the tissue-wise expression profile of AMP genes in response to probiotic administration and WSSV challenge.

# 6.2. Materials and methods

## *6.2.1. Experimental animals and rearing conditions*

Healthy adult *P. monodon* (20-25 g) were collected from a local shrimp farm in Vypeen, Kochi and maintained in aquarium tanks as mentioned in section 4.2.1.

## *6.2.2. Test diets*

Three experimental feeds were prepared by incorporating *Bacillus* MCCB101 ($10^3$ cells/animal), *Micrococcus* MCCB104 ($10^3$ cells/animal), and a combination of *Bacillus* + *Micrococcus* ($2x10^3$ cells/animal), to a standard shrimp diet (Higashi, India) (Fig. 6.1). The probiotic preparations were obtained from National Center for Aquatic Animal Health (NCAAH), CUSAT, India. Diet without supplementation of probiotics (FC) was used to feed the control group. Probiotic bacterial suspensions obtained were mixed with the standard shrimp diet, so that the animals receive 50 cells/g animal/day when administered individually and 100 cells/g animal /day in combination (*Bacillus* + *Micrococcus*).

## *6.2.3. Feeding experiment and WSSV challenge*

Shrimps were randomly divided into four groups (n=15) and were fed on their respective diets for 14 days. Feeding experiments were done in triplicates. Group 1 shrimps, fed on the standard shrimp diet without supplementation of probiotics, were used as the control (FC). Group 2 and 3 were maintained on the feed containing *Bacillus* (B) and *Micrococcus* (M) respectively and Group 4 on a diet containing *Bacillus* + *Micrococcus* (BM) (Table 6.1). Feeds were given twice daily (8AM and 7PM) at a rate of 10–15% body weight per day for 14 days. Physico-chemical parameters of the rearing water such as salinity, $NH_3$–N, $NO_2$–N, $NO_3$–N and dissolved oxygen were monitored regularly (APHA, 1995) and maintained at optimal levels by water exchange.

Six animals from each group were sampled after 14 days of probiotic supplementation for the pre-challenge data. On the $15^{th}$ day all the groups were orally challenged with WSSV by feeding WSSV infected frozen prawn tissue at the rate of 1g / animal. Thereafter the animals were maintained on their respective diets. After 48 h five animals each from all the groups were sampled for the post-challenge data and rest of the shrimps were maintained on respective diets for observing mortality, if any. During sampling only those shrimps in the intermoult stage were evaluated in the study. Survival in each treatment group was recorded daily for a period of seven days with dead animals removed promptly. Mortality by WSSV infection was confirmed by checking the characteristic white spots on the carapace of infected shrimps.

### *6.2.4. Haemolymph and other tissues collection*

Haemolymph and tissues including gill, muscle, heart, hepatopancreas and intestine were collected for total RNA isolation as described in section 5.2.4.

### *6.2.5. Total RNA isolation and reverse transcription*

Total RNA isolation and first strand cDNA synthesis were performed as described in section 2.2.3. to 2.2.6.

### *6.2.6. Semi-quantitative RT-PCR analysis of gene expression*

Expression of the target genes when supplemented with different probiotic bacteria pre- and post-challenge WSSV was determined by semi-quantitative RT-PCR analysis using 18S rRNA, β-actin and elongation factor (ELF) as the internal control (Marone et al., 2001) as described in section 4.2.6.

From the three different probiotics screened, the best one was selected based on AMP gene expression, intensity of WSSV infection as envisaged from the WSSV gene expression and post-challenge survival data. The best performed probiotic group along with control group was subjected to detailed tissue-wise expression profile analysis. PCR amplifications of the

tissue cDNA was performed for six AMP genes (ALF, crustin-1, crustin-2, crustin-3, penaeidin-3 and penaeidin-5) and two WSSV genes (latency related gene and VP28) in a 25 µl reaction volume as described in section 5.2.6.

### *6.2.7. Statistical analysis*

Statistical significance of the post challenge survival data of the control group and probiotic administered groups were determined by One-way ANOVA and Duncan's multiple comparison of the means using the software SPSS 10.0. A probability (*p*) value of less than 0.05 was considered significant.

## 6.3. Results

### *6.3.1. Expression profile of control genes in the haemocytes of P. monodon in response to the administration of probiotic bacteria pre- and post-challenge WSSV*

Expression profile of three control genes viz. 18S rRNA (Fig. 6.2), β-actin (Fig. 6.3) and ELF (Fig. 6.4) were analyzed. ELF was found to be the best reference gene.

### *6.3.2. Expression profile of AMP genes in the haemocytes of P. monodon in response to the administration of probiotic bacteria pre- and post-challenge WSSV*

Expression profile of all AMP genes viz. ALF, crustin-1, crustin-2, crustin-3, penaeidin-3 and penaeidin-5 in the haemocytes of *P. monodon* were found to vary on administration of the probiotic bacteria both pre- and post-challenge WSSV (Fig. 6.5–6.10). WSSV infection was found to modulate AMP gene expression in haemocytes of both the control and probiotic administered group of animals.

#### *6.3.2.1. Expression profile of ALF*

Noticeable variation in the expression of ALF could be observed on administration of probiotic bacteria. ALF was found to be constitutively

produced in the control group of shrimps at a minimal level. However, on administration of probiotic bacteria, the ALF gene was found to up-regulate considerably. In the pre-challenge condition maximum u-regulation of the ALF gene was supported by *Micrococcus* incorporated diet followed by the combination of *Bacillus* + *Micrococcus* and *Bacillus* (Fig.6.5 A & C).

On challenge with WSSV; the ALF gene was found to up-regulate considerably even in the control group of animals. The probiotic diet fed groups of shrimps viz. *Bacillus* + *Micrococcus* and *Micrococcus* was also found to up-regulate the ALF gene on WSSV challenge. *Bacillus* + *Micrococcus* treated group supported maximum up-regulation of the ALF gene post-challenge WSSV, followed by *Micrococcus* and the control group of shrimps (Fig.6.5 B & C). However, not much variation in the expression profile of ALF could be noticed post-challenge WSSV on administration of *Bacillus*.

***6.3.2.2. Expression profile of crustin-1***

Not much variation in the expression profile of crustin-1 could be detected on administration of probiotic bacteria pre-challenge WSSV. The level of expression of crustin-1 gene remained more or less similar except for a slight up-regulation in case of *Bacillus* and *Bacillus* + *Micrococcus* administered group (Fig.6.6 A & C).

On WSSV challenge, crustin-1 was found to down-regulate considerably in the control group of shrimps. However, when compared to the control group, crustin-1 gene was found to be up-regulated on administration of probiotic bacteria. *Bacillus* and *Micrococcus* administered group was found to support highest up-regulation of the crustin-1 gene post-challenge WSSV followed by *Bacillus* + *Micrococcus* (Fig.6.6 B & C).

***6.3.2.3. Expression profile of crustin-2***

Up-regulation of the crustin-2 gene could be noticed on administration of probiotic bacteria. Maximum up-regulation of the crustin-2

gene was supported by *Bacillus* administered group, followed by *Micrococcus* and *Bacillus* + *Micrococcus* administered group (Fig.6.7 A & C).

On WSSV challenge, crustin-2 followed the same pattern of expression as in case of crustin-1. Crustin-2 gene was found to be down-regulated in the control group of shrimps on challenge with WSSV. However, when compared to the control group, all experimental diet administered group supported up-regulation of the crustin-2 gene. *Bacillus* and *Micrococcus* administered group was found to support maximum up-regulation of the crustin-2 gene post-challenge WSSV (Fig.6.7 B & C).

***6.3.2.4. Expression profile of crustin-3***

Crustin-3 gene was found to be constitutively produced at a higher level in the haemocytes of *P. monodon*. Not much variation could be detected in the expression profile of crustin-3 gene on probiotic administration, except for a slight up-regulation in the treated groups, *Micrococcus* and *Bacillus* + *Micrococcus* (Fig.6.8 A & C).

On WSSV challenge, crustin-3 followed the same pattern of expression as in case of crustin-1 and -2. Crustin-3 gene was found to be down-regulated in the control group of shrimps on challenge with WSSV. However, when compared to the control group, all experimental diet administered group supported up-regulation of the crustin-3 gene. *Micrococcus* and *Bacillus* + *Micrococcus* administered group was found to support maximum up-regulation of the crustin-3 gene followed by *Bacillus* (Fig. 6.8 B & C).

***6.3.2.5. Expression profile of penaeidin-3***

Noticeable up-regulation of the penaeidin-3 gene could be detected on administration of *Bacillus* pre-challenge WSSV. However, not much variation in the expression profile of penaeidin-3 could be detected in case of other groups, except for a slight up-regulation in case of *Micrococcus* administered group (Fig.6.9 A & C).

On WSSV challenge, a noticeable down-regulation of the penaeidin-3 gene could be found in the control group of shrimps. However, considerable up-regulation of penaeidin-3 gene could be observed in case of probiotic administered groups. Maximum up-regulation of the penaeidin-3 gene was supported by *Bacillus* administered group of shrimps, followed by *Bacillus* + *Micrococcus* and *Micrococcus* (Fig.6.9 B & C).

#### *6.3.2.6. Expression profile of penaeidin-5*

Though much variation in the expression of penaeidin-5 could not be observed on administration of probiotic bacteria, slight up-regulation of the gene could be noticed in the probiotic administered groups (Fig.6.10 A & C).

On WSSV challenge, penaeidin-5 gene was found to be down-regulated in the control group of shrimps. However, when compared to the control group, the gene was found to be up-regulated in all the probiotic administered groups. *Bacillus* was found to support maximum up-regulation of the penaedin-5 gene post-challenge WSSV, followed by *Micrococcus* and *Bacillus* + *Micrococcus* (Fig.6.10 B & C).

### *6.3.3. Expression of WSSV genes in haemocytes of P. monodon in response to the administration of probiotic bacteria*

All WSSV gene transcripts could be detected in the haemocytes of the control group of shrimps confirming WSSV infection in this group. All the WSSV genes tested followed more or less similar pattern of expression for the various experimental groups analyzed, especially in case of the control group of shrimps. WSSV infection could be confirmed by the expression of DNA polymerase (Fig.6.11), endonuclease (Fig.6.12), immediate early gene (Fig.6.13), latency related gene (Fig.6.14), protein kinase (Fig.6.15), ribonucleotide reductase (Fig.6.16), thymidine kinase (Fig.6.17) and VP28 (Fig.6.18) genes of WSSV in the control group of shrimps.

However, probiotic administered group was found to be less infected by WSSV, as indicated by the expression profile of WSSV genes in these

treated group of shrimps. *Micrococcus* administered group of animals also gave positive amplifications for the WSSV genes viz. immediate early gene, ribonucleotide reductase and VP28 gene. Whereas, *Bacillus* + *Micrococcus* gave positive amplification only for immediate early gene of WSSV. Hence, these two probiotic administered group were found to be less infected with WSSV when compared to that of control group of shrimps. Interestingly, the *Bacillus* administered group of shrimps did not give any amplification for the WSSV gene studied.

Since *Bacillus* administered group was found to perform best in terms of AMP gene expression and WSSV gene expression, a detailed tissue-wise expression profile was performed for the above group of shrimps.

### *6.3.4. Expression profile of AMP genes in various tissues of P. monodon in response to the administration of probiotic Bacillus pre- and post-challenge WSSV*

AMP gene transcripts could be detected in all the target tissues at varying levels in the animals. In general gills and intestine were found to have maximum transcripts followed by muscle, hepatopancreas and heart. However, the expression profile of each was found to vary for the various tissue targets (Fig. 6.19 – 6.24).

#### *6.3.4.1. Expression profile of ALF*

Transcripts of ALF could be detected at high levels in the gills, intestine, hepatopancreas and muscle of the control group of *P. monodon*. In case of the control group of shrimps, gills and intestine supported maximum expression of the ALF gene followed by hepatopancreas and muscle. The ALF gene was found to be up-regulated on *Bacillus* administration pre-challenge WSSV, except in the hepatopancreas. Maximum up-regulation of the gene was found to be in the muscle of the *Bacillus* administered group (Fig. 6. 19 A & C).

ALF gene was found to be up-regulated on WSSV challenge even in the control group, especially in the heart. However, ALF was found to be down-regulated in the muscle and hepatopancreas of the control group of shrimps on WSSV challenge. In case of *Bacillus* treated group, a complete down-regulation of the ALF gene could be detected in all the target tissues. The only ALF expression detected in this group of shrimps was in the gills and intestine and hepatopancreas (Fig. 6. 19 B & D).

***6.3.4.2. Expression profile of crustin-1***

mRNAs of crustin-1 gene could be detected in all of the target tissues in the control group of animals prior to WSSV challenge. However, on *Bacillus* administration, a complete down-regulation of the crustin-1 gene could be detected in all the tissues. Gill, muscle, and intestine was found to support maximum expression of the crustin-1 gene in the control shrimps, whereas in case of *Bacillus* treated group of shrimps, expression could be detected only in the gills (Fig. 6. 20 A & C).

Not much variation could be observed in the pattern of expression of crustin-1 for the control and *Bacillus* treated group of shrimps post-challenge WSSV. On challenge with WSSV, a complete down-regulation of the crustin-1 gene could be observed in the various target tissues of both the control and *Bacillus* administered group of shrimps, with expressions limiting only to gills and intestine. When compared to the control group, *Bacillus* administered group supported up-regulation of the crustin-1 gene post-challenge WSSV in both the gills and intestine. Maximum level of expression was found to be in the intestine followed by gills. No transcripts of crustin-1 could be detected in muscle, hepatopancreas or heart of both the control group and *Bacillus* treated groups (Fig. 6. 20 B & D).

***6.3.4.3. Expression profile of crustin-2***

Crustin-2 was found to be expressed at very high levels in all the target tissues of the control group of shrimps pre-challenge WSSV. However,

the gene was found to be down-regulated in the *Bacillus* treated group prior to WSSV challenge. In *Bacillus* administered group, maximum expression was found in the gill, followed by intestine and muscle (Fig. 6. 21 A & C).

On challenge with WSSV, a general down-regulation of the crustin-2 gene could be noticed in all the target tissues of the control group. However, in case of *Bacillus* treated group of shrimps, the crustin-2 gene was found to be up-regulated considerably in all the tissues except in case of heart. Maximum expression was found in the gill and intestine (Fig. 6. 21 B & D).

***6.3.4.4. Expression profile of crustin-3***

Crustin-3 was found to be expressed in all the target tissues of the control group of shrimps pre-challenge WSSV. Maximum expression of the gene was found in gill and intestine. However, the gene was found to be completely down-regulated on administration of *Bacillus*, with expressions limiting to gill, intestine and muscle of the treated group (Fig. 6. 22 A & C).

On challenge with WSSV, complete down-regulation of the crustin-3 gene could be noticed in various target tissues. In the control group; gill was the only tissue that was found to express the crustin-3 gene, that too at very low level. However, when compared to the control, the *Bacillus* treated group up-regulated the crustin-3 gene, with maximum expression in the gill followed by intestine (Fig. 6. 22 B & D).

***6.3.4.5. Expression profile of penaeidin-3***

Penaeidin-3 was found to be expressed at very high levels in all the target tissues, except gills in the control group of shrimps prior to WSSV challenge. On administration of *Bacillus*, the penaeidin-3 gene was found to be up-regulated considerably in the gills. However, the gene was found to express at the same level in the muscle and intestine of the experimental animals. In case of hepatopancreas and heart, no expression of penaeidin-3 gene could be found (Fig. 6. 23 A & C).

On challenge with WSSV, a general down-regulation of the penaeidin-3 genes could be noticed in various target tissues. Penaeidin-3 transcripts could be found only in the gills and intestine of the WSSV challenged shrimps, intestine supporting maximum expression of the gene. But in case of *Bacillus* treated group, the gene was found to be up-regulated considerably in all the tissues, except heart. Gill was found to support maximum expression of the gene, followed by intestine, muscle and hepatopancreas (Fig. 6. 23 B & D).

#### *6.3.4.6. Expression profile of penaeidin-5*

Very low level of penaeidin-5 expression could be observed in the various tissues of gills, muscle and heart of the control group of shrimps. And no transcripts were detected in the hepatopancreas and intestine prior to WSSV challenge. Whereas in the *Bacillus* administered group of shrimps, up-regulation of the gene was noticed, with gill and muscle supporting maximum expression followed by intestine (Fig. 6. 24 A & C).

On challenge with WSSV, up-regulation of the penaeidin-5 gene could be noticed in the various target tissues of the control group of shrimps. Maximum expression was supported by the gills and intestine. *Bacillus* treated group also supported maximum up-regulation of the penaeidin-5 gene, and maximum expression was found in the intestine followed by gills and muscle (Fig. 6. 24 B & D).

### *6.3.5. Expression profile of WSSV genes in various tissues of P. monodon in response to the administration of probiotic Bacillus*

Since, all the WSSV genes studied in the haemocytes of *Bacillus* treated group, was found to follow a similar pattern of expression; only two WSSV genes were considered for studying the tissue-wise expression profile. In the control group both WSSV genes analyzed viz. the latency related gene (Fig. 6.25) and VP 28 gene (Fig. 6.26), gave amplifications and followed a similar pattern of expression for the various target tissues analyzed. Muscle was

found to support minimum expression the WSSV genes. Whereas, in the case of *Bacillus* administered group of shrimps, none of the target tissues were found to be infected with WSSV.

### *6.3.6. Post-challenge survival*

When compared to that of the control group of animals, probiotic bacteria administered groups gave higher survival rate on WSSV challenge. Among the probiotic treated groups, *Bacillus* administered group of shrimps showed significantly higher survival rate (73%), followed by *Micrococcus* (73%) and *Bacillus* + *Micrococcus* (56%) (Fig. 6.27).

## 6.4. Discussion

Although the exact mechanism is still not yet completely understood, several probiotic preparations are being applied in shrimp culture systems, to induce and build up protection against a wide range of diseases. In order to develop effective intervention strategies for disease control in shrimp culture, a scientific basis for the health modulators in shrimp is required. Several studies have been carried out on the enhancement of the immune response with respect to the application of probiotics. But there are hardly any studies on the effect of these probiotic products on the gene expression of AMPs. Also, the expression profile of immune genes during WSSV infection is also limited, especially AMPs. The response of AMP expression to bacterial challenge is supposed to be enigmatic and often does not follow the pattern expected for immune genes (Gross et al., 2001; Rojtinnakorn et al., 2002; Munoz et al., 2004; Lorgeril et al., 2005; Beale et al., 2008). Hence the present study was undertaken to study the expression profile of AMP genes in response to probiotic bacterial administration and WSSV challenge and thereby to analyze the mechanism of action of probionts in terms of AMP gene expression and to determine the antiviral properties of the major AMP families of shrimps.

*Bacillus* and *Micrococcus*, being the most widely used probiotic bacteria, were used in the present study. Since most of the commercially available probiotic products are in combination of various bacterial cultures, a probiotic combination containing *Bacillus* and M*icrococcus* was also used in the present study. The ultimate goal of this research was to determine if commercially available and candidate probionts used by the crustacean aquaculture industry might act by up-regulating the expression of AMP genes in the haemocytes of shrimps.

In the present study WSSV challenge was performed orally also so as to mimic the natural mode of WSSV infection. Sampling was performed at 48 hr after WSSV challenge, because, in the previous studies the immunological parameters were found to be generally at peak during that time, when orally challenged with WSSV.

Since haemocytes plays an important role in defense and have proved to be the site of production of AMP molecules (Smith et al., 2003; Li et al., 2005), in the present study, haemocytes were selected for studying the expression profile of major AMP families in response to the administration of probionts and WSSV challenge. Haemocytes contain high number of transcripts of the respective genes. Hence cDNA prepared from the haemocytes were diluted 5 times, so as to make it suitable for semi-quantitative RT-PCR analysis.

Analysis of the results showed that probiotic bacteria up-regulated the expression levels of all the AMP families of shrimps tested, both pre- and post-challenge WSSV (Fig. 6.5 – 6.10). It was also interesting to note that the expression of AMPs varied with the probiotic bacteria administered. In the present study, ALF was found to be up-regulated considerably on administration of probiotic bacteria under both pre- and post-challenge WSSV (Fig.6.5). It has been reported in several shrimp that ALF transcription

is induced upon bacterial challenge (Supungul et al., 2004; Liu et al., 2005; Nagoshi et al., 2006).

Also, the expression profile of ALF in response to gram-positive probiotic *Bacillus* was found to be similar to that of gram-negative *Vibrio* reported by Beale et al. (2008) in the lobster, *Homarus americanus.* Hence, we can say that the response of ALF to probiotic bacteria is similar to that of a pathogen. In terms of ALF expression, it may be explained that probiotic bacteria mimic a bacterial infection in shrimps that leads to an enhanced production of potent molecules like ALF, thereby enhancing the immune resistance of the host organism. de la Vega et al. (2008) have also showed an enhanced production of ALF in the haemocytes of *L. vannamei* on *V. penaeicida* infection. Recombinant *P. monodon* ALF showed antimicrobial activity against fungi, gram-positive and gram-negative bacteria (Somboonwiwat et al., 2005), and in the crayfish *P. lenuisculus* ALF has been implicated in the reduction of WSSV replication. Thus, it is possible to predict that ALF could function in shrimp as a broad spectrum antimicrobial peptide. The expression of ALF mRNA detected in unchallenged shrimp indicated that ALF is constitutively expressed and stored in shrimp haemocytes. The growing number of relatively conserved ALF genes identified with apparently conserved functions being characterized across taxa seems to indicate the likely importance of ALF in shrimp besides the AMPs in the penaeidin family (Gueguen et al., 2006; Somboonwiwat et al., 2008).

In the case of crustins also, up-regulation of the gene could be noticed (though a minor one) on probiotic administration pre-challenge WSSV (Fig. 6.6 – 6.8). This is in agreement with the previous works where crustins have been found to up-regulate with bacterial invasion in the shrimps (Jiravanichpaisal et al., 2007; Amparyup et al., 2008). Significant up-regulation of the crustins has also been reported after the administration of

gram-positive bacteria, *Planococcus citreus* in the shore crab *Carcinus maenas* (Brockton et al., 2008). Application of peptidoglycan in *Marsupenaeus japonicus* also resulted in the up-regulation of crustin gene in unchallenged shrimps (Rattanachai et al., 2004, 2005). In *P. monodon* also a five-fold up-regulation of the crustin transcripts has been reported following challenge with *Vibrio harveyii* (Amparyup et al., 2008). Contrasting results have also been obtained by Okumura et al. (2007) where, a down-regulation of the crustin gene was observed on administration of LPS in *Litopenaeus vannamei.* A down-regulation in the crustin transcripts has also been reported after 24h of challenge by *Vibrio alginolyticus* in *L. vannamei* (Jimenez-Vega et al., 2004; Vargas-Albores et al., 2004). Also in *P. monodon* crustins transcripts were found to be down-regulated after infection with gram-negative bacteria (Supungul et al., 2008). The variation in activity seen among these studies could simply be that these are different crustins from different species, and they act differently. The variable pattern in the expression of crustins after bacterial challenge is unlike that known for other arthropod AMPs (Bulet et al., 1999; Engstrom, 1999) and makes these molecules highly enigmatic (Smith et al., 2008).

The expression profiles of penaeidins were worth noticeable under pre-challenge conditions (Fig. 6.9 – 6.10). Expression profile of penaeidin-3 was found to up-regulate to higher levels on administration of probiotic *Bacillus*. However, administration of *Micrococcus* and the combination of *Bacillus* + *Micrococcus* was not found to influence the expression pattern of penaeidin-3. This might be due to the strong antibacterial activity especially against gram-positive bacteria already reported for penaeidins (Destoumieux et al., 2001). Slight up-regulation could be noticed for penaeidin-5 gene also, though to a lesser degree.

On WSSV challenge, and entirely different pattern of AMP gene expression could be noticed in all the groups. A general down-regulation of

all the target genes, except in case of ALF, could be noticed in the control group of organisms on WSSV challenge (Fig. 6.5 – 6.10). This is in agreement with the previous works where a general down-regulation of the immune genes has been reported with bacterial or viral infection (Burgents et al., 2004; Supungul et al., 2004; Vargas-Albores et al., 2004; Sun et al., 2008). In the control group of shrimps, ALF was the only AMP gene that was found to be up-regulated post-challenge WSSV (Fig. 6.5). This is in agreement with previous reports by Liu and co-workers (2006), where an up-regulation of ALF gene was noticed in *P. leniusculus* on WSSV challenge. Expression pattern of ALF post-challenge WSSV clearly indicates its possible antiviral activity. The apparent ability of ALF to so strongly interfere with viral replication warrants further exploration. In the crayfish *P. leniusculus* ALF has been implicated in the reduction of WSSV replication (Liu et al., 2006). Also, treatment of Pacific white shrimp *L. vannamei* with white spot syndrome virus resulted in up-regulation of an ALF gene in hepatopancreas (de la Vega et al., 2008). Previous research has also shown that injection of *Vibrio harveyi* into the giant tiger prawn *P. monodon* led to an increased abundance of ALF mRNA in haemocytes, while mRNAs for other antimicrobial proteins including penaeidin and crustin decreased (Supungul et al., 2004; Somboonwiwat et al., 2006). In the present study also, administration of probionts resulted in an enhanced production of ALF, so also WSSV challenge. These results suggest that there may be some overlap between the antiviral and the antibacterial response (Robalino et al., 2007; Beale et al., 2008).

All the AMPs analyzed in the present study were found to be up-regulated considerably on WSSV challenge in case of probiotic administration. This clearly shows that probiotic enhances the immune status in shrimps when maintained on probiotic diet. However, variations could be noticed in the expression profile of AMP genes with the type of

probiotic bacteria applied. Probionts administered individually and in combination were found to alter the expression of AMP genes. In case of ALF, combination of *Bacillus* + *Micrococcus* treatment supported maximum up-regulation of the gene post-challenge WSSV followed by *Micrococcus* and *Bacillus* treated groups. This might be due to the increase in cell number in the combination treatment (*Bacillus* + *Micrococcus*). All the three crustins were found to be up-regulated on probiotic administration post-challenge WSSV (Fig. 6.6 - 6.8). Also, all the three probiotic preparations up-regulated the crustin genes on WSSV challenge. Though several works have been reported on the expression profile of crustins in response to the invasion of gram-positive and gram-negative bacteria, expression profile of crustins in response to WSSV infection remains limited (Rattanachai et al., 2004, 2005; Jimenez-Vega et al., 2004; Vargas-Albores et al., 2004; Jiravanichpaisal et al., 2007; Okumura et al., 2007; Supungul et al., 2008; Amparyup et al., 2008).

Penaeidins showed a general up-regulation on probiotic treatment post challenge WSSV (Fig. 6.9 – 6.10). Both Penaeidin-3 and -5 were found to be up-regulated by the three probiotic preparations post-challenge WSSV. However, *Bacillus* administered group supported maximum up-regulation of both penaeidins, followed by the combination of *Bacillus* + *Micrococcus* and *Micrococcus*. This is in agreement with the results of Garcia et al. (2009) where an up-regulation of penaeidins and crustin gene have been observed in WSSV-infected pre-challenged *P. vannamei*.

Analysis of the WSSV gene expression profile also proved that probiotic administered groups were less infected with WSSV when compared to that of control. When control group exhibited high amount of transcripts of WSSV genes, confirming WSSV infection, probiotic administered groups were found to be less infected with WSSV. Also, none of the WSSV genes were found to amplify in the *Bacillus* administered group of shrimps.

Detailed tissue-wise analysis was performed for the probiotic that performed best in terms of AMP and WSSV gene expression. Hence, *Bacillus* was selected for the further analysis. For tissue-wise analysis cDNA was not diluted, since low number of AMP transcripts was present in these samples. Hence, in the present study the cDNA of haemocytes used were 5 times diluted than the tissue samples. Therefore, we can say that, haemocytes supported the maximum expression of AMP genes, when both haemocytes and tissues were considered.

Tissue-wise expression profile of AMPs showed the presence of mRNA transcripts of crustins and penaeidin-3 genes in all of the target tissues at varying levels in the animals prior to WSSV challenge (Fig. 6.19 – 6.24). When all the tissues analyzed were taken into consideration, haemocytes were found to support maximum expression of the AMP genes, followed by gills and intestine. Haemocytes are the main site of production of AMPs. Gills of bacteria infected crustaceans are known to accumulate haemocytes (Burnett et al., 2006), and thus it is not surprising that gills would exhibit high levels of mRNAs that may be derived from haemocytes. Also, since the probiotic bacteria are administered via diet, higher number of bacteria would have reached the intestine, which answers the higher expression of AMPs in intestine of the experimental animals.

ALF transcription has been reported to be tissue specific with high expression in haemocytes, gills and intestine of decapods crustaceans like *F. chinensis* (Liu et al., 2005), *L. vannamei* (de la Vega et al., 2008) and *Scylla paramamosain* (Imjongjirak et al., 2007). In the present study also, a similar pattern of ALF expression could be observed in case of *P. monodon*. These observations contrast with patterns of synthesis of many other AMPs in shrimp which is carried out primarily in haemocytes (Iwanaga and Kawabata, 1998; Munoz et al., 2002; Bachere et al., 2004). However, in shrimp

it is clear that ALF transcription, although tissue specific, occurs in multiple organs and could thereby provide systemic protection against pathogens.

In the present study, though a down-regulation of crustins in the tissues could be observed on *Bacillus* administration pre-challenge WSSV, ALF and penaeidins were found to be up-regulated. This may be because of the fact that, probiotic administration, in effect, mimics a microbial challenge in shrimps and a further WSSV challenge would have resulted in an increasing number of ALF and penaeidin positive haemocytes. Together, the results suggested that haemocytes left the circulation and migrated into shrimp tissues, presumably in order to control and prevent the spreading of the pathogen. A similar observation was seen in the expression of penaeidin in *L. vannamei* (Munoz et al., 2004; Somboonwiwat et al., 2008).

On WSSV challenge, the probiotic (*Bacillus*) treated group displayed higher up-regulation of all the AMP genes especially crustins and penaeidins. Maximum up-regulation was found to be in the gills and intestine of the treated group of shrimps. Previous studies on penaeidin expression has shown the expression levels of penaeidins to be higher in the haemocytes, gill, heart, and intestine since haemocytes infiltration are more in these tissues (Kang et al., 2004; Liu et al., 2005).

*Bacillus* administration was found to support maximum up-regulation of penaeidins than other AMPs under both pre- and post-challenge conditions. This might be because of the fact that penaeidins are up-regulated in the presence of bacteria (Bachere et al., 2000a; Cuthbertson et al., 2004; Chiou et al., 2005), showing its potential antibacterial activity. Up-regulation of penaeidins on WSSV challenge also seems to be pointing to its possible mode of antiviral defense. This up-regulation was very high on *Bacillus* administration, which shows that administration of probiotic bacteria could enhance the production of AMPs which in turn could result in better protection against WSSV infection.

Gills and intestine was found to be the tissues with maximum AMP transcripts. Transcripts observed in the gills and intestine were probably due to the infiltrating haemocytes (Liu et al., 2005). Gill and intestine are important defense barriers of shrimp as they have direct contact with water and food, respectively. Changes in AMP transcript concentration in the tissues shown might be due to the variation in the haemocyte count. Since haemocytes have been proved to be the sole site of production of AMPs in shrimps, expression of AMPs in tissues is believed to be due these infiltrating haemocytes. AMP-expressing haemocytes leave the blood circulation and most of the shrimp tissues via chemotaxis to migrate toward injured tissues. Massive accumulation of AMP-producing haemocytes and free AMPs are then seen around the site of infection and in the muscle tissue, which reveals a striking local anti-microbial response. WSSV which primarily affects the ectodermal and mesodermal tissues could be eliminated by an increased production of immune defense molecules in these tissues. The epithelial lining of gills and tissues are ectodermal in origin. Increased infiltration into these tissues thus in turn fights against WSSV attack delaying the process of infection in prawns.

ALF, Crustins and penaeidins have been proved to possess high antibacterial activity (Destoumieux et al., 2001; Smith et al., 2008; Somboonwiwat et al., 2008). The enhanced expression of crustins and penaeidins on administration of probiotic bacteria may be due to its high activity against gram-positive bacteria. This enhanced production of AMPs in turn gives added protection against other pathogenic microbes. Expression of WSSV genes also ensures that the level of WSSV infection is less in the probiotic treated groups when compared to the control group of shrimps (Fig. 6. 11- 6.18; Fig. 6.25 - 6.26). Several modes of action have been proposed for the probiotic bacteria (Antony and Philip, 2008). Action by enhanced production of AMPs could also be considered as one among them.

This work proves that probiotics enhance the production of AMPs as an effective means of protection in shrimps.

The absence of WSSV gene transcripts in *Bacillus* treated groups showed that the probionts play an important role in boosting the defense potential of the animal thwarting infection by the microbes. Under the challenged condition the AMP transcripts were high in probiotic treated groups compared to the control emphasizing the fact that probionts emerge as an important option as a prophylactic tool in aquaculture. The present study also indicates the antiviral property of AMPs stressing the importance of their up-regulation through the application of probiotics as a prophylactic strategy in aquaculture.

**Table.6.1. Experimental groups used in the present study**

| FEED CODE | FEED COMPOSITION |
|---|---|
| **FC** | Control feed |
| **B** | *Bacillus* (50 cells/g animal/day) incorporated diet |
| **M** | *Micrococcus* (50 cells/g animal/day) incorporated diet |
| **BM** | Combination of *Bacillus* + *Micrococcus* incorporated diet (100 cells/g animal/day) |

**(A)**

**(B)**

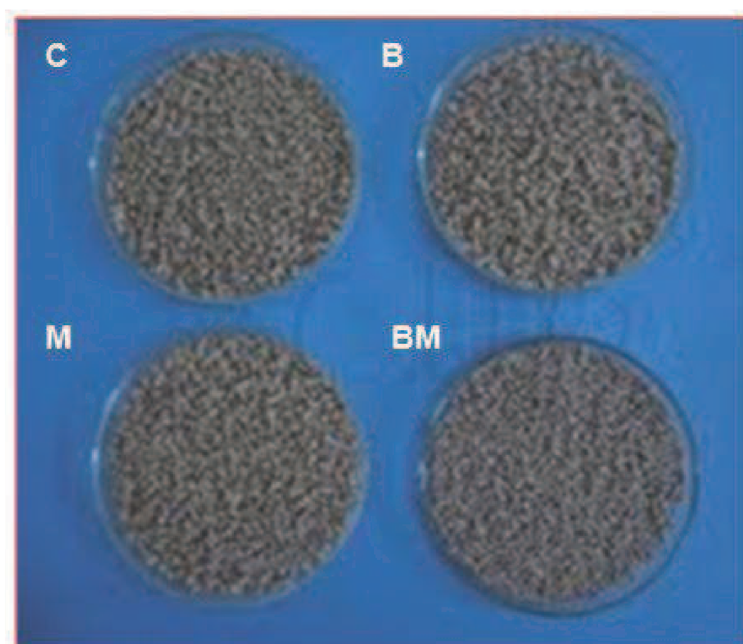
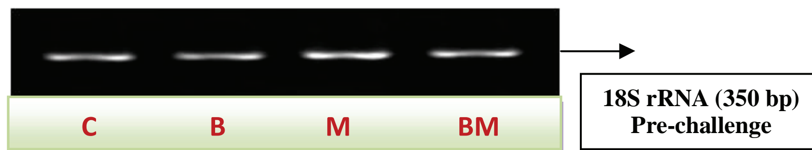



**Fig. 6.1. Experimental diets used in the study**

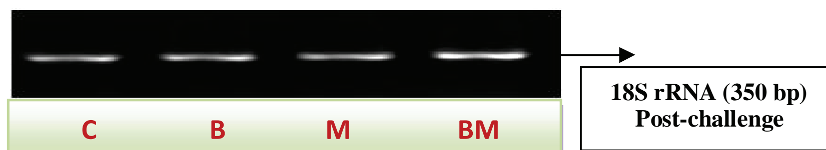
**(A) Probiotic preparations (Obtained from NCAAH, CUSAT) (B) Experimental feed**

*(C = Control feed; B = Bacillus MCCB101 incorporated feed; M = Micrococcus MCCB104 incorporated feed; BM = Combination of Bacillus MCCB101 + Micrococcus MCCB104 incorporated feed)*

(A)




(B)




(C)

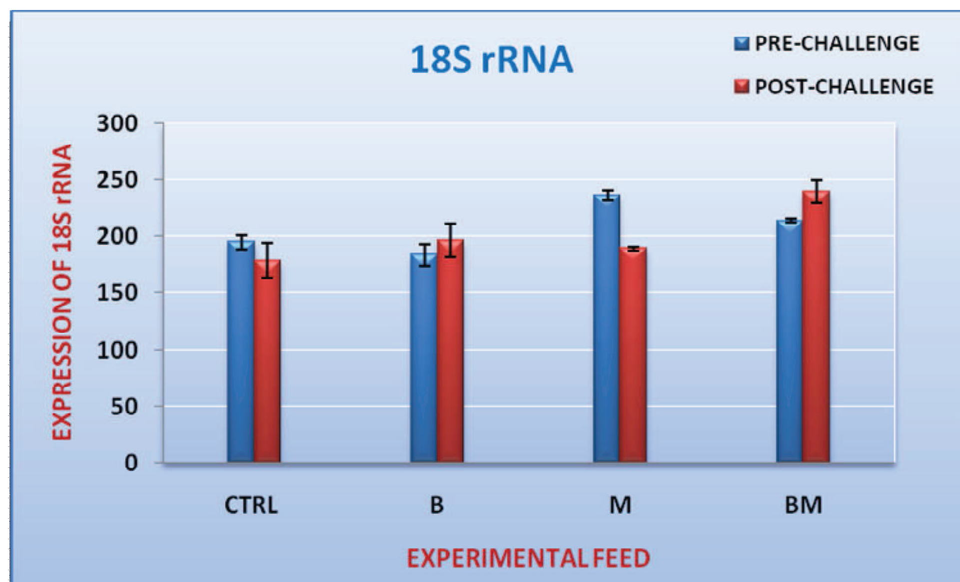



**Fig. 6.2. Expression profile of 18SrRNA (control gene) in the haemocytes of giant tiger shrimp, *P. monodon* in response to the administration of probiotic bacteria and WSSV challenge**

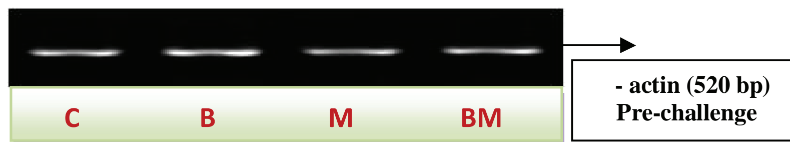
**(A) Agarose gel electrophoretogram - Pre-challenge**

**(B) Agarose gel electrophoretogram - Post-challenge WSSV**

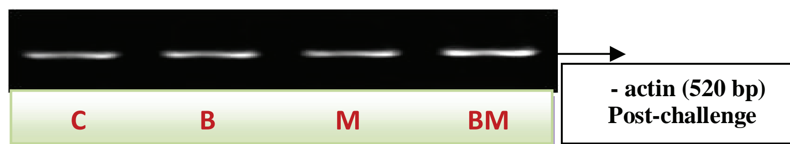
**(C) Graphical representation of the expression levels of 18S rRNA gene (x-axis = immunostimulants used, y-axis = Expression levels of the 18S rRNA gene)**

***(C – Control Feed, B – Bacillus MCCB101, M – Micrococcus MCCB104, BM – Combination of Bacillus + Micrococcus)***

(A)




(B)




(C)

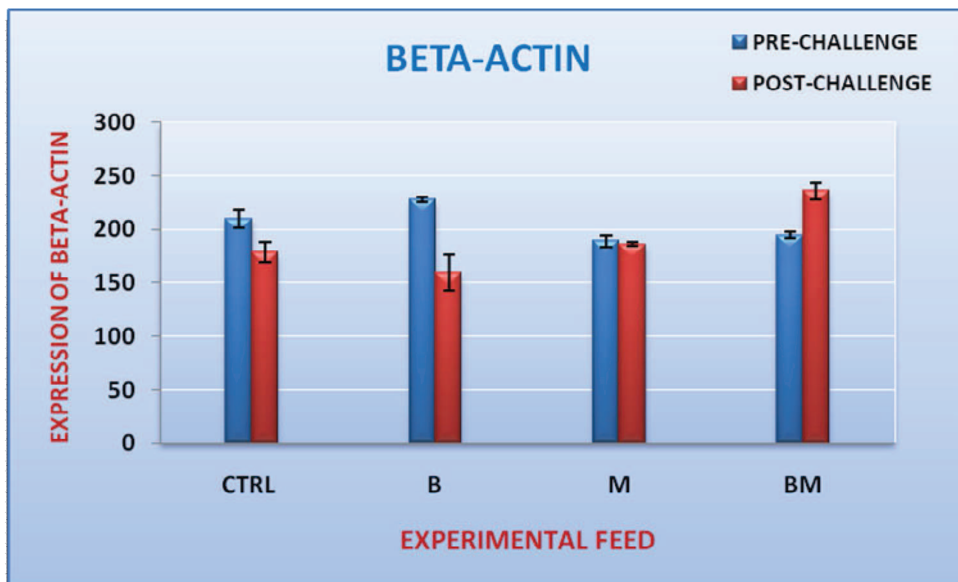



**Fig. 6.3. Expression profile of β-actin (control gene) in the haemocytes of giant tiger shrimp, *P. monodon* in response to the administration of probiotic bacteria and WSSV challenge**

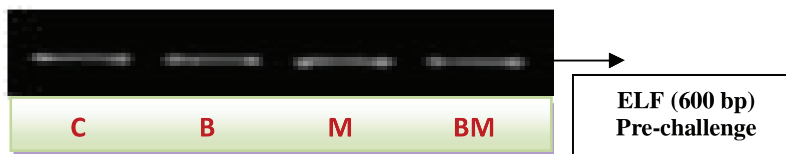
**(A) Agarose gel electrophoretogram - Pre-challenge**

**(B) Agarose gel electrophoretogram - Post-challenge WSSV**

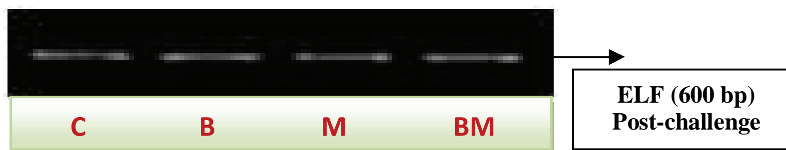
**(C) Graphical representation of the expression levels of β-actin gene (x-axis = immunostimulants used, y-axis = Expression levels of the β-actin gene)**

***(C – Control Feed, B – Bacillus MCCB101, M – Micrococcus MCCB104, BM – Combination of Bacillus + Micrococcus)***

(A)




(B)




(C)

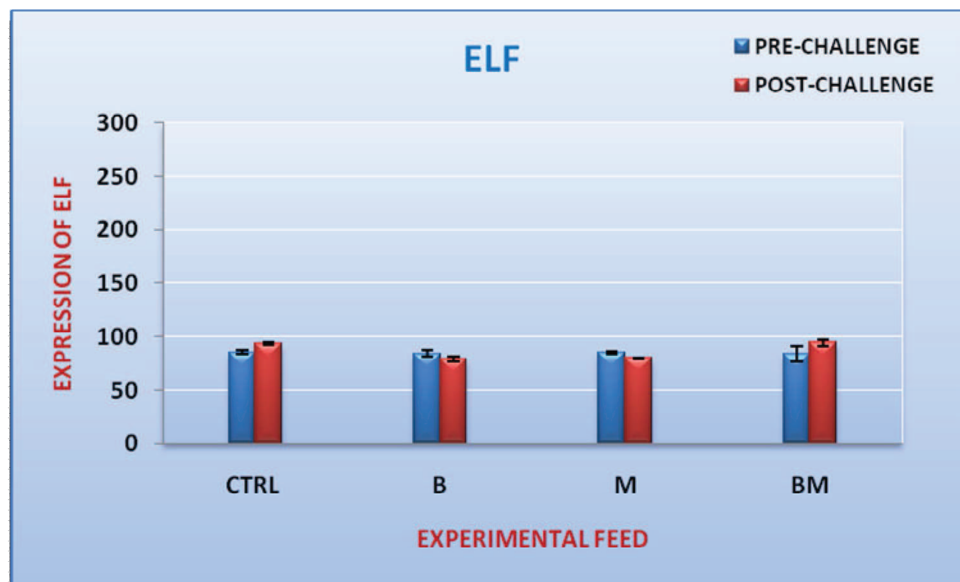



**Fig. 6.4. Expression profile of elongation factor (control gene) in the haemocytes of giant tiger shrimp, *P. monodon* in response to the administration of probiotic bacteria and WSSV challenge**

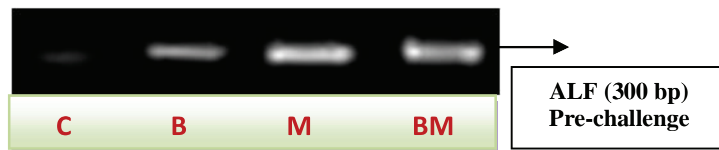
**(A) Agarose gel electrophoretogram - Pre-challenge**

**(B) Agarose gel electrophoretogram - Post-challenge WSSV**

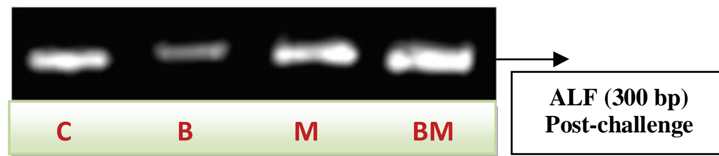
**(C) Graphical representation of the expression levels of elongation factor gene (x-axis = immunostimulants used, y-axis = Expression levels of the elongation factor gene)**

***(C – Control Feed, B – Bacillus MCCB101, M – Micrococcus MCCB104, BM – Combination of Bacillus + Micrococcus)***

(A)




(B)




(C)

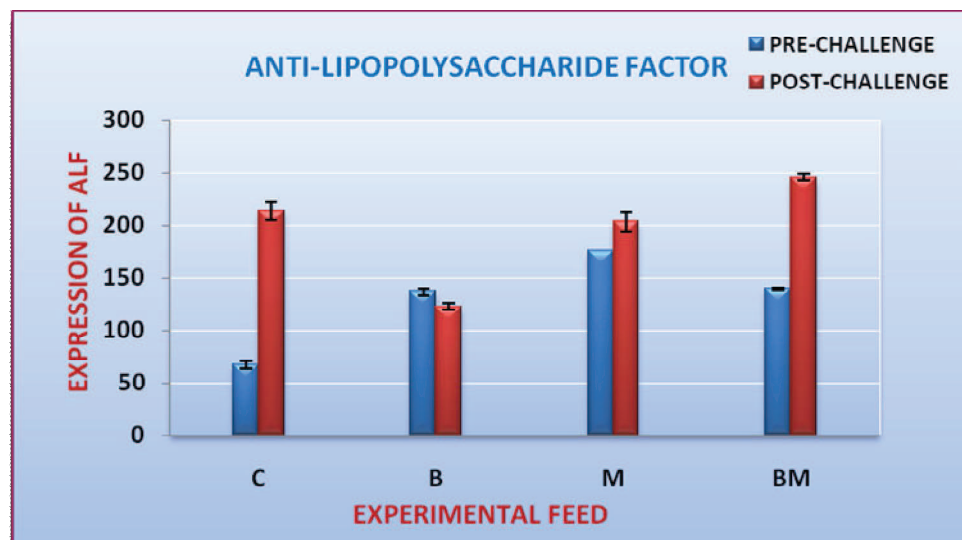



**Fig. 6.5. Expression profile of anti-lipopolysaccharide factor (AMP gene) in the haemocytes of giant tiger shrimp, *P. monodon* in response to the administration of probiotic bacteria and WSSV challenge**

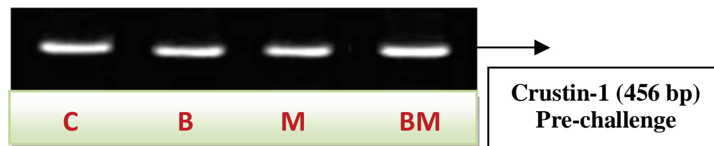
**(A) Agarose gel electrophoretogram - Pre-challenge**

**(B) Agarose gel electrophoretogram - Post-challenge WSSV**

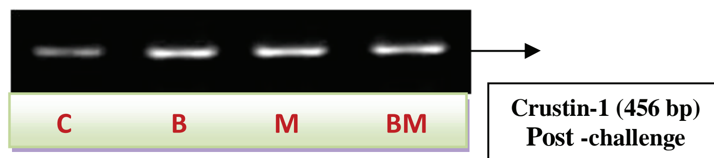
**(C) Graphical representation of the expression levels of anti-lipopolysaccharide factor gene (x-axis = immunostimulants used, y-axis = Expression levels of the anti-lipopolysaccharide factor gene)**

***(C – Control Feed, B – Bacillus MCCB101, M – Micrococcus MCCB104, BM – Combination of Bacillus + Micrococcus)***

(A)




(B)




(C)

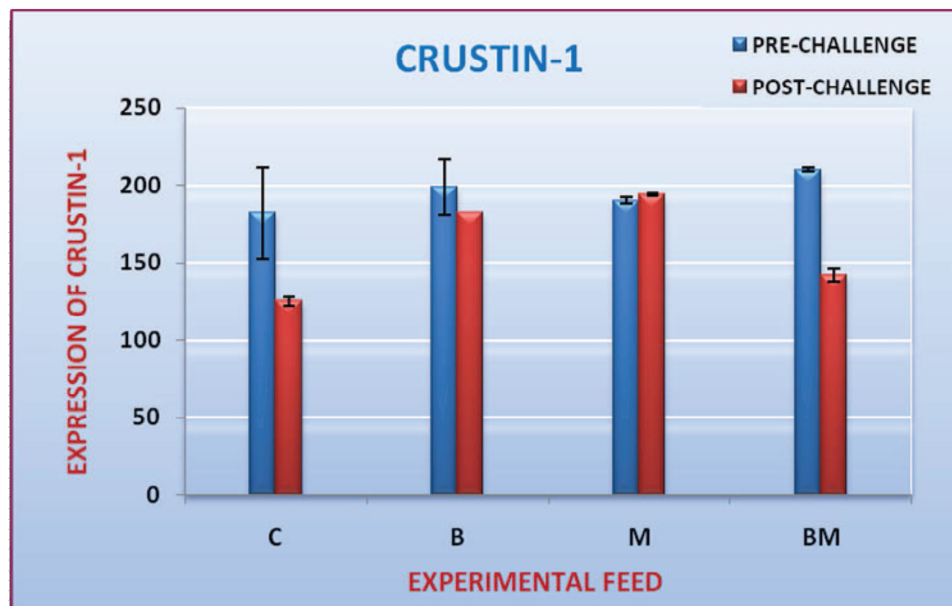



**Fig. 6.6. Expression profile of crustin-1 (AMP gene) in the haemocytes of giant tiger shrimp, *P. monodon* in response to the administration of probiotic bacteria and WSSV challenge**

**(A) Agarose gel electrophoretogram - Pre-challenge**

**(B) Agarose gel electrophoretogram - Post-challenge WSSV**

**(C) Graphical representation of the expression levels of crustin-1 gene (x-axis = immunostimulants used, y-axis = Expression levels of the crustin-1 gene)**

*(C – Control Feed, B – Bacillus MCCB101, M – Micrococcus MCCB104, BM – Combination of Bacillus + Micrococcus)*

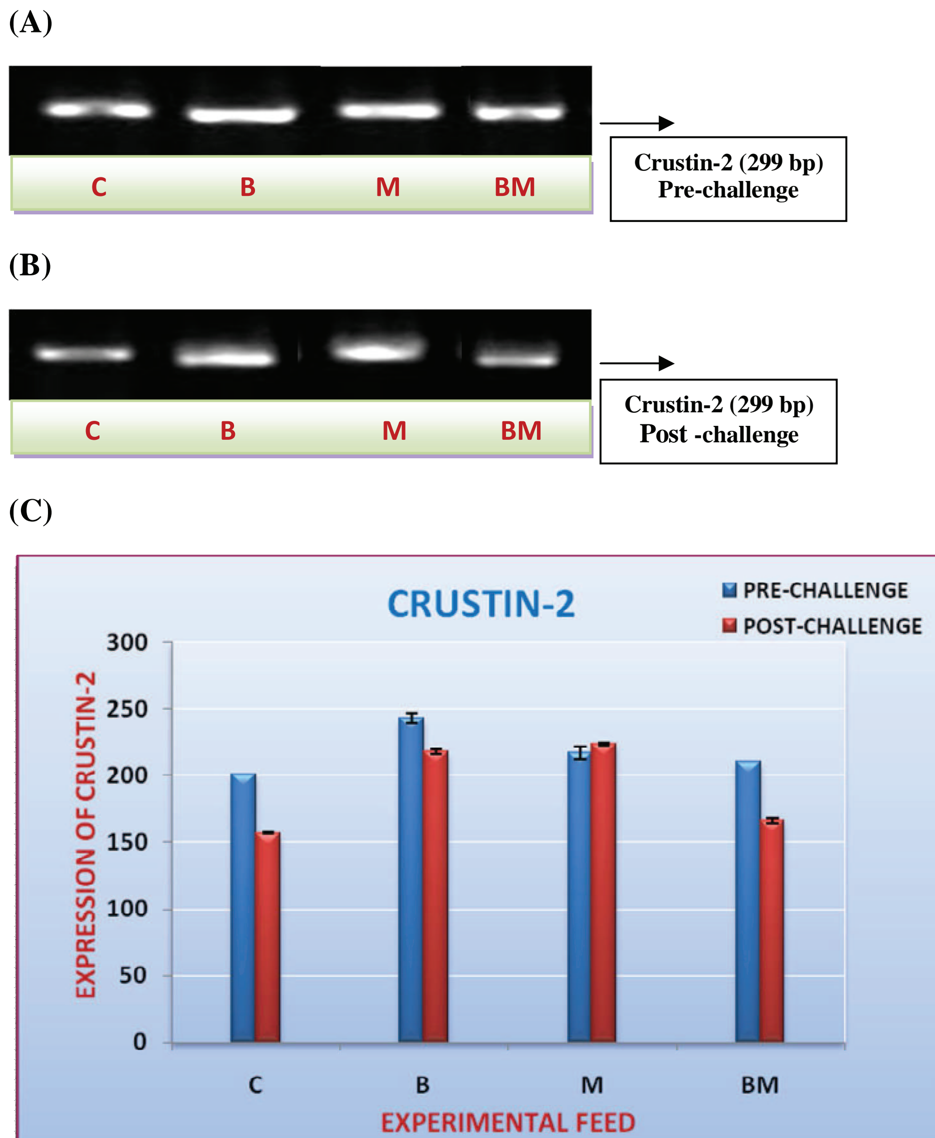



**Fig. 6.7. Expression profile of crustin-2 (AMP gene) in the haemocytes of giant tiger shrimp, *P. monodon* in response to the administration of probiotic bacteria and WSSV challenge**

**(A) Agarose gel electrophoretogram - Pre-challenge**

**(B) Agarose gel electrophoretogram - Post-challenge WSSV**

**(C) Graphical representation of the expression levels of crustin-2 gene (x-axis = immunostimulants used, y-axis = Expression levels of the crustin-2 gene)**

***(C – Control Feed, B – Bacillus MCCB101, M – Micrococcus MCCB104, BM – Combination of Bacillus + Micrococcus)***

(A)

(B)

(C)

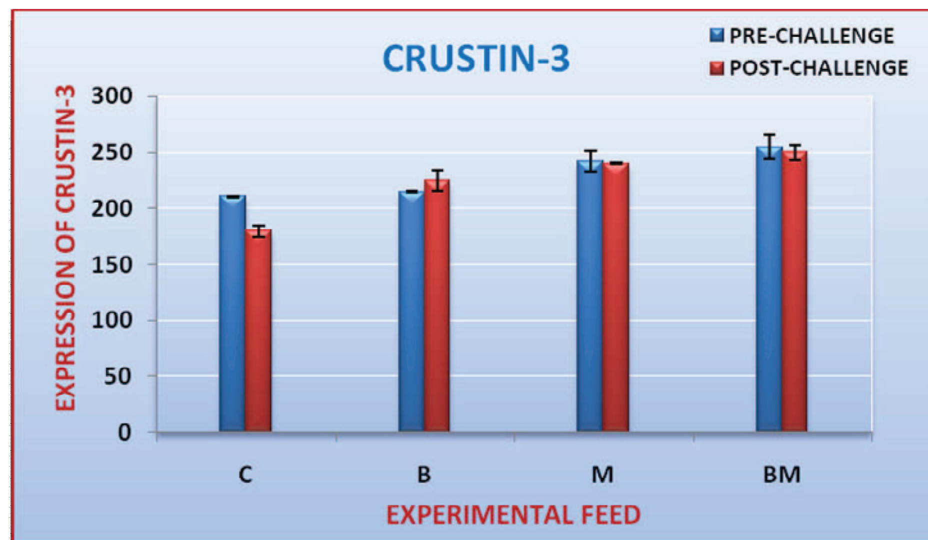



**Fig. 6.8. Expression profile of crustin-3 (AMP gene) in the haemocytes of giant tiger shrimp, *P. monodon* in response to the administration of probiotic bacteria and WSSV challenge**

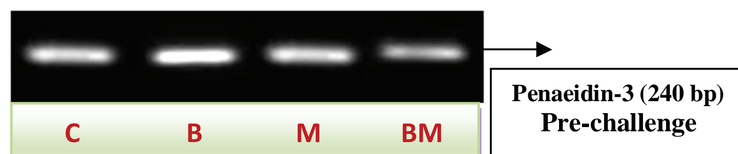
**(A) Agarose gel electrophoretogram - Pre-challenge**

**(B) Agarose gel electrophoretogram - Post-challenge WSSV**

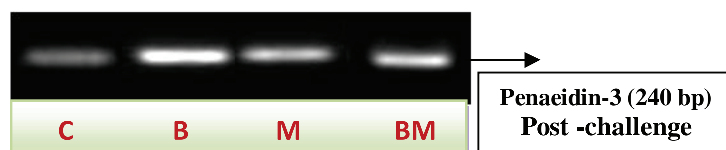
**(C) Graphical representation of the expression levels of crustin-3 gene (x-axis = immunostimulants used, y-axis = Expression levels of the crustin-3 gene)**

***(C – Control Feed, B – Bacillus MCCB101, M – Micrococcus MCCB104, BM – Combination of Bacillus + Micrococcus)***

(A)




(B)




(C)

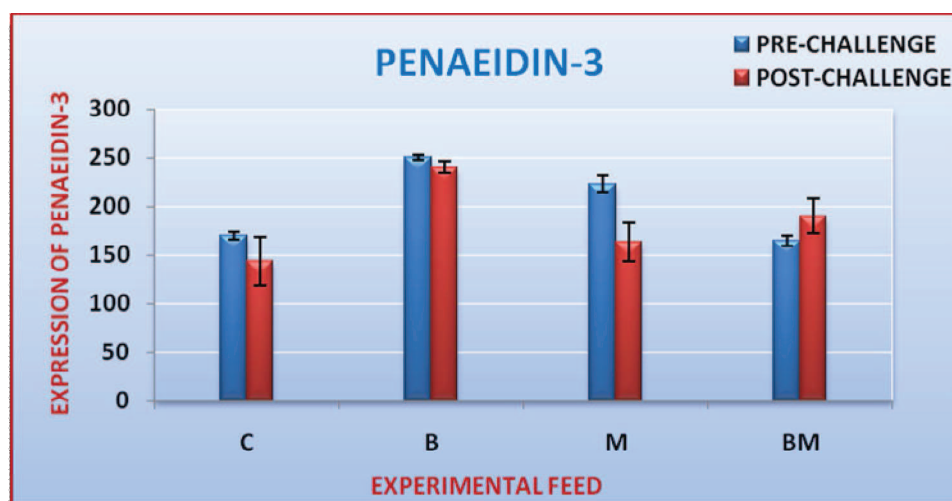



**Fig. 6.9. Expression profile of penaeidin-3 (AMP gene) in the haemocytes of giant tiger shrimp, *P. monodon* in response to the administration of probiotic bacteria and WSSV challenge**

**(A) Agarose gel electrophoretogram - Pre-challenge**

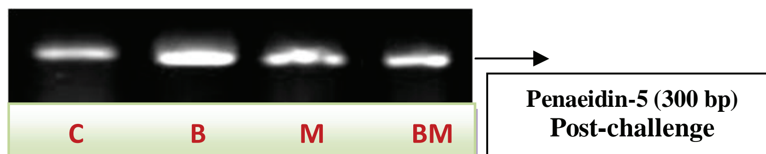
**(B) Agarose gel electrophoretogram - Post-challenge WSSV**

**(C) Graphical representation of the expression levels of penaeidin-3 gene (x-axis = immunostimulants used, y-axis = Expression levels of the penaeidin-3 gene)**

***(C – Control Feed, B – Bacillus MCCB101, M – Micrococcus MCCB104, BM – Combination of Bacillus + Micrococcus)***

(A)

(B)




(C)

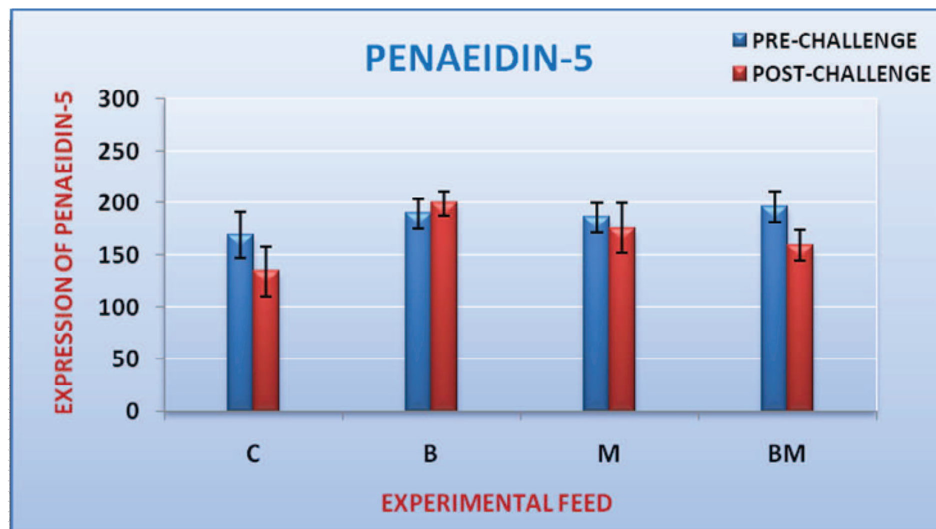



**Fig. 6.10. Expression profile of penaeidin-5 (AMP gene) in the haemocytes of giant tiger shrimp, *P. monodon* in response to the administration of probiotic bacteria and WSSV challenge**

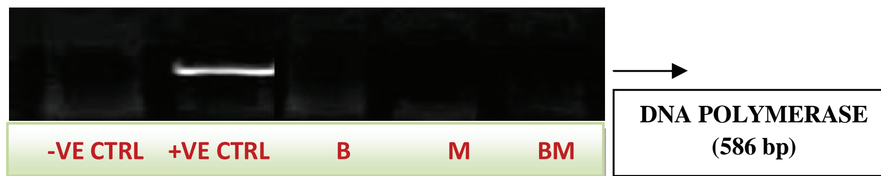
**(A) Agarose gel electrophoretogram - Pre-challenge**

**(B) Agarose gel electrophoretogram - Post-challenge WSSV**

**(C) Graphical representation of the expression levels of penaeidin-5 gene (x-axis = immunostimulants used, y-axis = Expression levels of the penaeidin-5 gene)**

***(C – Control Feed, B – Bacillus MCCB101, M – Micrococcus MCCB104, BM – Combination of Bacillus + Micrococcus)***

(A)




(B)

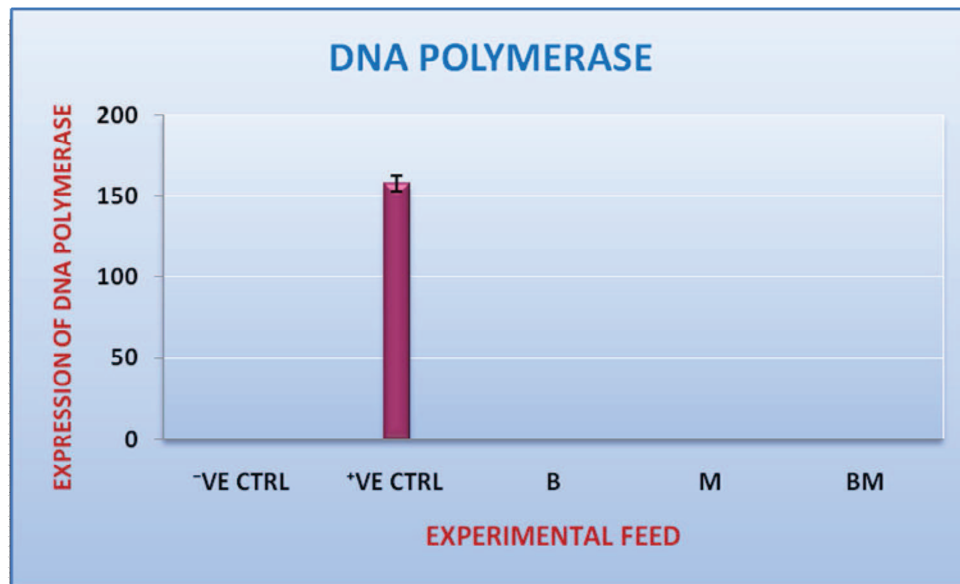


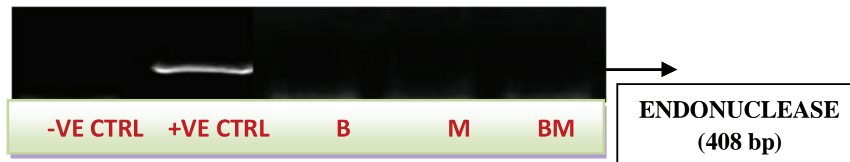

**Fig. 6.11. Expression profile of DNA polymerase (WSSV gene) in the haemocytes of giant tiger shrimp, *P. monodon* in response to the administration of probiotic bacteria and WSSV challenge**

**(A) Agarose gel electrophoretogram**

**(B) Graphical representation of the expression levels of DNA polymerase gene (x-axis = immunostimulants used, y-axis = Expression levels of the DNA polymerase gene)**

*(-ve control – Unchallenged shrimp, +ve control - WSSV Challenged shrimp, B – Bacillus MCCB101, M – Micrococcus MCCB104, BM – Combination of Bacillus + Micrococcus)*

(A)




(B)

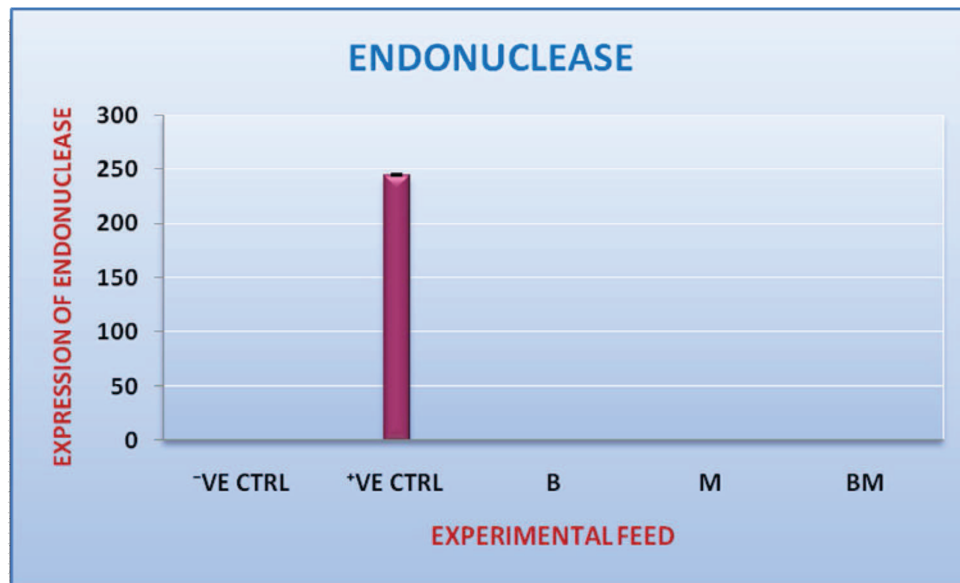



**Fig. 6.12. Expression profile of endonuclease (WSSV gene) in the haemocytes of giant tiger shrimp, *P. monodon* in response to the administration of probiotic bacteria and WSSV challenge**

**(A) Agarose gel electrophoretogram**

**(B) Graphical representation of the expression levels of endonuclease gene (x-axis = immunostimulants used, y-axis = Expression levels of the endonuclease gene)**

*(-ve control – Unchallenged shrimp, +ve control - WSSV Challenged shrimp, B – Bacillus MCCB101, M – Micrococcus MCCB104, BM – Combination of Bacillus + Micrococcus)*

(A)

(B)

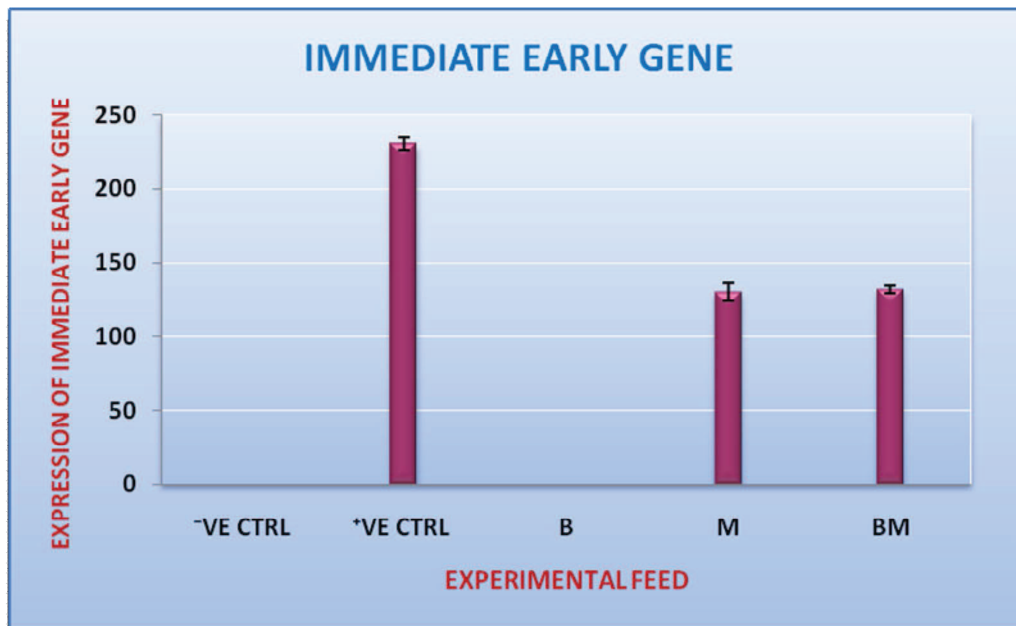



**Fig. 6.13. Expression profile of immediate early gene (WSSV gene) in the haemocytes of giant tiger shrimp, *P. monodon* in response to the administration of probiotic bacteria and WSSV challenge**

**(A) Agarose gel electrophoretogram**

**(B) Graphical representation of the expression levels of immediate early gene (x-axis = immunostimulants used, y-axis = Expression levels of the immediate early gene)**

*(-ve control – Unchallenged shrimp, +ve control - WSSV Challenged shrimp, B – Bacillus MCCB101, M – Micrococcus MCCB104, BM – Combination of Bacillus + Micrococcus)*

(A)

(B)

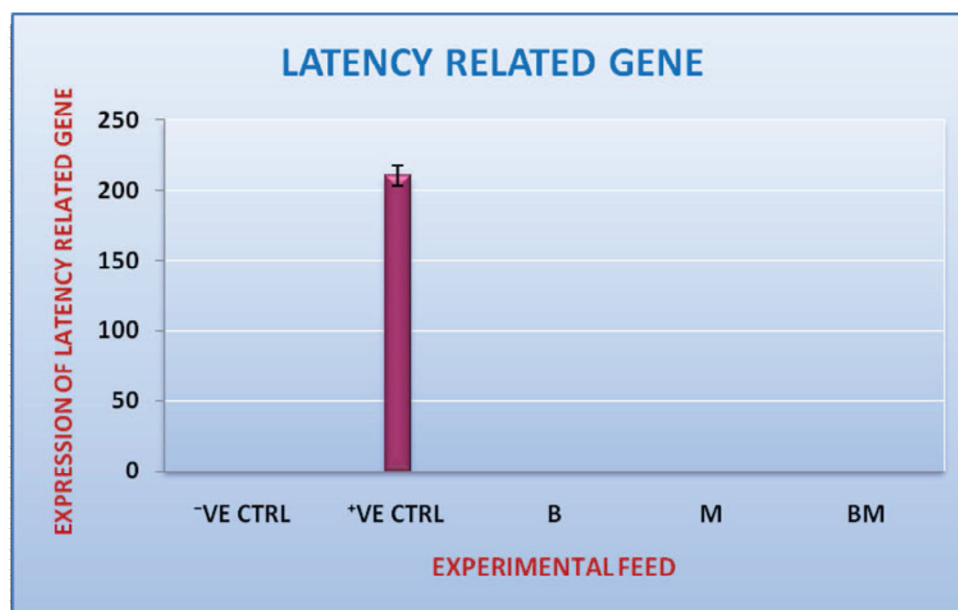


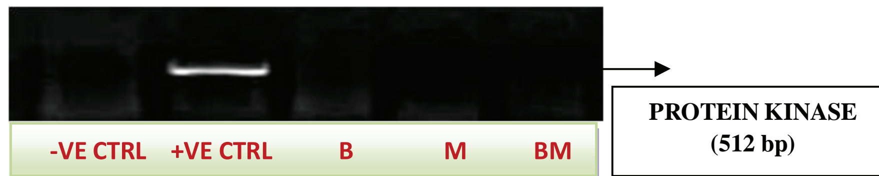

**Fig. 6.14. Expression profile of latency related gene (WSSV gene) in the haemocytes of giant tiger shrimp, *P. monodon* in response to the administration of probiotic bacteria and WSSV challenge**

**(A) Agarose gel electrophoretogram**

**(B) Graphical representation of the expression levels of latency related gene gene (x-axis = immunostimulants used, y-axis = Expression levels of the latency related gene)**

*(-ve control – Unchallenged shrimp, +ve control - WSSV Challenged shrimp, B – Bacillus MCCB101, M – Micrococcus MCCB104, BM – Combination of Bacillus + Micrococcus)*

(A)




(B)

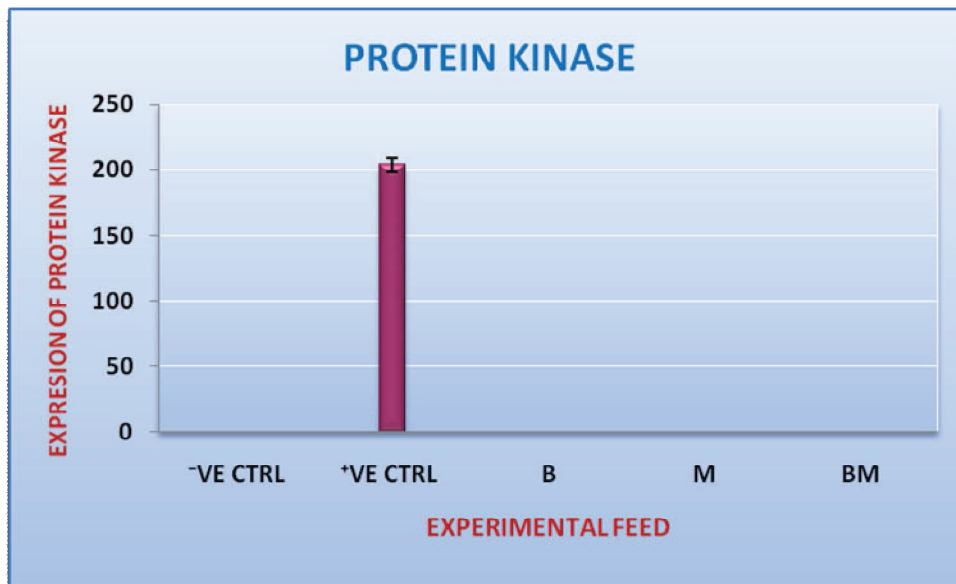


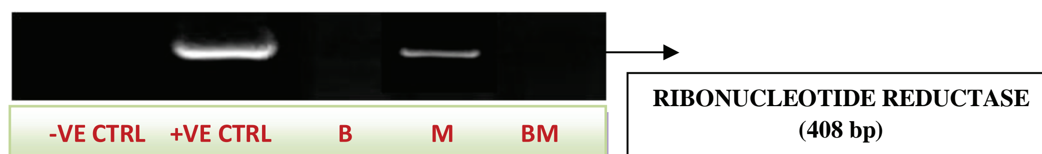

**Fig. 6.15. Expression profile of protein kinase (WSSV gene) in the haemocytes of giant tiger shrimp, *P. monodon* in response to the administration of probiotic bacteria and WSSV challenge**

**(A) Agarose gel electrophoretogram**

**(B) Graphical representation of the expression levels of protein kinase gene (x-axis = immunostimulants used, y-axis = Expression levels of the protein kinase gene)**

*(-ve control – Unchallenged shrimp, +ve control - WSSV Challenged shrimp, B – Bacillus MCCB101, M – Micrococcus MCCB104, BM – Combination of Bacillus + Micrococcus)*

(A)




(B)

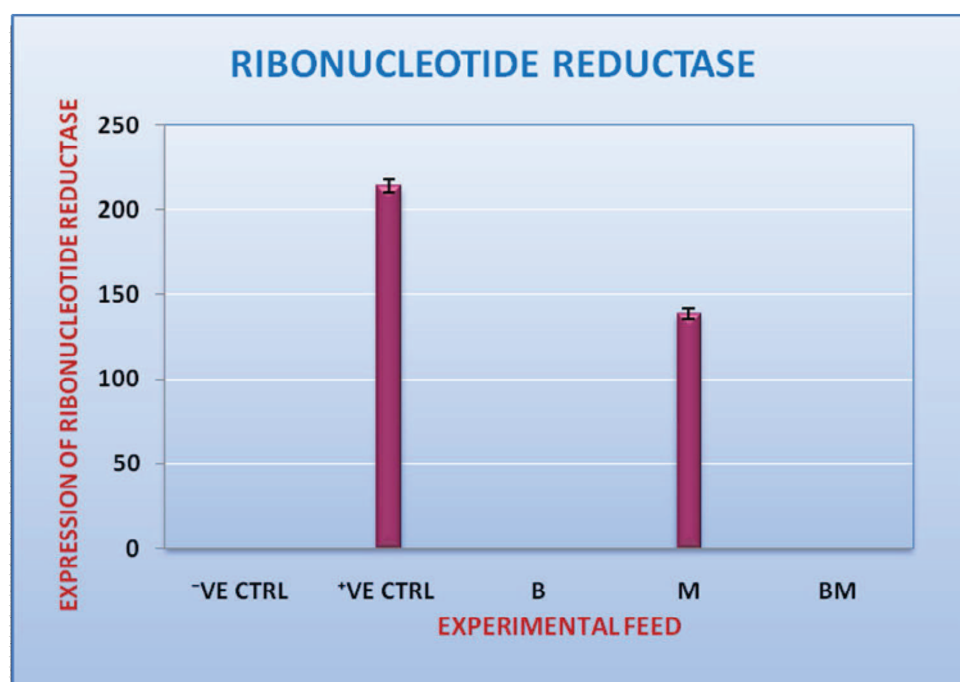


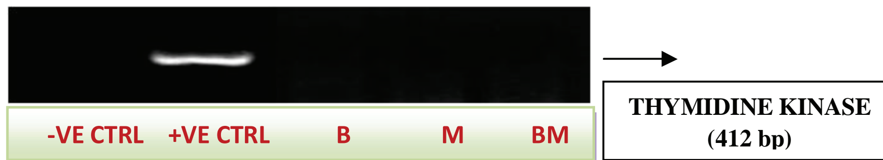

**Fig. 6.16. Expression profile of ribonucleotide reductase (WSSV gene) in the haemocytes of giant tiger shrimp, *P. monodon* in response to the administration of probiotic bacteria and WSSV challenge**

**(A) Agarose gel electrophoretogram**

**(B) Graphical representation of the expression levels of ribonucleotide reductase gene (x-axis = immunostimulants used, y-axis = Expression levels of the ribonucleotide reductase gene)**

*(-ve control – Unchallenged shrimp, +ve control - WSSV Challenged shrimp, B – Bacillus MCCB101, M – Micrococcus MCCB104, BM – Combination of Bacillus + Micrococcus)*

(A)




(B)

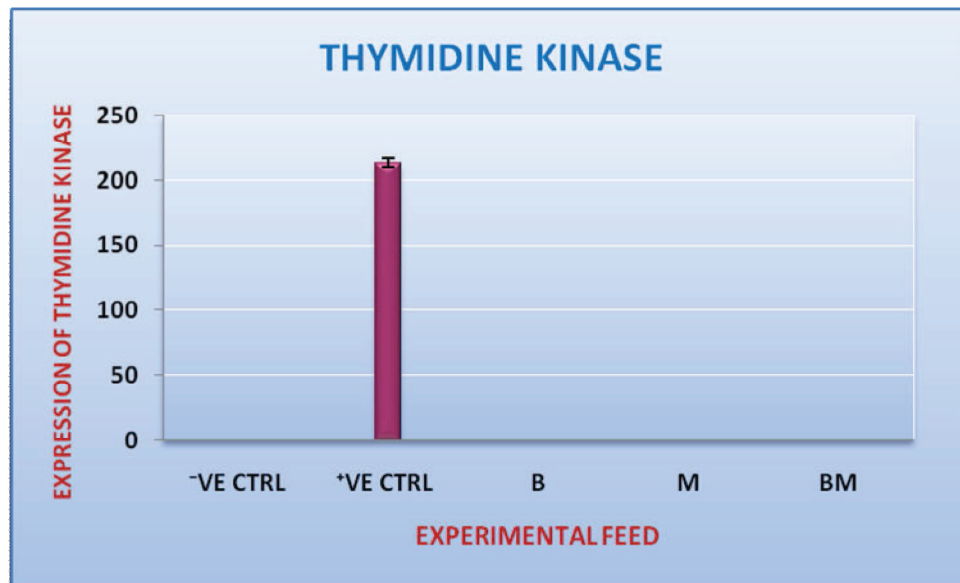


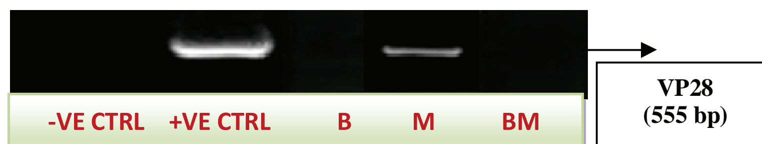

**Fig. 6.17. Expression profile of thymidine kinase (WSSV gene) in the haemocytes of giant tiger shrimp, *P. monodon* in response to the administration of probiotic bacteria and WSSV challenge**

**(A) Agarose gel electrophoretogram**

**(B) Graphical representation of the expression levels of thymidine kinase gene (x-axis = immunostimulants used, y-axis = Expression levels of the thymidine kinase gene)**

*(-ve control – Unchallenged shrimp, +ve control - WSSV Challenged shrimp, B – Bacillus MCCB101, M – Micrococcus MCCB104, BM – Combination of Bacillus + Micrococcus)*

(A)




(B)

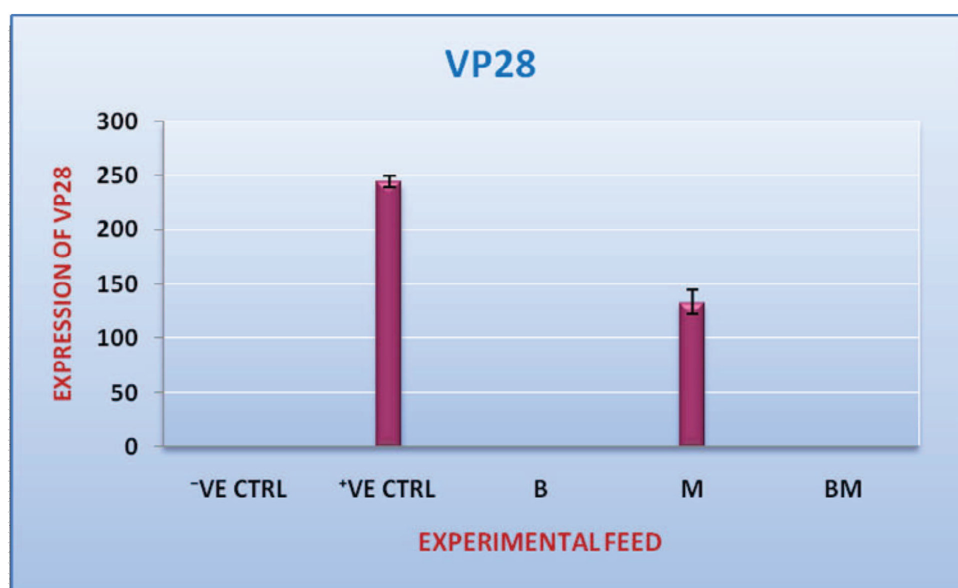



**Fig. 6.18. Expression profile of VP28 (WSSV gene) in the haemocytes of giant tiger shrimp, *P. monodon* in response to the administration of probiotic bacteria and WSSV challenge**

**(A) Agarose gel electrophoretogram**

**(B) Graphical representation of the expression levels of VP28 gene (x-axis = immunostimulants used, y-axis = Expression levels of the VP28 gene)**

*(-ve control – Unchallenged shrimp, +ve control - WSSV Challenged shrimp, B – Bacillus MCCB101, M – Micrococcus MCCB104, BM – Combination of Bacillus + Micrococcus)*

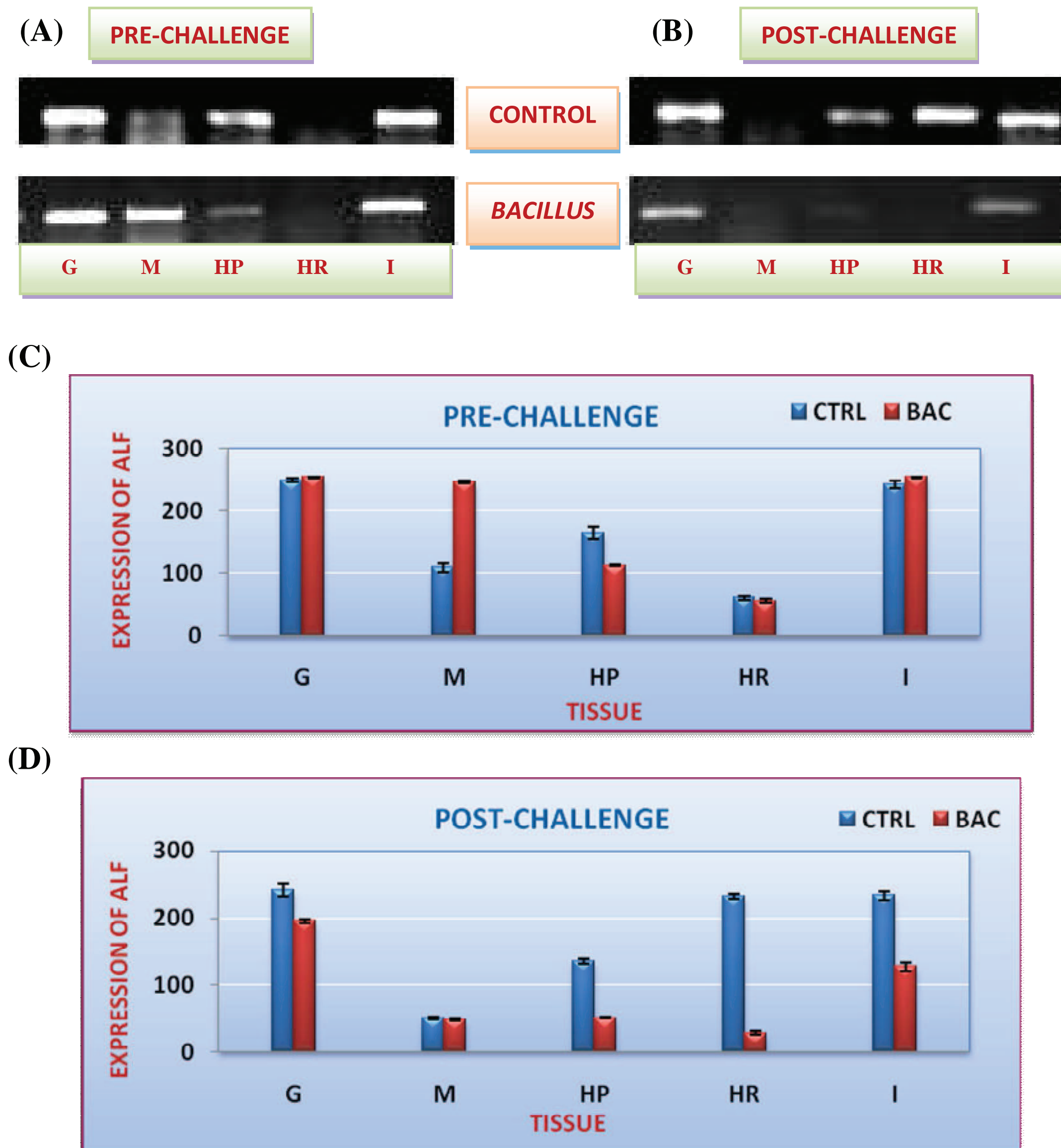



**Fig. 6.19. Tissue-wise expression profile of ALF (AMP gene) in various tissues of giant tiger shrimp, *P. monodon* in response to the administration of probiotic *Bacillus* and WSSV challenge**

**(A) Agarose gel electrophoretogram of control and *Bacillus* treated groups pre-challenge WSSV**

**(B) Agarose gel electrophoretogram of control and *Bacillus* treated groups Post-challenge WSSV**

**(C) & (D) Graphical representation of expression level of ALF gene pre- and post-challenge WSSV (x-axis = tissues analyzed, y-axis = expression level of ALF gene)**

***(G-Gill, M-Muscle, HP-Hepatopancreas, HR-Heart, I-Intestine)***

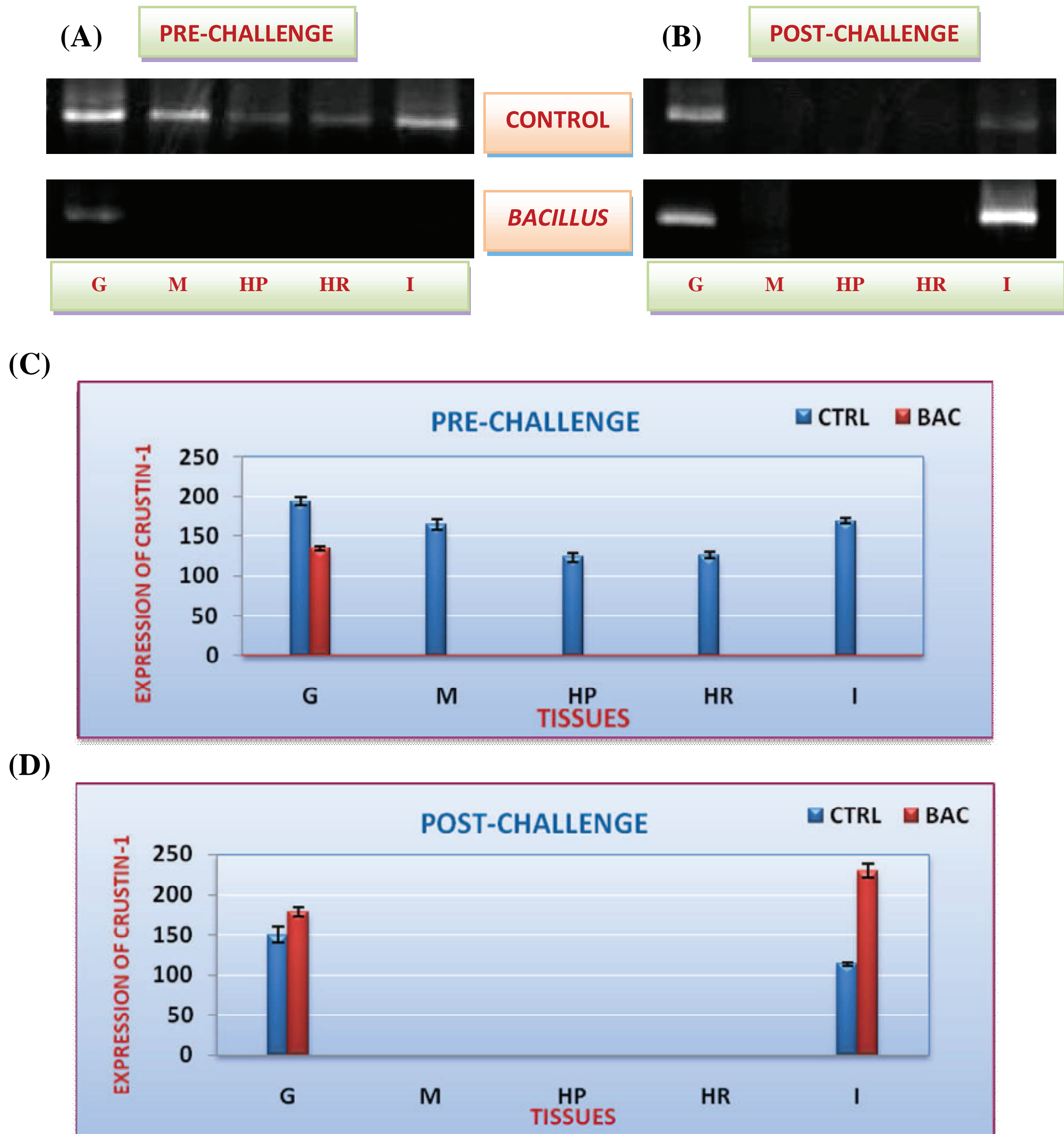



**Fig. 6.20. Tissue-wise expression profile of crustin-1 (AMP gene) in various tissues of giant tiger shrimp, *P. monodon* in response to the administration of probiotic *Bacillus* and WSSV challenge**

**(A) Agarose gel electrophoretogram of control and *Bacillus* treated groups pre-challenge WSSV**

**(B) Agarose gel electrophoretogram of control and *Bacillus* treated groups Post-challenge WSSV**

**(C) & (D) Graphical representation of expression level of crustin-1 gene pre- and post-challenge WSSV (x-axis = tissues analyzed, y-axis = expression level of crustin-1 gene)**

***(G-Gill, M-Muscle, HP-Hepatopancreas, HR-Heart, I-Intestine)***

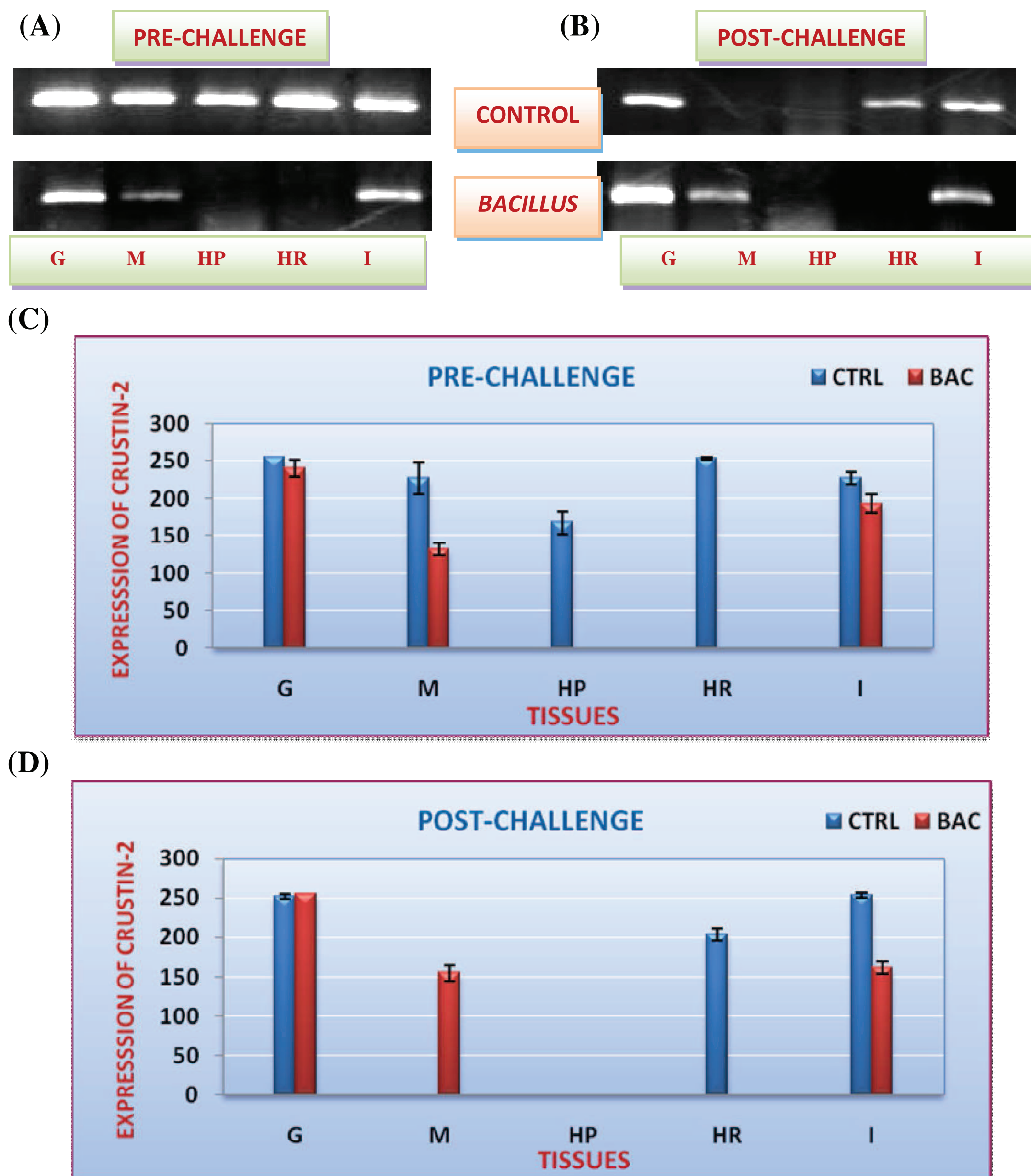



**Fig. 6.21. Tissue-wise expression profile of crustin-2 (AMP gene) in various tissues of giant tiger shrimp, *P. monodon* in response to the administration of probiotic *Bacillus* and WSSV challenge**

**(A) Agarose gel electrophoretogram of control and *Bacillus* treated groups pre-challenge WSSV**

**(B) Agarose gel electrophoretogram of control and *Bacillus* treated groups Post-challenge WSSV**

**(C) & (D) Graphical representation of expression level of crustin-2 gene pre- and post-challenge WSSV (x-axis = tissues analyzed, y-axis = expression level of crustin-2 gene)**

***(G-Gill, M-Muscle, HP-Hepatopancreas, HR-Heart, I-Intestine)***

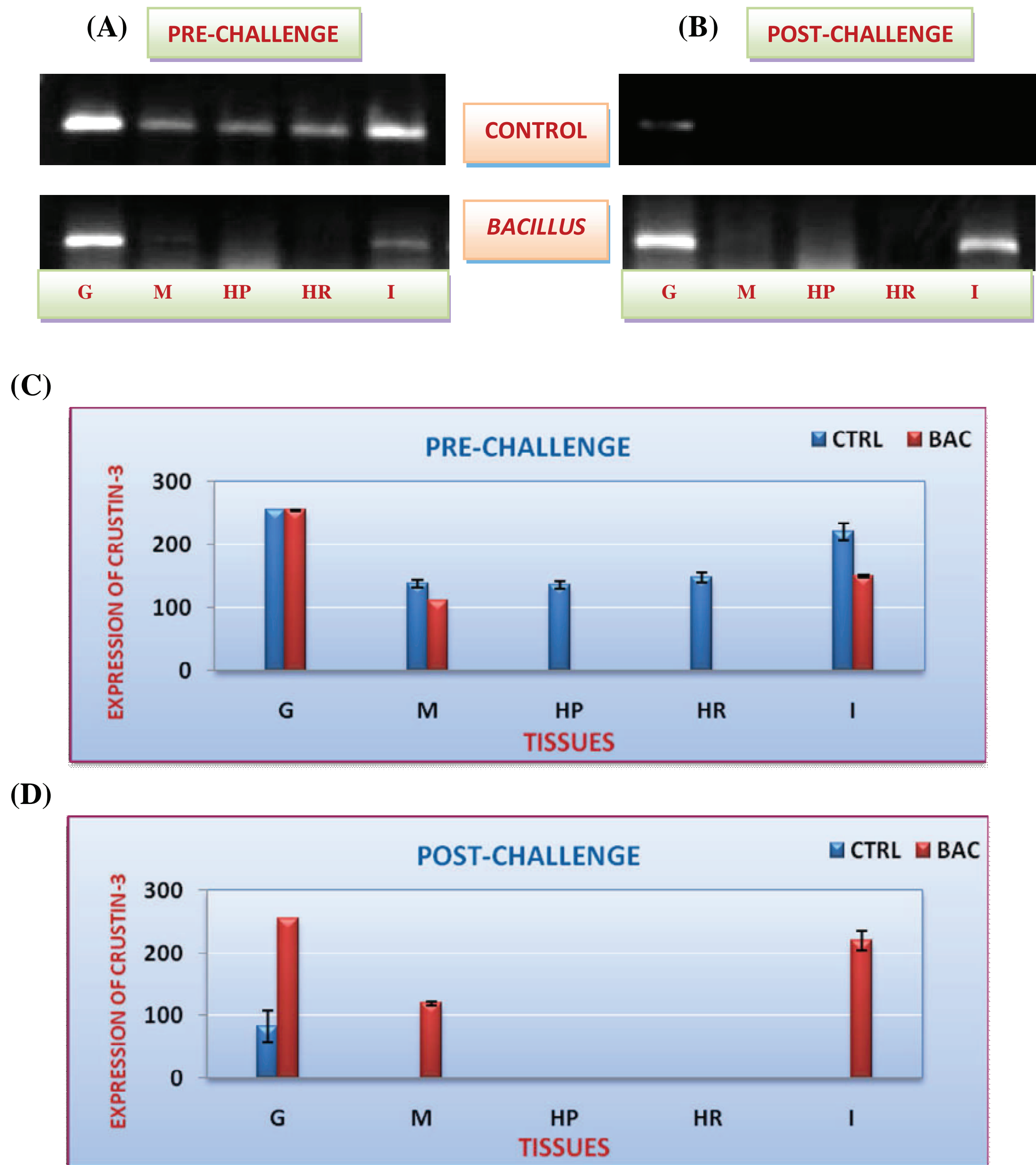



**Fig. 6.22. Tissue-wise expression profile of crustin-3 (AMP gene) in various tissues of giant tiger shrimp, *P. monodon* in response to the administration of probiotic *Bacillus* and WSSV challenge**

**(A) Agarose gel electrophoretogram of control and *Bacillus* treated groups pre-challenge WSSV**

**(B) Agarose gel electrophoretogram of control and *Bacillus* treated groups Post-challenge WSSV**

**(C) & (D) Graphical representation of expression level of crustin-3 gene pre- and post-challenge WSSV (x-axis = tissues analyzed, y-axis = expression level of crustin-3 gene)**

***(G-Gill, M-Muscle, HP-Hepatopancreas, HR-Heart, I-Intestine)***

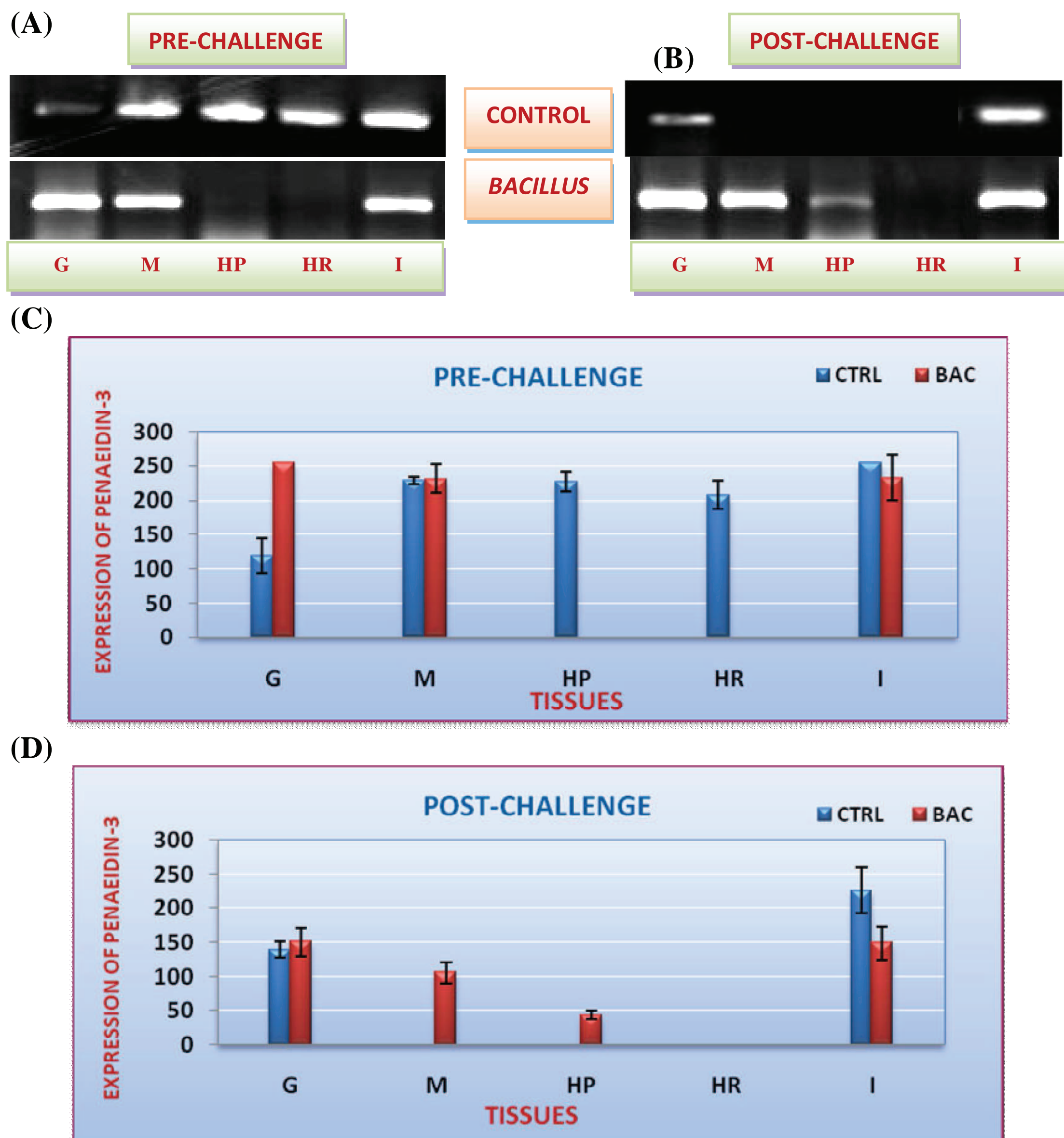



**Fig. 6.23. Tissue-wise expression profile of penaeidin-3 (AMP gene) in various tissues of giant tiger shrimp, *P. monodon* in response to the administration of probiotic *Bacillus* and WSSV challenge**

**(A) Agarose gel electrophoretogram of control and *Bacillus* treated groups pre-challenge WSSV**

**(B) Agarose gel electrophoretogram of control and *Bacillus* treated groups Post-challenge WSSV**

**(C) & (D) Graphical representation of expression level of penaeidin-3 gene pre- and post-challenge WSSV (x-axis = tissues analyzed, y-axis = expression level of penaeidin-3 gene)**

***(G-Gill, M-Muscle, HP-Hepatopancreas, HR-Heart, I-Intestine)***

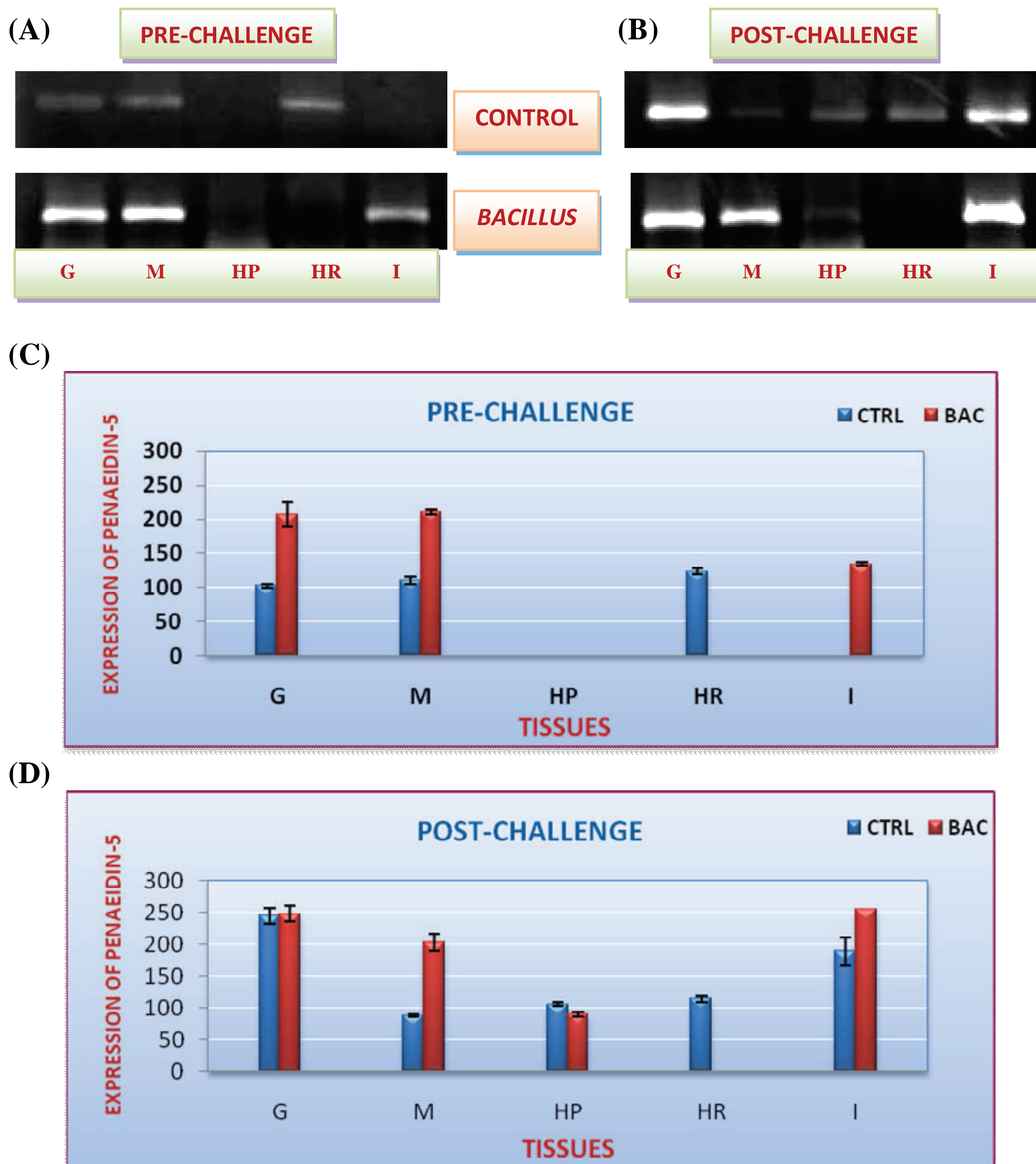



**Fig. 6.24. Tissue-wise expression profile of penaeidin-5 (AMP gene) in various tissues of giant tiger shrimp, *P. monodon* in response to the administration of probiotic *Bacillus* and WSSV challenge**

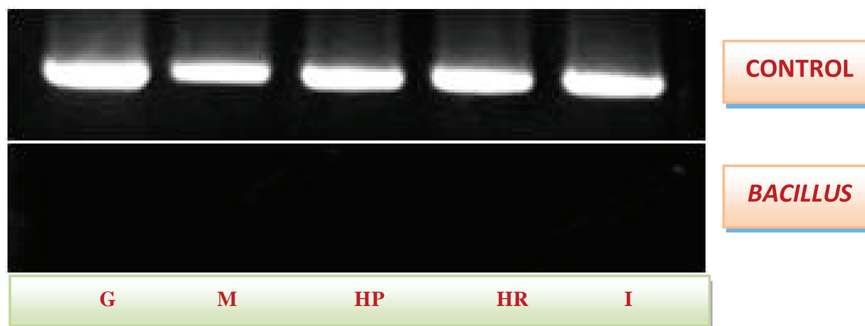
**(A) Agarose gel electrophoretogram of control and *Bacillus* treated groups pre-challenge WSSV**

**(B) Agarose gel electrophoretogram of control and *Bacillus* treated groups Post-challenge WSSV**

**(C) & (D) Graphical representation of expression level of penaeidin-5 gene pre- and post-challenge WSSV (x-axis = tissues analyzed, y-axis = expression level of penaeidin-5 gene)**

***(G-Gill, M-Muscle, HP-Hepatopancreas, HR-Heart, I-Intestine)***

**(A)**




**(B)**

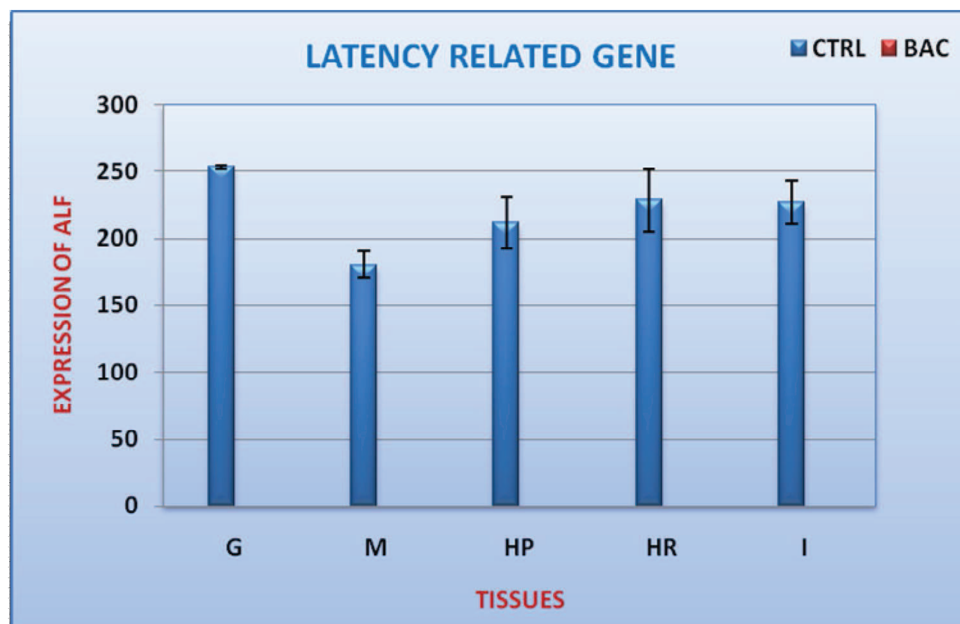



**Fig. 6.25. Tissue-wise expression profile of latency related gene (WSSV gene) in various tissues of giant tiger shrimp, *P. monodon* in response to the administration of probiotic *Bacillus* and WSSV challenge**

**(A) Agarose gel electrophoretogram of control and *Bacillus* treated groups**

**(B) Graphical representation of expression level of latency related gene (x-axis = tissues analyzed, y-axis = expression level of latency related gene)**

***(G-Gill, M-Muscle, HP-Hepatopancreas, HR-Heart, I-Intestine)***

(A)

(B)

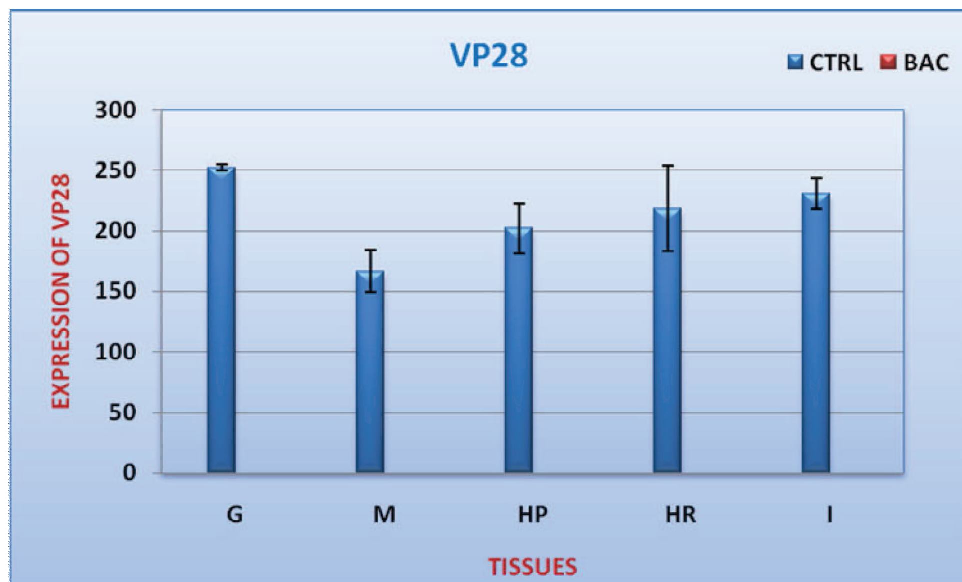



**Fig. 6.26. Tissue-wise expression profile of VP28 (WSSV gene) in various tissues of giant tiger shrimp, *P. monodon* in response to the administration of probiotic *Bacillus* and WSSV challenge**

**(A) Agarose gel electrophoretogram of control and *Bacillus* treated groups**

**(B) Graphical representation of expression level of VP28 gene (x-axis = tissues analyzed, y-axis = expression level of VP28 gene)**

***(G-Gill, M-Muscle, HP-Hepatopancreas, HR-Heart, I-Intestine)***

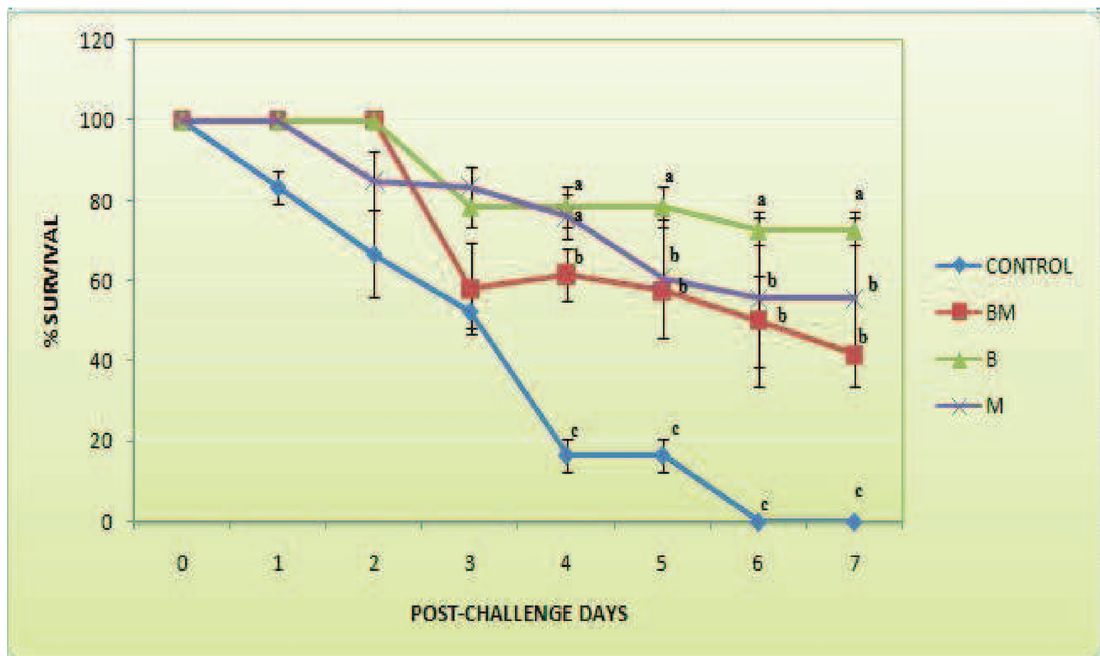



**Fig. 6.27. Post challenge survival of *P. monodon* fed with different probiotic bacteria incorporated diets and challenged with WSSV**

***(B – Bacillus MCCB101 administered group, M – Micrococcus MCCB104 administered group, BM – Combination of Bacillus MCCB101 + Micrococcus MCCB104 administered group)***

# *CHAPTER-7*

## *Summary & Conclusion*

## 7.1. Summary

Tropical shrimp culture is one of the fastest growing aquaculture sectors in the world. Since this production sector is highly affected by infectious pathogens, disease control is nowadays a priority. Application of various immunostimulants and probiotics to activate or boost the innate immune system has been widely accepted as a good alternative to reduce the widespread use of antibiotics. However, there is a reasonable degree of doubt about the efficacy of these stimulants, derived from microorganisms in promoting immunoprotection in cultured organisms. Also, effective prevention methods can be developed more efficiently when quantitative assays for the evaluation and monitoring of the health status of shrimps are available. Certainly several important issues remain to be resolved before their usefulness in crustacean aquaculture can be validated. In particular there is an urgent need to analyze their ability to up-regulate the expression of genes encoding defense proteins, as such studies are necessary to elucidate the mode of action of any promising compounds and thereby help to refine their use. Linked to this is the need for an extensive study of the defense responses of the host during WSSV infection. During the last two decades, much progress has been made in the understanding of the invertebrate innate immune system. The recent discovery of antimicrobial peptides in crustaceans provides new clues for understanding the fundamentals of crustacean immunity. Knowledge of AMP functions will be particularly important for further establishment of disease control in penaeid shrimp production. This class of proteins represents an exciting alternative approach to the concept of immunostimulation because the action of some is limited to prokaryotic material and any stimulant that causes an increase in

their expression will probably not cause damage to the crustacean host. In addition, evidence is accumulating that these compounds may have anti-viral properties. Compounds that effect up-regulation of genes coding for specific antimicrobial peptides could be explored by screening currently available immunostimulants. However, so far hardly any information is available on the expression of antimicrobial peptide (AMP) genes in shrimps in response to the administration of immunostimulants/probiotics and WSSV infection. Therefore, the present work was aimed at characterizing AMP genes in two important penaeid shrimps viz. *P.monodon* and *F. indicus* and studying the differential expression of AMP genes in response to immunostimulant/probiotic administration pre- and post-challenge WSSV.

The objectives of the study were:

- Molecular characterization and phylogenetic analysis of AMPs in *Penaeus monodon* and *Fenneropenaeus indicus*
- Time-course of AMP gene expression in response to WSSV challenge in *Penaeus monodon*
- Tissue-wise expression profile of AMP genes in *Penaeus monodon*
- Expression profile of AMP genes in response to the administration of immunostimulants in *Penaeus monodon* pre- and post-challenge WSSV
- Expression profile of AMP genes in response to the administration of probiotic bacteria in *Penaeus monodon* pre- and post-challenge WSSV

# Salient findings

- ✓ From *P. monodon*, seven AMP genes belonging to ALF, crustin and penaeidin family viz. ALF-1, ALF-2, crustin-1, crustin-2, crustin-3, penaeidin-3 and penaeidin-5 could be characterized at molecular level.

- ✓ From *F. indicus*, five AMP genes belonging to ALF, crustin and penaeidin family viz. ALF-1, ALF-2, Fi-crustin, penaeidin-5 and Fi-penaeidin and could be characterized at molecular level.

- ✓ The phylogenetic analysis performed for the AMPs of *P. monodon* and *F. indicus* revealed that the AMPs of shrimps were evolutionarily closely related to AMPs of other decapods species. The molecular phylogenetic tree implied that each AMP family possesses a same ancestral origin, which has subsequently diverged at different phases of evolution.

- ✓ Control genes viz. 18S rRNA, β-actin and elongation factor were also characterized from *P. monodon* and *F. indicus.*

- ✓ All the nucleotide sequences and the deduced amino acid sequences were submitted to GenBank under the following accession numbers:

  - ✓ *P. monodon*
    - ✓ ALF-1 - **GU732817**
    - ✓ ALF-2 - **HM588914**
    - ✓ Crustin-1 - **GQ334395**
    - ✓ Crustin-2 - **FJ535568**
    - ✓ Crustin-3 - **GQ334396**
    - ✓ Penaeidin-3 - **GU732819**
    - ✓ Penaeidin-5 - **GQ334397**

- ✓ β-actin – **GQ334394**
- ✓ ELF – **GU732818**

✓ *F. indicus*

- ✓ ALF-1 - **GU732814**
- ✓ ALF-2 - **HM366921**
- ✓ Fi-Crustin - **GQ469987**
- ✓ Penaeidin-5 - **HM243616**
- ✓ Fi-Penaeidin - **HM243617**
- ✓ β-actin – **GU732815**
- ✓ ELF – **GU732816**

✓ Among these, ALF (**GU732817**) and Crustin-2 (**FJ535568**) of *P. monodon* and Fi-crustin **(GQ469987),** ALF (**GU732814**), Penaeidin-5 (**HM243616)**, and Fi-penaeidin **(HM243617)** of *F. indicus* were found to be novel isoforms.

✓ Fi-crustin, Fi-penaeidin and Penaeidin-5 were the first AMPs to be reported from *F. indicus*

✓ Expression profiling of control genes revealed ELF to be the best control gene for gene expression studies in WSSV challenged shrimps

✓ In shrimp haemocytes, ALF and penaeidin-3 genes were found to be up-regulated during early hours (24-48 hrs) of WSSV challenge. However, ALF and crustin-3 were found to be up-regulated even during late hours of WSSV infection.

✓ Analysis of AMP gene expression profile during various hours of WSSV challenge, revealed the probable role of ALF, Crustin-3 and Penaeidin-3 in antiviral defense of shrimps

- ✓ Tissue-wise expression profile was found to be unique for the various AMP genes studied. All AMPs were found to be constitutively produced in various tissues of *P. monodon*. Maximum level of AMP expression was found in the haemocytes followed by gills, intestine, heart, muscle and hepatopancreas.
- ✓ On WSSV challenge a general down-regulation of all the AMP genes (except ALF) could be observed in the control group of shrimps
- ✓ Administration of immunostimulant and probiotic incorporated diet was found to alter the expression of AMP genes
- ✓ Even under WSSV challenged conditions, immunostimulant fed group up-regulated the AMP genes
- ✓ Though administration of both marine yeasts and beta-glucans up-regulated the AMP genes, marine yeasts (whole cell) were found to be better immunostimulant than β-1, 3 glucans
- ✓ *C. haemulonii* S27 yeast - proved to be the best immunostimulant in terms of AMP gene expression (especially ALF and penaeidin-3) and post-challenge survival
- ✓ Probionts administered individually and in combination were found to alter the expression of AMP genes
- ✓ Probiotics supported maximum up-regulation of ALF and Penaeidin-3 genes pre-challenge WSSV
- ✓ On WSSV challenge, probiotic bacteria was found to support up-regulation of all AMP genes

- ✓ Though all the three probiotic supplements were found to support up-regulation of AMP genes, *Bacillus* MCCB101 was found to perform best in terms of AMP gene expression and post-challenge survival.
- ✓ Shrimps maintained on marine yeast and probiotic diet showed delayed WSSV infection and hence the immunostimulants and probiotics were found to confer protection to some extent against WSSV infection.

# Conclusion

The constitutive production of AMPs in shrimps ensures that animals are able to protect themselves from low-level assaults by pathogens present in the environment. As these molecules play important roles in the shrimp immune defense system, the expression level of these AMPs are possible indicators of the immune state of shrimps. The present study also indicates the antiviral property of AMPs, especially ALF, stressing the importance of their up-regulation through the application of immunostimulants/probiotics as a prophylactic strategy in aquaculture. The present study shows that shrimp defense system is equipped enough to evade WSSV infection to a certain extent, when the animals were maintained on marine yeast and probiotic diet, whereas the control diet fed group succumbed to WSSV infection. This study reveals that marine yeast and probiotic supplemented diet can delay the process of WSSV infection and confer greater protection to the animals. Particularly, the protection conferred by marine yeast, *C. haemulonii* S27 and *Bacillus* MCCB101 were highly promising imparting greater hope to the aquaculture community to overcome the prevailing disease problems in aquaculture. It may be inferred from the present study that up-regulation of AMP genes could be effected by the application of immunostimulants and probiotics. Also, AMP expression profile could be used as an effective tool for screening immunostimulants and probiotics for

application in shrimp culture. Ultimately, it is likely that no single compound or strategy will provide a solution to the problem of disease within aquaculture and that, in reality, a suite of techniques will be required including the manipulation of the rearing environment, addition of probionts as a matter of routine during culture, and the use of immunostimulants and other supplements during vulnerable growth phases. Finally, the development of good management practices, the control of environmental variables, genetic improvement in the penaeid species, understanding of host-virus interaction, modulation of the shrimp immune system, supported by functional genomics and proteomics of this crustacean, as a whole suggests that the control of WSSV is not far.

## Future prospects

- Heterologous expression of the AMPs would be highly promising in the production of potent AMPs for therapeutic applications
- Anti-WSSV property of the AMPs and its mode of action need to be studied in detail
- Protection conferred by marine yeast, *C. haemulonii* S27 is highly promising. Detailed research on the factors responsible for this protection need to be investigated

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Zhang, J., Li, F., Wang, Z., Xiang, J. 2007. Cloning and recombinant expression of a crustin-like gene from Chinese shrimp, *Fenneropenaeus chinensis*. J. Biotech. 127, 605-614.

Zhang, J., Li, F., Wang, Z., Zhang, X., Zhou, Q., Xiang, J. 2006. Cloning, expression and identification of ferritin from Chinese shrimp, *Fenneropenaeus chinensis.* J. Biotech. 125, 173–184.

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Zhang, L., Rozek, A., Hancock, R.E.W. 2001. Interaction of cationic antimicrobial peptides with model membranes. J. Biol. Chem. 276, 35714– 35722.

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Zhao, H., Rinaldi, A.C., Rufo, A., Bozzi, A., Kinnunen, P.K.J., di Giulio, A. 2002. Structural and charge requirements for antimicrobial peptide insertion into biological and model membranes. In pore-forming peptides and protein toxins Menestrina, G., Dalla Serra, M. and Lazarovici, P., (Eds.), Harwood Academic Publishers, Reading, UK. Pp. 151-177.

Zhao, Z.Y., Yin, Z.X., Weng, S.P., Guan, H.J., Li, S.D., Xing, K., Chan, S.M., He, J.G. 2007. Profiling of differentially expressed genes in hepatopancreas of white spot syndrome virus-resistant shrimp *Litopenaeus vannamei* by suppression subtractive hybridisation. Fish Shellfish Immunol. 225, 520-534.

# *GENBANK ACCESSIONS*

# Penaeus monodon anti-lipopolysaccharide factor-like mRNA, partial sequence

**GenBank: GU732817.1**

---

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LOCUS       GU732817     264 bp    mRNA    linear   INV 18-MAY-2010
DEFINITION  Penaeus monodon anti-lipopolysaccharide factor-like
            mRNA, partial sequence.
ACCESSION   GU732817
VERSION     GU732817.1  GI:296034223
KEYWORDS    .
SOURCE      Penaeus monodon (black tiger shrimp)
  ORGANISM  Penaeus monodon
            Eukaryota; Metazoa; Arthropoda; Crustacea; Malacostraca;
            Eumalacostraca; Eucarida; Decapoda; Dendrobranchiata;
            Penaeoidea;Penaeidae; Penaeus.
REFERENCE   1  (bases 1 to 264)
  AUTHORS   Antony,S.P., Bright Singh,I.S. and Philip,R.
  TITLE     Direct Submission
  JOURNAL   Submitted (08-FEB-2010) Department of Marine Biology,
            Microbiology and Biochemistry, Cochin University of
            Science and Technology, Fine Arts Avenue, Kochi, Kerala
            682016, India
FEATURES             Location/Qualifiers
     source          1..264
                     /organism="Penaeus monodon"
                     /mol_type="mRNA"
                     /db_xref="taxon:6687"
     misc_feature    <1..>264
                     /note="similar to anti-lipopolysaccharide
                     factor;antimicrobial peptide"
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       61 tacacgttag cctgtgttgg cttctccccg gatgtcctac caggctagga tgtggtgccc
      121 aggctggagg gcactcggag gatattccaa caaggcttgt cggtccgggg gagctggaga
      181 cacaacaaaa tacttctttc ggaacgcttt ccacaaaggt ctcatctctg aacaggaggc
      241 caaccagtgc ctcccaccca ttga
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# Penaeus monodon anti-lipopolysacharide factor mRNA, partial cds

**GenBank: HM588914.1**

---

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LOCUS       HM588914     295 bp    mRNA    linear   INV 01-AUG-2010
DEFINITION  Penaeus monodon anti-lipopolysacharide factor mRNA,
            partial cds.
ACCESSION   HM588914
VERSION     HM588914.1  GI:301350790
KEYWORDS    .
SOURCE      Penaeus monodon (black tiger shrimp)
  ORGANISM  Penaeus monodon
            Eukaryota; Metazoa; Arthropoda; Crustacea; Malacostraca;
            Eumalacostraca; Eucarida; Decapoda; Dendrobranchiata;
            Penaeoidea;Penaeidae; Penaeus.
REFERENCE   1  (bases 1 to 295)
  AUTHORS   Antony,S.P., Bright Singh,I.S. and Philip,R.
  TITLE     Molecular characterization of an anti-lipopolysacharide
            factor (ALF)from the giant tiger shrimp, Penaeus monodon
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 295)
  AUTHORS   Antony,S.P., Bright Singh,I.S. and Philip,R.
  TITLE     Direct Submission
  JOURNAL   Submitted (24-JUN-2010) Department of Marine Biology,
            Microbiology and Biochemistry, Cochin University of
            Science and Technology, Fine Arts Avenue, Kochi, Kerala
            682016, India
FEATURES             Location/Qualifiers
     source          1..295
                     /organism="Penaeus monodon"
                     /mol_type="mRNA"
                     /db_xref="taxon:6687"
     CDS             <1..>295
                     /note="antimicrobial peptide"
                     /codon_start=2
                     /product="anti-lipopolysacharide factor"
                     /protein_id="ADK74771.1"
                     /db_xref="GI:301350791"

/translation="QCQAQGWEAVAAAVASKIVGLWRNEKTELLGHECKFTVKPYLKRFQVYYKGRMWCPGWTAIRGEASTR
             SQSGVAGKTAKDFVRKAFQKGLISQQEANQ"
ORIGIN
        1 acagtgccag gctcaagggt gggaggctgt ggcagcggcc gtcgccagca agatcgtggg
       61 gttgtggagg aacgaaaaaa ctgaacttct cggccacgag tgcaagttca ccgtcaagcc
      121 ttatttgaag agattccagg tgtactacaa ggggaggatg tggtgcccag gctggacggc
      181 catcagagga gaagccagca cacgcagtca gtccggggta gctggaaaga cagccaaaga
      241 cttcgttcgg aaagctttcc agaaaggtct catctctcaa caggaggcca accag
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# Penaeus monodon crustin-like antimicrobial peptide type 2 mRNA, complete cds

**GenBank: GQ334395.1**

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LOCUS       GQ334395     456 bp    mRNA    linear   INV 03-AUG-2009
DEFINITION  Penaeus monodon crustin-like antimicrobial peptide type
            2 mRNA,complete cds.
ACCESSION   GQ334395
VERSION     GQ334395.1  GI:254832575
KEYWORDS    .
SOURCE      Penaeus monodon (black tiger shrimp)
  ORGANISM  Penaeus monodon
            Eukaryota; Metazoa; Arthropoda; Crustacea; Malacostraca;
            Eumalacostraca; Eucarida; Decapoda; Dendrobranchiata;
            Penaeoidea;Penaeidae; Penaeus.
REFERENCE   1  (bases 1 to 456)
  AUTHORS   Antony,S.P., Philip,R. and Bright Singh,I.S.
  TITLE     Enhanced production of crustins and penaeidins in the
            hemocytes of giant tiger shrimp Penaeus monodon on
            administration of yeast and glucans
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 456)
  AUTHORS   Antony,S.P., Philip,R. and Bright Singh,I.S.
  TITLE     Direct Submission
  JOURNAL   Submitted (27-JUN-2009) Dept. of Marine Biology,
            Microbiology and Biochemistry, Cochin University of
            Science and Technology, Fine Arts Avenue, Kochi, Kerala
            682016, India
FEATURES             Location/Qualifiers
     source          1..456
                     /organism="Penaeus monodon"
                     /mol_type="mRNA"
                     /db_xref="taxon:6687"
     CDS             28..453
                     /codon_start=1
                     /product="crustin-like antimicrobial peptide
                     type 2"
                     /protein_id="ACT82963.1"
                     /db_xref="GI:254832576"

/translation="MLKFVVLSVVAVAVVHAQDKGNADTRFLGGVGVPGGGVPGVGVPGVGGGFLPGVPGHGGVVPGGGGLL
            PGGQFECNYCRTRYGYVCCKPGRCPQIRETCPGLRKGIPICRQDTDCFGSDKCCFDTCLNDTVCKPI
            VLGSEG"
ORIGIN
        1 cgcacagccg agagaaacac tatcaagatg ctaaagtttg tagtattatc cgttgtcgct
       61 gtggctgtgg tacacgcgca ggataaaggc aatgccgata ctcgcttcct aggtggagtt
      121 ggagttcctg gaggtggagt tcctggagtt ggagttcctg gagttggagg tggattcctg
      181 ccgggggttc ctgggcatgg tggcgttgtt cctggaggcg gtggccttct ccctggaggt
      241 caattcgagt gcaattactg cagaacgagg tacggatacg tatgctgcaa gcccggcagg
      301 tgtccacaga ttcgcgaaac ctgcccaggc ctcagaaagg gtatcccgat ctgccgtcag
      361 gacactgact gcttcggctc cgacaaatgc tgcttcgaca cctgcttgaa cgacaccgtc
      421 tgcaaaccca tcgtgctggg ttctgaggga taggcc
```

# Penaeus monodon crustin-like antimicrobial peptide mRNA, partial cds

**GenBank: FJ535568.1**

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LOCUS       FJ535568     299 bp    mRNA    linear   INV 14-JAN-2009
DEFINITION  Penaeus monodon crustin-like antimicrobial peptide mRNA,
            partial cds.
ACCESSION   FJ535568
VERSION     FJ535568.1  GI:219880962
KEYWORDS    .
SOURCE      Penaeus monodon (black tiger shrimp)
  ORGANISM  Penaeus monodon
            Eukaryota; Metazoa; Arthropoda; Crustacea; Malacostraca;
            Eumalacostraca; Eucarida; Decapoda; Dendrobranchiata;
            Penaeoidea;Penaeidae; Penaeus.
REFERENCE   1  (bases 1 to 299)
  AUTHORS   Antony,S.P., Philip,R. and Bright Singh,I.S.
  TITLE     Molecular characterization and expression profile of a
            crustin-like antimicrobial peptide in giant tiger
            shrimp, Penaeus monodon
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 299)
  AUTHORS   Antony,S.P., Philip,R. and Bright Singh,I.S.
  TITLE     Direct Submission
  JOURNAL   Submitted (09-DEC-2008) Department of Marine Biology,
            Microbiology and Biochemistry, Cochin University of
            Science and Technology, Fine Arts Avenue, Kochi, Kerala
            682016, India
FEATURES             Location/Qualifiers
     source          1..299
                     /organism="Penaeus monodon"
                     /mol_type="mRNA"
                     /db_xref="taxon:6687"
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rev_seq:
                     caaagattcaactaaataaacag"
     CDS             <1..110
                     /note="immune-related"
                     /codon_start=3
                     /product="crustin-like antimicrobial peptide"
                     /protein_id="ACL51682.1"
                     /db_xref="GI:219880963"

/translation="SHDFKCAGLDKCCFDRCLGEHVCKPPSFYGRNVKG"
ORIGIN
        1 gttcccacga cttcaagtgt gctggcctcg ataagtgttg cttcgacagg tgtttgggag
       61 aacacgtgtg caagcctcct tctttctacg gaaggaatgt taaaggatga tgagaataaa
      121 caaaagacca actgacagac aaccgatgat ttggaattta ggaccacgaa tgttcaatct
      181 actgttatgt caagtaccaa gcaatctgag agtactatta tctgtaaaaa ataaataaac
      241 aaataataaa tgtaacaggg aatgaactac tcctttctgt ttatttagtt gaatctttg
```

# Penaeus monodon crustin-like antimicrobial peptide type 1 mRNA, partial cds

**GenBank: GQ334396.1**

---

```
LOCUS       GQ334396     239 bp    mRNA    linear   INV 03-AUG-2009
DEFINITION  Penaeus monodon crustin-like antimicrobial peptide type
            1 mRNA,partial cds.
ACCESSION   GQ334396
VERSION     GQ334396.1  GI:254832577
KEYWORDS    .
SOURCE      Penaeus monodon (black tiger shrimp)
  ORGANISM  Penaeus monodon
            Eukaryota; Metazoa; Arthropoda; Crustacea; Malacostraca;
            Eumalacostraca; Eucarida; Decapoda; Dendrobranchiata;
            Penaeoidea;Penaeidae; Penaeus.
REFERENCE   1  (bases 1 to 239)
  AUTHORS   Antony,S.P., Philip,R. and Bright Singh,I.S.
  TITLE     Enhanced production of crustins and penaeidins in the
            hemocytes of giant tiger shrimp Penaeus monodon on
            administration of yeast and glucans
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 239)
  AUTHORS   Antony,S.P., Philip,R. and Bright Singh,I.S.
  TITLE     Direct Submission
  JOURNAL   Submitted (27-JUN-2009) Department of Marine Biology,
            Microbiology and Biochemistry, Cochin University of
            Science and Technology, Fine Arts Avenue, Kochi, Kerala
            682016, India
FEATURES             Location/Qualifiers
     source          1..239
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                     /db_xref="taxon:6687"
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type 1"
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                     /db_xref="GI:254832578"

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              TDCFGSDKCCFDTCLNDTVCKPIVAGSQG"
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       61 gcccggcagg tgtccacaga ttcgcgaaac ctgcccaggc ctcagaaagg gtgtcccgat
      121 ctgccgtcag gacactgact gcttcggctc cgacaaatgc tgcttcgaca cctgcttgaa
      181 cgacaccgtc tgcaaaccca tcgtggcagg ttctcaggga taggcctgca tgttcgact
```

# Penaeus monodon penaeidin-like mRNA, partial sequence

**GenBank: GU732819.1**

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LOCUS       GU732819    138 bp    mRNA    linear   INV 18-MAY-2010
DEFINITION  Penaeus monodon penaeidin-like mRNA, partial sequence.
ACCESSION   GU732819
VERSION     GU732819.1  GI:296034226
KEYWORDS    .
SOURCE      Penaeus monodon (black tiger shrimp)
  ORGANISM  Penaeus monodon
            Eukaryota; Metazoa; Arthropoda; Crustacea; Malacostraca;
            Eumalacostraca; Eucarida; Decapoda; Dendrobranchiata;
            Penaeoidea;Penaeidae; Penaeus.
REFERENCE   1  (bases 1 to 138)
  AUTHORS   Antony,S.P., Bright Singh,I.S. and Philip,R.
  TITLE     Penaeidin-3 like antimicrobial peptide from black tiger
            shrimp,Penaeus monodon
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 138)
  AUTHORS   Antony,S.P., Bright Singh,I.S. and Philip,R.
  TITLE     Direct Submission
  JOURNAL   Submitted (08-FEB-2010) Department of Marine Biology,
            Microbiology and Biochemistry, Cochin University of
            Science and Technology, Fine Arts Avenue, Kochi, Kerala
            682016, India
FEATURES             Location/Qualifiers
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                     /mol_type="mRNA"
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peptide"
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      121 tgaagaaagt gcaaccct
```

# Penaeus monodon penaeidin-like antimicrobal peptide mRNA, partial cds

**GenBank: GQ334397.1**

---

```
LOCUS       GQ334397    169 bp    mRNA    linear   INV 03-AUG-2009
DEFINITION  Penaeus monodon penaeidin-like antimicrobal peptide
            mRNA, partial cds.
ACCESSION   GQ334397
VERSION     GQ334397.1  GI:254832579
KEYWORDS    .
SOURCE      Penaeus monodon (black tiger shrimp)
  ORGANISM  Penaeus monodon
            Eukaryota; Metazoa; Arthropoda; Crustacea; Malacostraca;
            Eumalacostraca; Eucarida; Decapoda; Dendrobranchiata;
            Penaeoidea; Penaeidae; Penaeus.
REFERENCE   1  (bases 1 to 169)
  AUTHORS   Antony,S.P., Philip,R. and Bright Singh,I.S.
  TITLE     Enhanced production of crustins and penaeidins in the
            hemocytes of giant tiger shrimp Penaeus monodon on
            administration of yeast and glucans
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 169)
  AUTHORS   Antony,S.P., Philip,R. and Bright Singh,I.S.
  TITLE     Direct Submission
  JOURNAL   Submitted (27-JUN-2009) Department of Marine Biology,
            Microbiology and Biochemistry, Cochin University of
            Science and Technology, Fine Arts Avenue, Kochi, Kerala
            682016, India
FEATURES             Location/Qualifiers
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                     /mol_type="mRNA"
                     /db_xref="taxon:6687"
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                     /protein_id="ACT82965.1"
                     /db_xref="GI:254832580"

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       61 atgcgtctcg tggtctgcct ggtcttcctg gcctccttcg ccctggtctg ccaagcccaa
      121 gggtaccagg gtggttacac acgcccgttc cccagaccac cctatgggg
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# Penaeus monodon beta-actin mRNA, partial cds

**GenBank: GQ334394.1**

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LOCUS       GQ334394      583 bp    mRNA    linear   INV 03-AUG-2009
DEFINITION  Penaeus monodon beta-actin mRNA, partial cds.
ACCESSION   GQ334394
VERSION     GQ334394.1  GI:254832573
KEYWORDS    .
SOURCE      Penaeus monodon (black tiger shrimp)
  ORGANISM  Penaeus monodon
            Eukaryota; Metazoa; Arthropoda; Crustacea; Malacostraca;
            Eumalacostraca; Eucarida; Decapoda; Dendrobranchiata;
            Penaeoidea; Penaeidae; Penaeus.
REFERENCE   1  (bases 1 to 583)
  AUTHORS   Antony,S.P., Philip,R. and Bright Singh,I.S.
  TITLE     Enhanced production of crustins and penaeidins in the
            hemocytes of giant tiger shrimp Penaeus monodon on
            administration of yeast and glucans
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 583)
  AUTHORS   Antony,S.P., Philip,R. and Bright Singh,I.S.
  TITLE     Direct Submission
  JOURNAL   Submitted (27-JUN-2009) Department of Marine Biology,
            Microbiology and Biochemistry, Cochin University of
            Science and Technology, Fine Arts Avenue, Kochi, Kerala
            682016, India
FEATURES             Location/Qualifiers
     source          1..583
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     CDS             <1..>583
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                     /protein_id="ACT82962.1"
                     /db_xref="GI:254832574"

/translation="LVVDNGSGMVKAGFAGDDAPRAVFPSIVGRARHQGVMVGMGQKDAYVGDEAQSK
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              REKMTQIMFESFSVPATYITIQAVLSLYASGRTTGEVCDSGDGVTHFVPVYEGF
              ALPHAILRLDLAGRDLTHYLMKIMTERGYSFT"
ORIGIN
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      121 ggtcagaagg acgcctacgt cggtgatgag gcccagagca agcgtggtat cctcaccctc
      181 aagtacccca ttgagcacgg tatcatcacc aactgggatg acatggagaa gatctggtac
      241 cacaccttct acaatgagct ccgtgttgcc cctgaggagt gccccactct cctcactgag
      301 gctcccctca accccaaggc caaccgtgag aagatgactc agatcatgtt cgagtccttc
      361 agcgtccctg ccacttacat caccatccag gccgtgctct ccctctacgc ctccggtcgt
      421 actaccggtg aggtttgcga ctctggtgat ggtgtgactc actttgtccc cgtctatgaa
      481 ggtttcgctc ttccccatgc tattctccgt ctcgacttgg ctggtcgtga ccttacccac
      541 tacctcatga agatcatgac tgagcgtggc tactccttca cca
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# Penaeus monodon elongation factor mRNA, partial cds

**GenBank: GU732818.1**

---

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LOCUS       GU732818      111 bp    mRNA    linear   INV 18-MAY-2010
DEFINITION  Penaeus monodon elongation factor mRNA, partial cds.
ACCESSION   GU732818
VERSION     GU732818.1  GI:296034224
KEYWORDS    .
SOURCE      Penaeus monodon (black tiger shrimp)
  ORGANISM  Penaeus monodon
            Eukaryota; Metazoa; Arthropoda; Crustacea; Malacostraca;
            Eumalacostraca; Eucarida; Decapoda; Dendrobranchiata;
            Penaeoidea; Penaeidae; Penaeus.
REFERENCE   1  (bases 1 to 111)
  AUTHORS   Antony,S.P., Bright Singh,I.S. and Philip,R.
  TITLE     Direct Submission
  JOURNAL   Submitted (08-FEB-2010) Department of Marine Biology,
            Microbiology and Biochemistry, Cochin University of
            Science and Technology, Fine Arts Avenue, Kochi, Kerala
            682016, India
FEATURES             Location/Qualifiers
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                     /mol_type="mRNA"
                     /db_xref="taxon:6687"
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                     /product="elongation factor"
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                     /db_xref="GI:296034225"

/translation="SQSVEMHHEALTEAVPGDNVGFNVKNVSVKDLKREF"

ORIGIN
        1 tgagtcagtc tgtggagatg caccacgaag ctcttaccga ggctgtccct ggtgacaacg

       61 ttggcttcaa cgtgaagaac gtgtctgtaa aggatctgaa acgggaattc g
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# Fenneropenaeus indicus anti-lipopolysaccharide factor-like mRNA, partial sequence

**GenBank: GU732814.1**

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LOCUS       GU732814      255 bp    mRNA    linear   INV 18-MAY-2010
DEFINITION  Fenneropenaeus indicus anti-lipopolysaccharide factor-
like mRNA,
            partial sequence.
ACCESSION   GU732814
VERSION     GU732814.1  GI:296034219
KEYWORDS    .
SOURCE      Fenneropenaeus indicus
  ORGANISM  Fenneropenaeus indicus
            Eukaryota; Metazoa; Arthropoda; Crustacea; Malacostraca;
            Eumalacostraca; Eucarida; Decapoda; Dendrobranchiata;
            Penaeoidea; Penaeidae; Fenneropenaeus.
REFERENCE   1  (bases 1 to 255)
  AUTHORS   Antony,S.P., Bright Singh,I.S. and Philip,R.
  TITLE     Direct Submission
  JOURNAL   Submitted (08-FEB-2010) Department of Marine Biology,
            Microbiology and Biochemistry, Cochin University of
            Science and Technology, Fine Arts Avenue, Kochi, Kerala
            682016, India
FEATURES             Location/Qualifiers
     source          1..255
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                     /mol_type="mRNA"
                     /db_xref="taxon:29960"
     misc_feature    <1..>255
                     /note="similar to anti-lipopolysaccharide
factor;
                     antimicrobial peptide"

ORIGIN
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       61 gtcaagcctt acattaagag gttccagttg aactacaagg ggaggatgtg gtgcccagct
      121 ggacggccat cagaggagaa gccagaacac gcagtcattc cggggtggct ggacggacag
      181 cccaaaactt attcggaaac tttccaaaaa ggtttcatct ctcaacaggg ggcccccaat
      241 ggctcacctc aaagt
```

# Fenneropenaeus indicus anti-lipopolysacharide factor mRNA, complete cds

**GenBank: HM366921.1**

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LOCUS       HM366921      360 bp    mRNA    linear   INV 08-AUG-2010
DEFINITION  Fenneropenaeus indicus anti-lipopolysacharide factor
mRNA, complete
            cds.
ACCESSION   HM366921
VERSION     HM366921.1  GI:302138012
KEYWORDS    .
SOURCE      Fenneropenaeus indicus
  ORGANISM  Fenneropenaeus indicus
            Eukaryota; Metazoa; Arthropoda; Crustacea; Malacostraca;
            Eumalacostraca; Eucarida; Decapoda; Dendrobranchiata;
            Penaeoidea; Penaeidae; Fenneropenaeus.
REFERENCE   1  (bases 1 to 360)
  AUTHORS   Antony,S.P., Bright Singh,I.S. and Philip,R.
  TITLE     Molecular characterization of anti-lipopolysaccharide
factor from
            the Indian white shrimp, Fenneropenaeus indicus
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 360)
  AUTHORS   Antony,S.P., Bright Singh,I.S. and Philip,R.
  TITLE     Direct Submission
  JOURNAL   Submitted (27-MAY-2010) Department of Marine Biology,
            Microbiology and Biochemistry, Cochin University of
            Science and Technology, Fine Arts Avenue, Kochi, Kerala
            682016, India
FEATURES             Location/Qualifiers
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      121 gggctgtgga ggaacgagaa aactgaactc ctgggccacg agtgcaagtt caccgtcaag

      181 ccttacatta agaggttcca gttgaactac aaggggagga tgtggtgccc aggctggacg

      241 gccatcagag gagaagccag aacacgcagt cattccgggg tggctggacg gacggcccaa

      301 gacttcgttc ggaaagcttt ccagaaaggt ctcatctctc aacaggaggc caaccagtga
```

# Fenneropenaeus indicus crustin-like antimicrobial peptide mRNA, complete cds

**GenBank: GQ469987.1**

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LOCUS       GQ469987      371 bp    mRNA    linear   INV 02-JUN-2010
DEFINITION  Fenneropenaeus indicus crustin-like antimicrobial
peptide mRNA,
            complete cds.
ACCESSION   GQ469987
VERSION     GQ469987.1  GI:258618630
KEYWORDS    .
SOURCE      Fenneropenaeus indicus
  ORGANISM  Fenneropenaeus indicus
            Eukaryota; Metazoa; Arthropoda; Crustacea; Malacostraca;
            Eumalacostraca; Eucarida; Decapoda; Dendrobranchiata;
            Penaeoidea; Penaeidae; Fenneropenaeus.
REFERENCE   1  (bases 1 to 371)
  AUTHORS   Antony,S.P., Bright Singh,I.S. and Philip,R.
  TITLE     Molecular characterization of a crustin-like, putative
            antimicrobial peptide, Fi-crustin, from the Indian white
shrimp,
            Fenneropenaeus indicus
  JOURNAL   Fish Shellfish Immunol. 28 (1), 216-220 (2010)
   PUBMED   19837171
REFERENCE   2  (bases 1 to 371)
  AUTHORS   Antony,S.P., Philip,R. and Bright Singh,I.S.
  TITLE     Direct Submission
  JOURNAL   Submitted (08-AUG-2009) Department of Marine Biology,
            Microbiology and Biochemistry, Cochin University of
            Science and Technology, Fine Arts Avenue, Kochi, Kerala
            682016, India
FEATURES             Location/Qualifiers
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                KPIVLGSEG"
ORIGIN
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      121 gggcatggcg gcattgcccc tggattcgaa tgcaattact gcagaacgag gtatgggtac
      181 gtatgctgca agcccggcag gtgtccaccg gttcgcgata cctgcccagg catcaggaac
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      361 acgccacgta t
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# Fenneropenaeus indicus penaeidin-like antimicrobial peptide mRNA, complete cds

**GenBank: HM243617.1**

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LOCUS       HM243617      186 bp    mRNA    linear   INV 03-JUL-2010
DEFINITION  Fenneropenaeus indicus penaeidin-like antimicrobial
peptide mRNA,
            complete cds.
ACCESSION   HM243617
VERSION     HM243617.1  GI:299780319
KEYWORDS    .
SOURCE      Fenneropenaeus indicus
  ORGANISM  Fenneropenaeus indicus
            Eukaryota; Metazoa; Arthropoda; Crustacea; Malacostraca;
            Eumalacostraca; Eucarida; Decapoda; Dendrobranchiata;
            Penaeoidea; Penaeidae; Fenneropenaeus.
REFERENCE   1  (bases 1 to 186)
  AUTHORS   Swapna,A.P., Bright Singh,I.S. and Philip,R.
  TITLE     Molecular characterization of a putative antimicrobial
peptide,
            Fi-penaeidin from the Indian white shrimp,
Fenneropenaeus indicus
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 186)
  AUTHORS   Swapna,A.P., Bright Singh,I.S. and Philip,R.
  TITLE     Direct Submission
  JOURNAL   Submitted (19-MAY-2010) Department of Marine Biology,
            Microbiology and Biochemistry, Cochin University of
            Science and Technology, Fine Arts Avenue, Kochi, Kerala
            682016, India
FEATURES             Location/Qualifiers
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# Fenneropenaeus indicus penaeidin-like antimicrobial peptide mRNA, partial cds

**GenBank: HM243616.1**

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LOCUS       HM243616      320 bp    mRNA    linear   INV 03-JUL-2010
DEFINITION  Fenneropenaeus indicus penaeidin-like antimicrobial
peptide mRNA,
            partial cds.
ACCESSION   HM243616
VERSION     HM243616.1  GI:299780317
KEYWORDS    .
SOURCE      Fenneropenaeus indicus
  ORGANISM  Fenneropenaeus indicus
            Eukaryota; Metazoa; Arthropoda; Crustacea; Malacostraca;
            Eumalacostraca; Eucarida; Decapoda; Dendrobranchiata;
            Penaeoidea; Penaeidae; Fenneropenaeus.
REFERENCE   1  (bases 1 to 320)
  AUTHORS   Swapna,A.P., Bright Singh,I.S. and Philip,R.
  TITLE     Molecular characterization of a putative penaeidin-like
            antimicrobial peptide from the Indian white shrimp,
Fenneropenaeus
            indicus
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 320)
  AUTHORS   Swapna,A.P., Bright Singh,I.S. and Philip,R.
  TITLE     Direct Submission
  JOURNAL   Submitted (19-MAY-2010) Department of Marine Biology,
            Microbiology and Biochemistry, Cochin University of
            Science and Technology, Fine Arts Avenue, Kochi, Kerala
            682016, India
FEATURES             Location/Qualifiers
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      121 ttgctcttca tgccgcctta ttaccttcaa ttacgctgtt gcttgttgct ggcggttagg
      181 acgctgttgt tctgcagaga ggattatctg gccgatggag agacaacgaa accctggctt
      241 tgcaacgtgt tatttggact catatatcaa gagactgcaa ccttgtttga actgtatttt
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## Fenneropenaeus indicus beta-actin mRNA, partial cds

**GenBank: GU732815.1**

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DEFINITION  Fenneropenaeus indicus beta-actin mRNA, partial cds.
ACCESSION   GU732815
VERSION     GU732815.1  GI:296034220
KEYWORDS    .
SOURCE      Fenneropenaeus indicus
  ORGANISM  Fenneropenaeus indicus
            Eukaryota; Metazoa; Arthropoda; Crustacea; Malacostraca;
            Eumalacostraca; Eucarida; Decapoda; Dendrobranchiata;
            Penaeoidea; Penaeidae; Fenneropenaeus.
REFERENCE   1  (bases 1 to 547)
  AUTHORS   Antony,S.P., Bright Singh,I.S. and Philip,R.
  TITLE     Direct Submission
  JOURNAL   Submitted (08-FEB-2010) Department of Marine Biology,
            Microbiology and Biochemistry, Cochin University of
            Science and Technology, Fine Arts Avenue, Kochi, Kerala
            682016, India
FEATURES             Location/Qualifiers
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      181 ccaactggga cgacatggag aagatctggc atcatacctt ctacaacgag ctccgcgtgg
      241 cccccgagga gcaccccgtc ctgctgaccg aggctcccct caaccccaag gccaaccgcg
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      421 acggcgtgtc ccacaccgtg cccatctacg agggctacgc cctgccccac gccatcctgc
      481 gtctggactt ggccggccgc gacctcacag actacctgat gaagatcctg accgagcgtg
      541 gctactc
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# Fenneropenaeus indicus elongation factor-like mRNA, partial sequence

**GenBank: GU732816.1**

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LOCUS       GU732816      234 bp    mRNA    linear   INV 18-MAY-2010
DEFINITION  Fenneropenaeus indicus elongation factor-like mRNA,
partial
            sequence.
ACCESSION   GU732816
VERSION     GU732816.1  GI:296034222
KEYWORDS    .
SOURCE      Fenneropenaeus indicus
  ORGANISM  Fenneropenaeus indicus
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            Eumalacostraca; Eucarida; Decapoda; Dendrobranchiata;
            Penaeoidea; Penaeidae; Fenneropenaeus.
REFERENCE   1  (bases 1 to 234)
  AUTHORS   Antony,S.P., Bright Singh,I.S. and Philip,R.
  TITLE     Direct Submission
  JOURNAL   Submitted (08-FEB-2010) Department of Marine Biology,
            Microbiology and Biochemistry, Cochin University of
            Science and Technology, Fine Arts Avenue, Kochi, Kerala
            682016, India
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      181 ctcaaccacc ctggccagat ccaggctggc tactcacctg tgcttgattg ccac
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# *PUBLICATIONS*

# *List of Publications*

### *International:*

1. **Antony, S.P.,** Singh I.S.B., Philip, R. **2010**. Molecular characterization of a crustin-like, putative antimicrobial peptide, Fi-crustin, from the Indian White Shrimp, *Fenneropenaeus indicus*. **Fish and Shellfish Immunology**. 28, 216-220.
2. **Antony, S.P.,** Singh, I.S.B., Sudheer, N.S., Vrinda, S., Priyaja, P., Philip, R. **2011**. Molecular characterization and expression profile of a crustin-like antimicrobial peptide in the haemocytes of the giant tiger shrimp, *Penaeus monodon*, in response to various immunostimulants and challenge with WSSV. **Immunobiology**. 216, 184-194.

### *National (Seminars/Symposia presentations):*

1. **Antony, S.P.,** Philip, R., Singh, I.S.B. **2008**. Antimicrobial peptides from tiger prawn, *Penaeus monodon*. Proceedings of the **National Seminar on Bioactive compounds from Marine Organisms.** Menon, N.N., Hatha, A.A.M., Philip, B., Saramma, A.V. (Eds.). Pp. 77-84.
2. **Antony, S.P.,** Singh, I.S.B., Philip, R. **2010**. Immune Gene Expression in *Penaeus monodon* in Response to the Administration of Immunostimulants, Probiotics and White Spot Syndrome Virus Challenge. Proceedings of 22nd **Kerala Science Congress**. 28-31 January, 2010, KFRI, Peechi. Yasodharan, E.P. (Ed. in chief). Pp. 106-107.
3. **Antony SP,** Philip R., and Singh ISB. **2010**. Antimicrobial peptides in shrimps: A new frontier for microbial infection control. Abstracts of the 1st Kerala **Women's Science Congress**. 10-12 August, 2010, St. Teresa's College, Ernakulam. Editors: MS Kala, L Amthew, G. Bindu, R. Subramaian. Pp 33.

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Short sequence report

# Molecular characterization of a crustin-like, putative antimicrobial peptide, Fi-crustin, from the Indian white shrimp, *Fenneropenaeus indicus*

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WAP domain
Innate immunity

Antimicrobial peptides are important innate immune defense, especially in those animals which lack adaptive immunity [1–8]. Due to their small size, amphipathic structure and cationic character they can rapidly diffuse to the point of infection [9], a mechanism that presumably makes it easier to circumvent microbial resistance against the peptides [10]. Besides providing an immediate and broad-spectrum microbicidal activity, AMPs can kill bacteria in micromolar range, are promptly synthesized at low metabolic cost, and are easily stored in large amounts and readily available shortly after an infection [11–13]. Many AMPs show a remarkable specificity for prokaryotes with low toxicity for eukaryotic cells; a phenomenon which has favored their investigation and exploitation as potential new antibiotics [14]. AMP gene expression and distribution are regulated through haemocyte reactions [15]. Transcripts of crustin-encoding genes have also been observed in gills, heart and intestine [16–18] but as these tissues are highly vascularised, it is assumed the transcripts from these organs are due primarily to the haemocytes.

In penaeid shrimps, four main families of AMPs have been currently described and characterized from the haemocytes: penaeidins, crustins, anti-lipopolysaccharide factors (ALFs) and lysozymes. Penaeidins are mainly active against Gram-positive bacteria, filamentous fungi [19], viruses and protozoans [20] whereas ALFs have a broader antimicrobial spectrum including Gram-negative bacteria [21,22]. Conversely, crustins are reported to have a more-restricted activity spectrum, affecting mainly marine Gram-positive bacteria [17,23,24] Crustins, a widely distributed family of AMPs was first isolated from the shore crab, *Carcinus maenas* as an 11.5 kDa peptide [23]. Crustins are cationic, cysteine-rich antimicrobial AMPs having molecular weight of 7–14 kDa, with an isoelectric point in the range of 7.0–8.7, and contain one whey-acidic protein (WAP) domain at the carboxy terminus [25]. Crustins have been proved to be an important antimicrobial protein in the plasma and haemocyte granules of crustaceans and described as a component of the innate immune system [8]. These AMPs are dominantly synthesized and stored in haemocytes [4,8,16,18,23,24,26–30] and their release from haemocytes is induced by bacterial infection [15,27,31]. Crustin mechanisms of action and function are still largely unknown, although they contain a whey-acidic protein (WAP) domain common to proteinase inhibitory activities as well as antimicrobial activities [8].

Many full-length cDNA and several ESTs of crustins have been described in a wide range of penaeid prawns including *Litopenaeus vannamei* [8,24,30,32], *Litopenaeus setiferus* [24,32,33], *Penaeus monodon* [16,17,29,30,34–37], *Marsupenaeus japonicus* [17,38], *Litopenaeus schmitti* [33], *Fenneropenaeus chinensis* [17,29], *Farfantepenaeus brasiliensis* [33], *Farfantepenaeus paulensis* [33] and *Farfantepenaeus subtilis* [33]. However, no antimicrobial peptide sequences have been reported from *Fenneropenaeus indicus*. In the current study a crustin cDNA has been characterized from the Indian White Shrimp, *F. indicus*.

Healthy adult *F. indicus* (8–10 g body weight) were purchased from a local shrimp farm in Vypeen, Kochi. They were transferred to aquaria of 500 l capacity and acclimatized for one week under laboratory conditions. Prawns were fed with a standard feed (Higashimaru, India). Aeration was provided in all tanks during the experiment and bioreactor was set in all the aquaria for the removal of ammonia and nitrate. Only shrimps in the intermoult stage were sampled during the study.

Haemolymph was collected from the rostral sinus using specially designed capillary tubes (RNase-free) rinsed using pre-cooled anticoagulant solution (RNase-free, 10% sodium citrate, pH 7.0). Total RNA was extracted from the haemocytes using TRI

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```
atg cta aag ttt gta gta tta tcc gtt gtc gcc gtg
 M   L   K   F   V   V   L   S   V   V   A   V

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 A   V   V   Q   S   Q   E   D   T   R   F   L

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ccg ggg gtt cca ggg cat ggc ggc att gcc cct gga
 P   G   V   P   G   H   G   G   I   A   P   G

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 F   E   C   N   Y   C   R   T   R   Y   G   Y

gta tgc tgc aag ccc ggc agg tgt cca ccg gtt cgc
 V   C   C   K   P   G   R   C   P   P   V   R

gat acc tgc cca ggc atc agg aac aga ccc ccg atc
 D   T   C   P   G   I   R   N   R   P   P   I

tgc cgt cag gac act gag tgc ttc ggc tcc gac aag
 C   R   Q   D   T   E   C   F   G   S   D   K

tgc tgc tac gac acc tgc ttg aac gac acc gtc tgc
 C   C   Y   D   T   C   L   N   D   T   V   C

aaa ccc atc gtg ctg ggt tct gag gga tag gcc aaa
 K   P   I   V   L   G   S   E   G   *

acg cca cgt at
```

**Fig. 1.** Nucleotide and amino acid sequences of Fi-crustin from the haemocyte of the Indian white shrimp, *Fenneropenaeus indicus* (GenBank Accession No. GQ469987). The underlined amino acid residues indicate a putative signal sequence. Cysteine residues that participate in the formation of intramolecular disulphide bonds are bold printed. An asterisk is the stop codon.

Reagent (Sigma) following the manufacture's protocol. RNA was quantified by spectrophotometry at 260 and 280 nm. Only RNAs with absorbance ratios ($A_{260}$:$A_{280}$) greater than 1.8 were used for the present work. First strand cDNA was generated in a 20 µl reaction volume containing 5 µg total RNA, 1x RT buffer, 2 mM dNTP, 2 µM oligo d($T_{20}$), 20 U of RNase inhibitor and 100 U of M-MLV reverse transcriptase (New England Biolabs). The reaction was conducted at 42 °C for 1 h followed by an inactivation step at 85 °C for 15 min. PCR amplification of 1 µl of cDNA was performed in a 25 µl reaction volume containing 1x standard Taq buffer (10 mM Tris–HCl, 50 mM KCl, pH 8.3), 3.5 mM $MgCl_2$, 200 µM dNTPs, 0.4 µM each primer and 1U Taq DNA polymerase (New England Biolabs). Amplification was performed using the primers, Crus F (5′- cgca cagccgagagaaacactatcaagat −3′) and Crus R (5′ggcctatccctcagaa cccagcacg −3′). The thermal profile used was 94 °C for 2 min followed by 35 cycles of 94 °C for 15 s, 55 °C for 30 s and 68 °C for 30 s and a final extension at 68 °C for 10 min. PCR product was analyzed by electrophoresis in 1.5% agarose gels in TBE buffer, stained with ethidium bromide and visualized under UV light. Purified PCR products were sequenced at Xcelris, India.

The sequence homology and the deduced amino acid sequence comparisons were carried out using BLAST algorithm at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/blast). Gene translation and prediction of deduced protein were performed with ExPASy (http://www.au.expasy.org/). The signal peptide was predicted by SignalP program (http://www.au.expasy.org/). The multiple sequence alignments were performed on amino acid sequences of known crustin or crustin-like peptides from shrimps using CLUSTALW and GENDOC computer programs. Amino acid sequences of shrimp crustins were retrieved from the NCBI GenBank and phylogenetic tree was constructed by the Neighbor-Joining (NJ) method and the Maximum Likelihood method (ML) based on amino acid sequences. Phylogenetic tree was drawn based on the sequences with WAP domain using MEGA version 4.0 [39]. The nucleotide sequence and deduced amino acid sequence was submitted to GenBank (GQ469987).

A 371 bp fragment cDNA encoding 122 amino acids and an ORF of 117 amino acids was obtained from the mRNA of *F. indicus* haemocyte by RT-PCR (Fig. 1). BLAST analysis of the nucleotide sequence revealed the relation of Fi-crustin to that of crustins from *F. chinensis* and *P. monodon* (Table 1). Multiple alignment and the bootstrap distance tree calculated for the resulting crustin sequences of BLAST analysis confirmed that Fi-crustin possessed more similarity to that of *F. chinensis* crustin than to the *P. monodon* crustins (Fig. 2). The ORF encoded 117 amino acid residues with a predicted molecular weight (MW) of 10.61 kDa and theoretical isoelectric point (pI) of 7.59 as predicted by the PROTPARAM software. The analysis with the Signal P software revealed the presence of a signal peptide with 17 amino acids at the N-terminal region of the Fi-crustin (Fig. 1).

The deduced amino acid sequence of Fi-crustin was found to be rich in amino acid residues Glycine (14.5%) and Valine (12.8%). At the N-terminal of the mature peptide, Fi-crustin contained a number of glycine-rich repeats between positions 25 and 49. Following the repeat region is a cys-rich region just like that of the crustin-like peptide from *M. japonicus* and *F. chinensis*; however they have no proline-rich domain compared with those of *M. japonicus* and hence is more similar to crustin of *F. chinensis* [17,38]. The C-terminal segment included a high proportion of Cysteine-rich region (10.3%), which contained 12 Cysteine residues that participate in the formation of disulphide bonds. The 12 cysteines in Fi-crustin are considered to be important for maintaining the tertiary structure of the peptide just as that reported in *L. setiferus*, *L. vannamei* and *F. chinensis* crustins [32,17].

As predicted by the ScanProsite program, a whey-acidic protein (WAP) domain signature exists in the C-terminal. According to the previous reports on the crustin-like proteins, the four-disulfide core domain has proved to play important roles in the biological function of crustins [17]. The position of the conserved cysteines for the 'four-disulfide core' domain is $Cys^{68}$-$Cys^{98}$, $Cys^{75}$-$Cys^{102}$, $Cys^{85}$-$Cys^{97}$, and $Cys^{91}$-$Cys^{108}$. In addition, searching the Prosite database, analysis of Fi-crustin revealed the existence of WAP-type 'four-disulfide core' domain signature, C1-(Xn)-C2 (Xn)-C3-(X5)-C4-(X5)-C5-C6-(X3-5)-C7-(X3-4)-C8 [24] and Fi-crustin followed the same pattern with 4 residues between $C_7$ and $C_8$ ($C_7$ $X_4$ $C_8$). Several other consensus sequences also appear in the 4-DSC domain: (1) the consensus KXGXCP containing C1; (2) a conserved aspartate (D) residue between C3 and C4; (3) KCC with C5 and C6; (4) CXXP with C8. [24] Fi-crustin follows this pattern as 'CXP with $C_8$' instead of 'CXXP with $C_8$'.

The amino acid sequence of Fi-crustin was also compared with crustins of decapod crustaceans and it revealed maximum identity to that of *F. chinensis,* and *P. monodon* (Fig. 3). Multiple alignments of

**Table 1**
Result of BLAST analysis of Fi-crustin (GQ469987).

| Accession No. | Description of the AMP | Query coverage | *E* value | Max identity |
|---|---|---|---|---|
| DQ097703 | *Fenneropenaeus chinensis* crustin-like protein fc-1 mRNA, complete cds | 94% | 1e-78 | 91% |
| GQ334395 | *Penaeus monodon* crustin-like antimicrobial peptide type 2 mRNA, complete cds | 70% | 2e-80 | 97% |
| EF654659 | *P. monodon* crustin-like antimicrobial peptide gene, complete cds | 70% | 2e-75 | 97% |
| DQ097704 | *F. chinensis* crustin-like protein fc-2 mRNA, complete cds | 58% | 2e-27 | 96% |

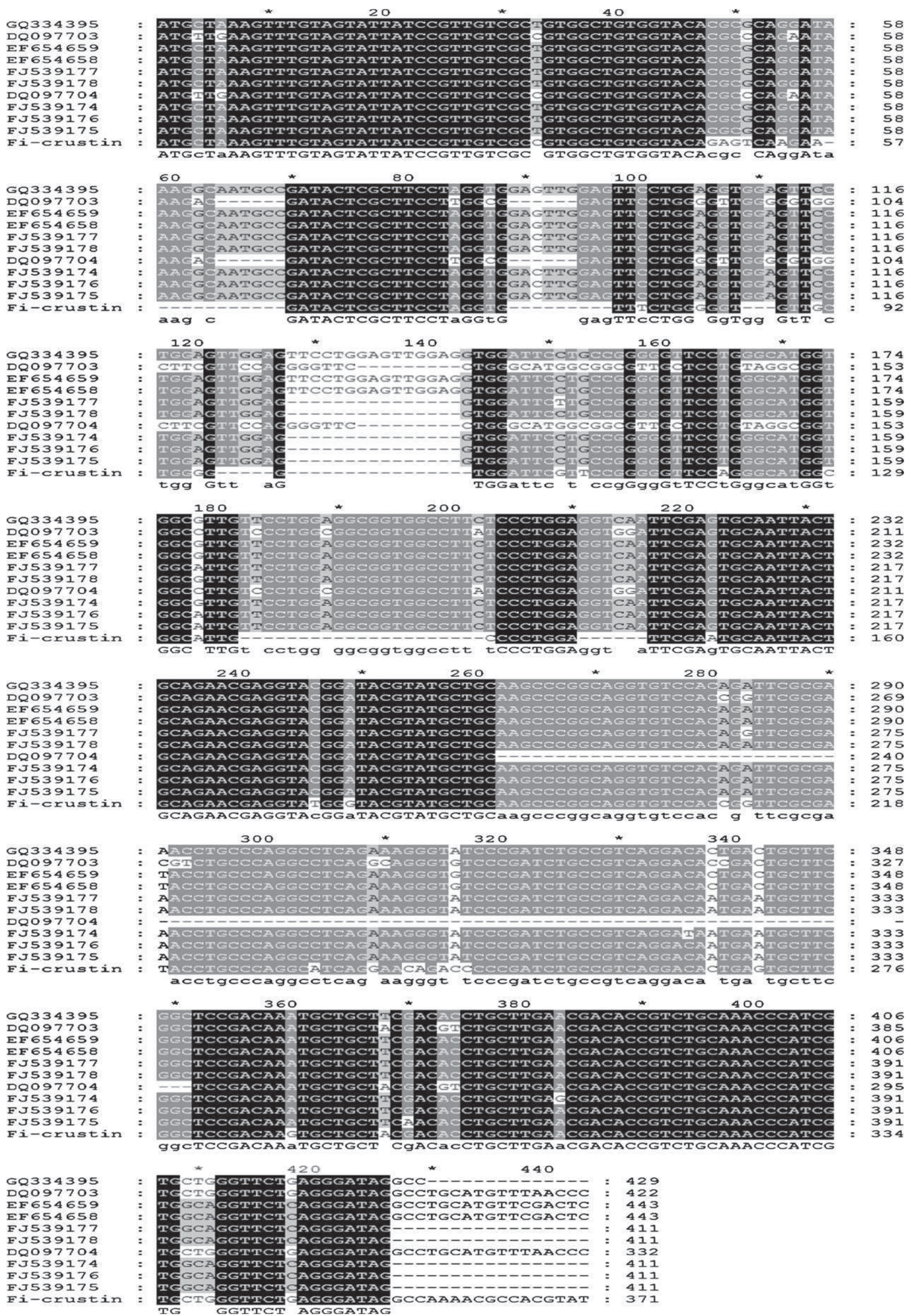

**Fig. 2.** Multiple alignment of nucleotide sequence of the *Fenneropenaeus indicus* crustin-like antimicrobial peptide, Fi-crustin (GQ469987) with other shrimp crustins (*Penaeus monodon* GQ334395, *Fenneropenaeus chinensis* DQ097703, *P. monodon* EF654659, *P. monodon* EF654658, *P. monodon* FJ539177, *P. monodon* FJ539178, *F. chinensis* DQ097704, *P. monodon* FJ539174, *P. monodon* FJ539175, *P. monodon* FJ539176) obtained using GenDoc programme Version 2.7.0. Black and grey indicates conserved sequences.

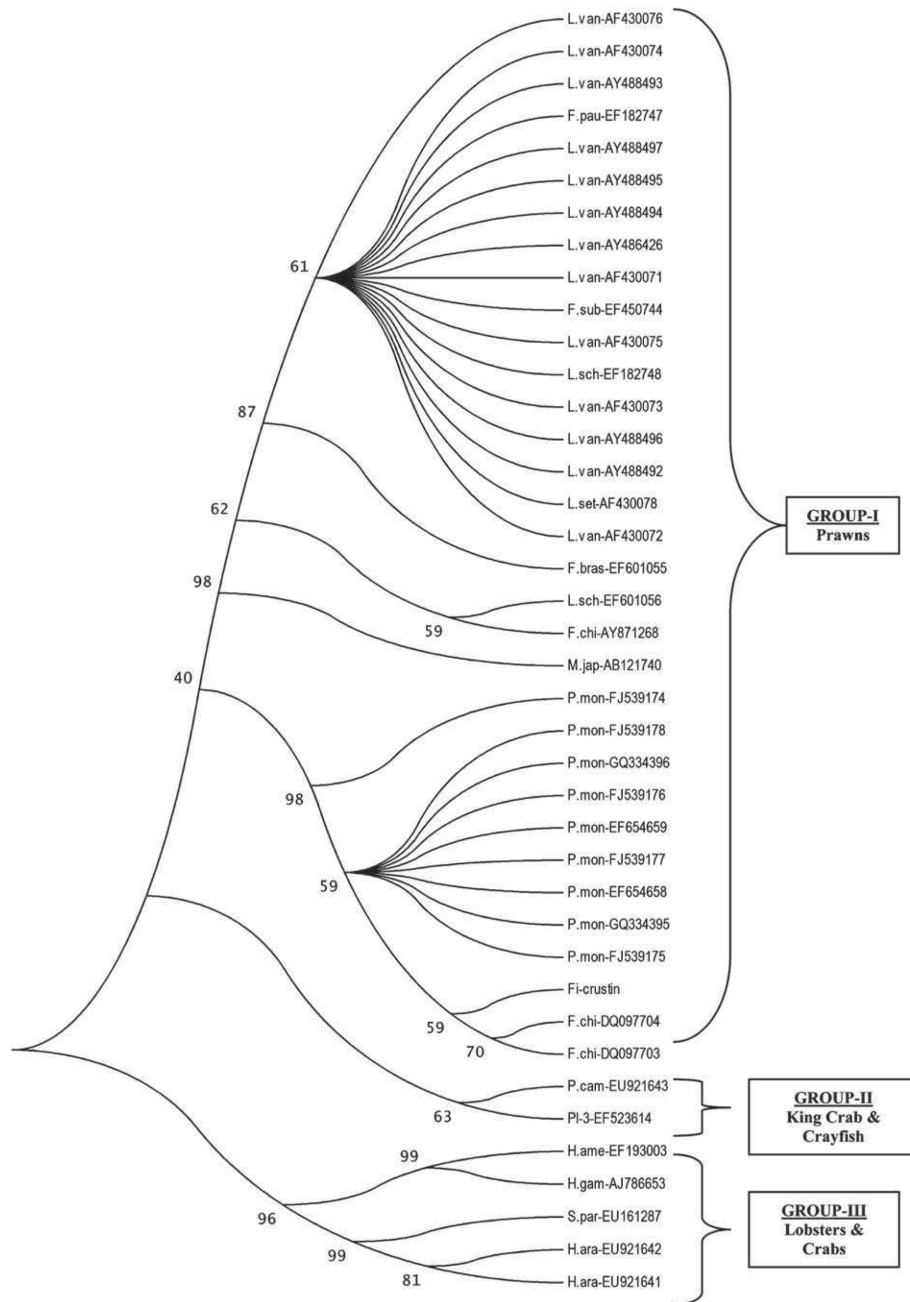


**Fig. 3.** A bootstrapped neighbor-joining tree obtained using MEGA version 4.0 illustrating relationships between the deduced amino acid sequence of the *Fenneropenaeus indicus* crustin-like AMP, Fi-crustin (GQ469987) with other crustins of decapod crustaceans (*Litopenaeus vannamei* AF430071, *L. vannamei* AF430074, *L. vannamei* AY488497, *L. vannamei* AY488492, *Litopenaeus setiferus* AF430078, *Farfantepenaeus paulensis* EF182747, *L. vannamei* AY488493, *L. vannamei* AF430073, *L. vannamei* AY486426, *L. vannamei* AF430072, *Farfantepenaeus subtilis* EF450744, *Litopenaeus schmitti* EF182748, *L. vannamei* AY488496, *L. vannamei* AY488495, *L. vannamei* AF430075, *L. vannamei* AF430076, *L. vannamei* AY488494, *Farfantepenaeus brasiliensis* EF601055, *L. schmitti* EF601056, *Fenneropenaeus chinensis* AY871268, *Marsupenaeus japonicus* AB121740, *Penaeus monodon* FJ539174, *P. monodon* EF654659, *P. monodon* FJ539178, *P. monodon* EF654658, *P. monodon* GQ334395, *P. monodon* GQ334396, *P. monodon* FJ539175, *P. monodon* FJ539177, *F. chinensis* DQ097704, *F. chinensis* DQ097703, *Paralithodes camtschaticus* EU921643, *Pacifastacus leniusculus* 1 EF523614, *Homarus americanus* EF193003, *Homarus gammarus* AJ786653, *Scylla paramamosain* EU161287, *Hyas araneus* EU921642, *Hyas araneus* EU921641). Values at the node indicate the percentage of times that the particular node occurred in 1000 trees generated by bootstrapping the original deduced protein sequences.

amino acid sequences of crustins also showed that the deduced amino acids of Fi-crustin shared relatively high identities with those of *F. chinensis* and *P. monodon*. Multiple alignment of the nucleotide sequences of Fi-crustin and other shrimp crustins showed high similarity in the signal peptide region, major gaps could be observed in the ensuing region. The 58–70th nucleotide sequences were found to be absent in the Fi-crustin when compared to *P. monodon* crustin sequence. A similar gap could be observed for the *F. chinensis* crustins between the 64–70th position. Similarly, another missing sequence region could be observed for Fi-crustin between the 88–98th position that matches with a similar gap for the *F. chinensis* crustins at the 88–93rd position, when compared to *P. monodon*. Great variation between the sequences of *P. monodon* and *Fenneropenaeus* sp. could be observed at the 122nd–144th position. Other major missing sequences of the Fi-crustins were found at 182–202nd and also between 211th and 216th position. *F. chinensis* showed a major gap for the nucleotide sequences at 262–352nd position whereas *P. monodon* and Fi-crustin did not.

The phylogenetic relationships between Fi-crustin and other crustins with WAP domain are shown in Fig. 3. The tree topologies revealed the relationships of Fi-crustin with other invertebrate crustin-like peptides. The molecular phylogenetic tree based on amino acid sequences suggests that all the crustin members possess the same ancestral origin, which has subsequently diverged at different phases of evolution. Out of all the species, crustins of prawns are found to be evolutionarily distantly related to crustins of other decapod species. The tree could be broadly classified into three major groups, one major group (Group I) which included the crustins of prawns; another (Group II) with that of king crab/crayfish crustins and Group III containing the lobster/crab crustins. The bootstrap distance tree calculated for the crustin sequences clearly indicate that the Fi-crustin possessed great similarity to crustins isolated from *F. chinensis* and *P. monodon*. Great variability could also be noticed in the crustin sequences of various decapods.

This is the first report of an antimicrobial peptide from Indian white prawn, *F. indicus* .The reported AMP belonged to the class of crustins with its characteristic WAP domain and showed 91% similarity to *F. chinensis* crustins. The phylogenetic tree analysis showed that the crustins diverged from an ancestral sequence to three major groups i.e. Group 1 with prawns, Group II with Cray fishes/King crabs and Group III with Lobsters/Crabs. Under prawn – crustins, three sub groups were noticed 1) *L. vannamei* 2) *P. monodon* and 3) *Fenneropenaeus* sp. The wide distribution of crustins in crustaceans indicates the importance of these antimicrobial peptides in the innate immune system.

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# Molecular characterization of a crustin-like antimicrobial peptide in the giant tiger shrimp, *Penaeus monodon*, and its expression profile in response to various immunostimulants and challenge with WSSV

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ABSTRACT

A crustin-like antimicrobial peptide from the haemocytes of giant tiger shrimp, *Penaeus monodon* was partially characterized at the molecular level and phylogenetic analysis was performed. The partial coding sequence of 299 bp and 91 deduced amino acid residues possessed conserved cysteine residues characteristic of the shrimp crustins. Phylogenetic tree and sequence comparison clearly confirmed divergence of this crustin-like AMP from other shrimp crustins. The differential expression of the crustin-like AMP in *P. monodon* in response to the administration of various immunostimulants *viz*., two marine yeasts (*Candida haemulonii* S27 and *Candida sake* S165) and two β-glucan isolates (extracted from *C. haemulonii* S27 and *C. sake* S165) were noted during the study. Responses to the application of two gram-positive probiotic bacteria (*Bacillus* MCCB101 and *Micrococcus* MCCB104) were also observed. The immune profile was recorded pre- and post-challenge white spot syndrome virus (WSSV) by semi-quantitative RT-PCR. Expressions of seven WSSV genes were also observed for studying the intensity of viral infection in the experimental animals. The crustin-like AMP was found to be constitutively expressed in the animal and a significant down-regulation could be noted post-challenge WSSV. Remarkable down-regulation of the gene was observed in the immunostimulant fed animals pre-challenge followed by a significant up-regulation post-challenge WSSV. Tissue-wise expression of crustin-like AMP on administration of *C. haemulonii* and *Bacillus* showed maximum transcripts in gill and intestine. The marine yeast, *C. haemulonii* and the probiotic bacteria, *Bacillus* were found to enhance the production of crustin-like AMP and confer significant protection to *P. monodon* against WSSV infection.

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## Introduction

Antimicrobial peptides (AMPs), widely distributed in the whole living kingdom, play an important role in the immunological defense especially in those organisms which lack adaptive immunity (Boman 1995; Dimarcq et al. 1998; Hancock and Lehrer 1998; Zasloff 2002; Otvos 2002; Bulet et al. 2004). AMPs are promptly synthesized at low metabolic cost, easily stored in large amounts and readily available shortly after an infection, to rapidly kill a broad range of microbes (Hancock 1997, 2001; Prenner et al. 1999a,b). Due to their small size, amphipathic structure and cationic character, AMPs can rapidly diffuse to the point of infection (Brogden 2005). AMPs can kill bacteria in micromolar concentration supporting a non-receptor mediated mechanism as their mode of action. Many antibacterial peptides show a remarkable specificity for prokaryotes and low toxicity for eukaryotic cells; a phenomenon which has favored their investigation and exploitation as potential new antibiotics (Zasloff 1992).

Crustins, a widely distributed family of AMPs were first isolated from the shore crab, *Carcinus maenas* as an 11.5 kDa peptide by Relf et al. (1999). Crustins are cationic, cysteine-rich AMPs with a molecular weight of 7–14 kDa and isoelectric point of 7.0–8.7 and one whey-acidic protein (WAP) domain at the carboxy terminus (Smith et al. 2008). Several isoforms of crustins have been described in a wide range of penaeid prawns including *Litopenaeus vannamei* (Bartlett et al. 2002), *Litopenaeus setiferus* (Bartlett et al. 2002), *Penaeus monodon* (Supungul et al. 2004; Chen et al. 2004; Amparyup et al. 2008), *Marsupenaeus japonicus* (Rattanachai et al. 2004), *Litopenaeus schmitti* (Rosa et al. 2007), *Fenneropenaeus chinensis* (Zhang et al. 2007), *Farfantepenaeus brasiliensis* (Rosa et al. 2007), *Farfantepenaeus paulensis* (Rosa et al. 2007), *Farfantepenaeus subtilis* (Rosa et al. 2007) and *Fenneropenaeus indicus* (Antony et al. 2010).

White spot syndrome virus (WSSV) is one of the most devastating shrimp pathogens, and it has caused serious damage to the worldwide shrimp culture industry (Takahashi et al. 1994; Wang et

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**Table 1**
Rearing conditions and water quality parameters.

| | |
|---|---|
| Tank capacity | 500 l |
| Stocking density | 12 nos. |
| Feeding level | 10–15% of body weight |
| Feeding frequency | Twice daily |
| Water temperature | 24–27 °C |
| pH | 7.5–8 |
| Salinity | 15–18‰ |
| Ammonia | 0.01–0.02 mg l$^{-1}$ |
| Nitrite | 0.00–0.01 mg l$^{-1}$ |
| Nitrate | Below detectable level |
| Alkalinity | 50–60 |
| Dissolved oxygen | 6–7 mg l$^{-1}$ |

al. 1995). WSSV is an enveloped virus with a large, double-stranded, circular DNA genome (~300 kb) containing approximately 180 putative open reading frames (ORFs), most of which have no homology with any known genes or proteins in public databases (Chou et al. 1995; Wongteerasupaya et al. 1995; Wang et al. 1995; Chang et al. 1996; Chen et al. 1997; Yang et al. 2001). Proper husbandry and management of farms with the application of immunostimulants, probiotics and bioremediators can save the industry from the onslaught of diseases to a certain extent.

The selection of suitable compounds with potent immunostimulatory property present a bewildering task. Clearly the over-riding criteria for the selection of suitable immunostimulants are cost, ease of administration, efficacy and low toxicity to the host (Smith et al. 2003). In most cases the initiation of the defense reactions in shrimps is triggered by the presence of pathogen-associated molecular patterns (PAMPs), which include bacterial cell wall components such as lipopolysaccharide (LPS) and peptidoglycan (PG), β-1,3 glucan of fungal cell wall and double-stranded RNA of viruses (Lee and Soderhall 2002). The exact mechanism of action of immunostimulants and the antiviral defense mechanism of crustaceans is poorly understood at the molecular level. AMPs provide a useful way of assessing and studying innate immunity at the biochemical and molecular level.

In the current study, a crustin-like AMP-cDNA from giant tiger shrimp *Penaeus monodon* was cloned and partially characterized at the molecular level. The expression profile of the crustin-like AMP gene in response to various immunostimulants/probiotic bacteria and on challenge with white spot syndrome virus (WSSV) was also analyzed using semi-quantitative RT-PCR. The expression of seven WSSV genes were also analyzed for confirmation of WSSV infection.

## Materials and methods

### *Experimental animals and rearing conditions*

Healthy adult *P. monodon* (20–25 g body weight) PCR negative for WSSV were purchased from a local shrimp farm in Vypeen, Kochi. They were transferred to aquarium tanks of 500 l capacity and acclimatized for 1 week under laboratory conditions. Shrimps were fed standard diet (Higashimaru, India) twice daily during acclimatization period. Constant aeration was provided in all tanks during the experiment. Bioreactor was set in all the aquarium tanks for the effective removal of ammonia and nitrate. Physico-chemical parameters such as salinity, pH, alkalinity, ammonia, nitrite, nitrate, dissolved oxygen and temperature were monitored regularly (Table 1).

### *Immunostimulants/probiotics used*

Two marine yeasts, *Candida haemulonii* S27 and *Candida sake* S165 (isolated from the Arabian Sea and maintained in the Microbiology Laboratory of Department of Marine Biology, Microbiology and Biochemistry, CUSAT); two glucan preparations from *Candida haemulonii* S27 and *Candida sake* S165; two gram-positive bacterial probionts, *Bacillus* MCCB 101 and *Micrococcus* MCCB 104 and a Combination of these two probionts (*Bacillus* MCCB 101 + *Micrococcus* MCCB 104) (obtained from National Center for Aquatic Animal Health (NCAAH), CUSAT) were tested for its efficacy as immunostimulants by observing the expression profile of the crustin-like gene (Table 2). The two marine yeasts, *C. haemulonii* S27 and *C. sake* S 165 have been proved to be good source of immunostimulants by Prabha (2007) and Sajeevan et al. (2006), respectively. *Bacillus* and *Micrococcus* are commercial probionts used in shrimp culture (Antony and Philip 2008).

**Table 2**
Immunostimulants/probiotics used for studying the expression profile of the crustin gene.

| Sl. no. | Immunostimulants/probiotics | Diet code |
|---|---|---|
| | β-1,3 glucans | |
| 1 | *Candida haemulonii* S27 glucan | CHG |
| 2 | *Candida sake* S165 glucan | CSG |
| | Marine yeasts | |
| 3 | *Candida haemolunii* S27 whole cell | CHY |
| 4 | *Candida sake* S165 whole cell | CSY |
| | Gram-positive bacteria | |
| 5 | *Bacillus* MCCB101 | B |
| 6 | *Micrococcus* MCCB104 | M |
| 7 | *Bacillus* MCCB101 and *Micrococcus* MCCB104 – combination | BM |

### *Experimental diets used*

The experimental feeds were prepared by incorporating the different immunostimulants/probiotics to a standard shrimp diet (Higashimaru, India) which was used as the control feed. Seven different types of experimental diets were prepared: i.e., two glucan diets (CHG and CSG), two yeast diets (CHY and CSY) and three probiont incorporated diets (B, M and BM). The two glucan diets (CHG and CSG) were prepared by incorporating 0.2% glucan (extracted from *C. haemulonii* S27 and *C. sake* S165) with the standard diet based on previous studies (Sajeevan et al. 2006, 2009). For yeast diets (CHY and CSY), the two marine yeast (wet weight) biomass (*C. haemulonii* S27and *C. sake* S165) were incorporated at a concentration of 10% (w/w) in the standard diet. In the case of probiotic diets, *Micrococcus* (M) and *Bacillus* (B) biomass were prepared and mixed with the standard diet at $10^3$ cells/g feed .The probiotic combination diet (BM) was prepared by incorporating *Micrococcus* and *Bacillus* at $10^3$ cells each per gram diet ($2 \times 10^3$ cells/g). All feed preparations were kept at −20 °C until used.

### *Feeding experiment and WSSV challenge*

Shrimps were randomly divided into eight groups and were fed the experimental diets for 14 days. Group 1 shrimps fed standard shrimp diet served as the control. Group 2 was fed the experimental diet CHG (0.2% *C. haemulonii* glucan) and group 3, CSG (0.2% *C. sake* S165 glucan). Group 4 was fed experimental diet CHY (10% *C. haemulonii* S27 biomass) and group 5, CSY (10% *C. sake* S165 biomass) (Sajeevan et al. 2006). Group 6, 7, and 8 were fed experimental feeds B (*Bacillus* MCCB101 incorporated diet (50 cells/g animal/day)); M (*Micrococcus* MCCB104 (50 cells/g animal/day)) and BM (*Bacillus* MCCB101 + *Micrococcus* MCCB104 (100 cells/g animal/day)), respectively. The animals were fed twice daily with the experimental diet, except the glucan diets which was given only once in seven days and the control diet on the rest of the days as per the optimized feeding schedule of Sajeevan et al. (2009). Five animals from each group were sampled after 14 days. Only those in

**Table 3**
Primers used for the study.

| ORF/gene | Primer sequence (5′–3′) | Annealing temp. (°C) | Amplicon size (bp) | References |
|---|---|---|---|---|
| Crustin | F – tgttcccacgacttcaagtgtgc<br>R – caaagattcaactaaataaacag | 60 | 299 | Chen et al. (2004) |
| β-actin | F – cttgtggttgacaatggctccg<br>R – tggtgaaggagtagccacgctc | 60 | 520 | Zhang et al. (2007) |
| 18S rRNA | F – ttgtacgaggatcgagtgga<br>R – atgctttcgcagtaggtcgt | 52 | 350 | Supungul et al. (2004) |
| Latency related gene | F – cttgtgggaaaagggtcctc<br>R – tcgtcaaggcttacgtgtcc | 53 | 647 | Liu et al. (2005) |
| VP28 | F – ctgctgtgattgctgtattt<br>R – cagtgccagagtaggtgac | 54 | 555 | Liu et al. (2005) |
| DNA polymerase | F – tgggaagaaagatgcgagag<br>R – ccctccgaacaacatctcag | 54 | 586 | Liu et al. (2005) |
| Endonuclease | F – tgacgaggaggattgtaaag<br>R – ttatggttctgtatttgagg | 50 | 408 | Liu et al. (2005) |
| Thymidine kinase | F – gagcagccatacgggtaaac<br>R – gcgagcgtctaccttaatc | 54 | 412 | Liu et al. (2005) |
| Protein kinase | F – tggagggtggggaccaacggacaaaac<br>R – caaattgacagtagagaattttgcac | 55 | 512 | Liu et al. (2005) |
| Ribonucleotide reductase | F – atctgctagtccctgcacac<br>R – aaagaggtggtgaaggcacg | 53 | 408 | Liu et al. (2005) |

**Table 4**
Result of BLAST analysis of crustin-like AMP nucleotide (FJ535568).

| Closest species | Accession number | *E*-value | % identity |
|---|---|---|---|
| *Farfantepenaeus paulensis* | EF182747 | 1e−31 | 94% |
| *Litopenaeus vannamei* | AF430072 | 5e−31 | 94% |
| *Farfantepenaeus subtilis* | EF450744 | 7e−30 | 93% |
| *Farfantepenaeus brasiliensis* | EF601055 | 7e−30 | 93% |

the intermoult stage were sampled during the study. On the 15th day all the groups were challenged with WSSV by feeding WSSV infected *P. monodon* tissue at the rate of 1 g/animal. The animals were maintained on their respective diets post-challenge WSSV. After 48 h five animals each from all the groups were sampled for the gene expression analysis.

*Haemolymph and tissue collection*

Haemolymph was collected from the rostral sinus using specially designed capillary tubes (RNase-free) rinsed using pre-cooled anticoagulant solution (RNase-free, 10% sodium citrate, pH 7.0). Tissues including gill, muscle, heart, hepatopancreas and intestine were collected. Haemolymph and the tissues were suspended in TRI reagent (Sigma) for total RNA isolation.

*Total RNA isolation and reverse transcription*

Total RNA was extracted from the haemocytes and the target tissues using TRI Reagent (Sigma) following manufacture's protocol. RNA was quantified and the purity was checked by spectrophotometry at 260 and 280 nm. Only RNAs with absorbance ratio $(A_{260}:A_{280}) \geq 1.8$ were used for further experiments. First strand cDNA was generated in a 20 μl reaction volume containing 5 μg total RNA, 1× RT buffer, 2 mM dNTP, 2 μM oligo d($T_{20}$), 20 U of RNase inhibitor and 100 U of M-MLV reverse transcriptase (New England Biolabs, USA). The reaction was conducted at 42 °C for 1 h followed by an inactivation step at 85 °C for 15 min.

*Semi-quantitative RT-PCR analysis of gene expression*

Expression of the target gene when supplemented with different immunostimulants was determined by semi-quantitative RT-PCR analysis using β-actin and 18S rRNA as the internal control pre and post-challenge WSSV (Marone et al. 2001). PCR amplification of 1 μl of cDNA was performed in a 25 μl reaction volume containing 1× Standard Taq buffer (10 mM Tris–HCl, 50 mM KCl, pH 8.3), 3.5 mM $MgCl_2$, 200 μM dNTPs, 0.4 μM each primer and 1 U Taq DNA polymerase (New England Biolabs). Amplification was performed using the target gene primers, Crustin (Forward – 5′-tgttcccacgacttcaagtgtgc-3′ and Reverse – 5′-caaagattcaactaaataaacag-3′) and β-actin (Forward –

```
gttcccacgacttcaagtgtgctggcctcgataagtgttgcttcgacaggtgtttgggagaa
  S  H  D  F  K  C  A  G  L  D  K  C  C  F  D  R  C  L  G  E
cacgtgtgcaagcctccttctttctacggaaggaatgttaaaggatgatgagaataaaca
 H  V  C  K  P  P  S  F  Y  G  R  N  V  K  G  *  *  E  *  T
aaagaccaactgacagacaaccgatgatttggaatttaggaccacgaatgttcaatctac
 K  D  Q  L  T  D  N  R  *  F  G  I  *  D  H  E  C  S  I  Y
tgttatgtcaagtaccaagcaatctgagagtactattatctgtaaaaaataaataaacaa
 C  Y  V  K  Y  Q  A  I  *  E  Y  Y  Y  L  *  K  I  N  K  Q
ataataaatgtaacagggaatgaactactcctttctgtttatttagttgaatctttg
 I  I  N  V  T  G  N  E  L  L  L  S  V  Y  L  V  E  S  L
```

**Fig. 1.** Nucleotide and amino acid sequences of crustin-like AMP from the haemocyte of the Giant tiger shrimp, *Penaeus monodon* (GenBank accession no. FJ535568). Asterisk indicates stop codon.

5′-cttgtggttgacaatggctccg-3′, Reverse – 5′-tggtgaaggagtagccacgctc-3′) as the internal control. As rRNA is considered as a reliable reference for quantitative RT-PCR (Bustin 2002), 18S rRNA was also included as an internal control (Forward – 5′-ttgtacgaggatcgagtgga-3′, Reverse – 5′-atgctttcgcagtaggtcgt-3′). The thermal profile used was an initial denaturation at 94 °C for 2 min followed by 27 cycles of denaturation at 94 °C for 15 s, extension at 68 °C for 30 s and a final extension at 68 °C for 10 min. for the target genes. Annealing temperature varied for the different genes as given in Table 3.The PCR cycles had been optimized so that the target gene and housekeeping gene amplification were at logarithmic phase. The PCR reaction of each sample was carried out in triplicate. PCR product was analyzed by electrophoresis using 1.5% agarose gel in TBE buffer, stained with ethidium bromide and visualized under UV light. The intensity of the gel bands were measured using Image J analysis software. The relative expression level of tiger shrimp crustin-like AMP mRNA was expressed as the ratio of tiger shrimp crustin mRNA to β-actin mRNA.

*Cloning and sequencing*

PCR product was cloned into the pGEM-T Easy vector system (Promega). Recombinant clones were identified and plasmid with the insert was isolated and purified using Gen Pure Plasmid isolation kit (Sigma) and was sequenced at Microsynth, Switzerland.

*Sequence analysis*

The sequence homology and the deduced amino acid sequence comparisons were carried out using BLAST algorithm at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/blast). Gene translation and prediction of deduced protein were performed with ExPASy (http://www.au.expasy.org/). Two multiple sequence alignments were performed with amino acid sequences of known crustin/crustin-like peptides from shrimps and crustaceans separately (NCBI GenBank) using CLUSTALW and GENDOC version 2.7. Phylogenetic and molecular evolutionary analysis were carried out and the consensus trees were compared by bootstrap employing MEGA version 4.0 (Tamura et al. 2007). The neighbour-joining (NJ) tree obtained was validated with the minimum evolution tree. 1000 bootstraps were performed for the NJ tree to check for repeatability of the results. ScanProsite program was used for confirming WAP domain signature.

*PCR analysis of WSSV genes*

Seven genes *viz.* DNA polymerase, endonuclease, latency related gene, protein kinase, ribonucleotide reductase, thymidine kinase and VP28 required for WSSV metabolism and infection were selected for the study (Table 3). Expression profiles of these genes when supplemented with different immunostimulants were studied. Shrimps fed standard diet and challenged with WSSV served as the positive control and the unchallenged shrimps as the negative control. PCR amplification of 1 μl of cDNA was performed in a 25 μl reaction volume as described above.

## Results

*Crustin-like AMP gene in P. monodon*

A partial mRNA transcript of 299 bp, belonging to the crustin family of AMPs was obtained from the mRNA of *P. monodon* haemocyte by RT-PCR (Fig. 1). The nucleotide sequence of the tiger shrimp crustin cDNA was submitted to GenBank under the accession number FJ535568. The sequencing was performed in both directions and sequence was analyzed by homology searches against GenBank data for both nucleotide and amino acid similarity using BLAST program which showed that the crustin-like AMP shared maximum similarity with other crustins of *F. paulensis* (EF182747) (94%), *L. vannamei* (AF430072) (94%), *F. subtilis* (EF450744) (93%) and *F. brasiliensis* (EF601055) (93%) (Table 4). The sequence also showed high similarity to the crustin (No GenBank Submission) isolated from *P. monodon* by Chen and co-workers (Chen et al. 2004).

Multiple alignment of the deduced amino acid sequence of the crustin-like AMP using GenDoc program showed highly significant homology with other crustins isolated from prawns (Fig. 2A). Multiple alignments showed three conserved regions indicating the $C_4$–$C_8$ cysteine residues of WAP domain (Fig. 2A). Multiple alignment of the deduced amino acid sequence of the crustin-like AMP with all known crustins showed a characteristic conserved “KCC” region with the $C_5$ and $C_6$ conserved cysteine residues, characteristic of the WAP domain of crustins. This region was found to be conserved in all the isolated crustins of prawns, lobsters, crabs and crayfishes (Fig. 3A) (Bartlett et al. 2002).

*Phylogenetic analysis of crustin family*

All known crustins were retrieved from the GenBank and phylogenetic analysis of both the nucleotide and amino acid sequences of the crustin-like AMPs were performed. The crustins of both shrimps and that of all crustaceans were subjected to phylogenetic analysis using MEGA 4.0 software.

Phylogenetic analysis of crustins isolated from shrimps showed three branches (Fig. 2B). Group I included crustins isolated from the shrimps, *L. vannamei*, *L. setiferus*, *F. paulensis*, *F. subtilis*, *L. schmitti* and crustin-3 of *F. chinensis*. The deduced amino acid sequence of the crustin-like AMP of *P. monodon* in this study belonged to this group at 93% similarity. Group II included the five crustin isoforms isolated and characterized from *M. japonicus* and Group III consisted of crustins from *F. chinensis* (crustin 1 and 2) and *P. monodon* (crustin 1).

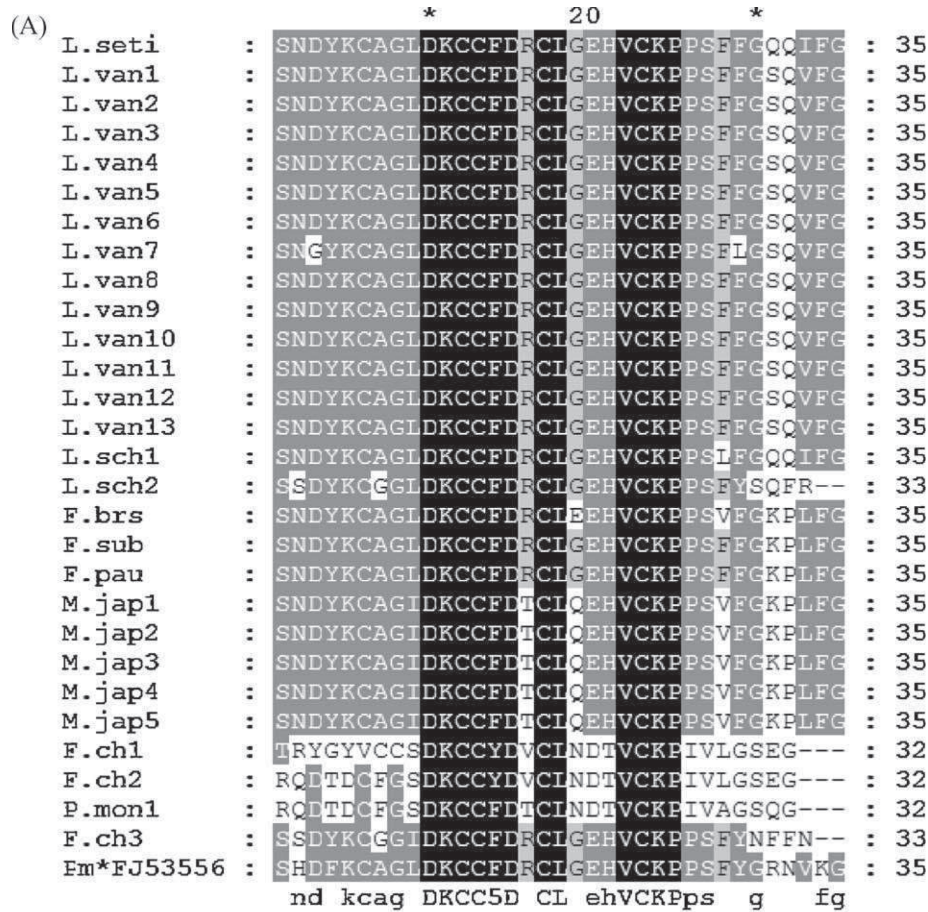
Phylogenetic analysis of crustins isolated and characterized from the entire crustacean group showed five major branches (Fig. 3B). Group I included all the crustins from prawns and Group II included all the crustins from lobsters, *H. americanus* and *H. gammarus.* Group III consisted of crustins from the crayfish *P. leniusculus* whereas the crustins from crabs, *Scylla paramamosain* and *Hyas araneus* formed Group IV.

*Expression of crustin-like AMP genes in response to various immunostimulants*

Crustin gene was found to be up-regulated significantly when *P. monodon* was fed probiotic bacteria, *Bacillus* MCCB101, *Micrococcus* MCCB104 (Fig. 4) and a combination of both *Bacillus* MCCB101 and *Micrococcus* MCCB104 incorporated diets. But the application of yeasts and glucans showed significant down-regulation in the expression profile of crustin-like AMP gene before WSSV challenge.

*Expression of crustin-like AMP genes in response to various immunostimulants post-challenge WSSV*

All the test diets induced up-regulation of the crustin gene post-challenge WSSV. Marine yeasts *viz. C. haemulonii* S27 (Group 2) and *C. sake* S165 (Group 3) and their cell wall glucans up-regulated the crustin gene significantly. Considerable up-regulation of the gene could be noticed in the case of probiotic fed groups also. In the control group, a down-regulation of the crustin gene could be noticed post-challenge WSSV (Fig. 4).



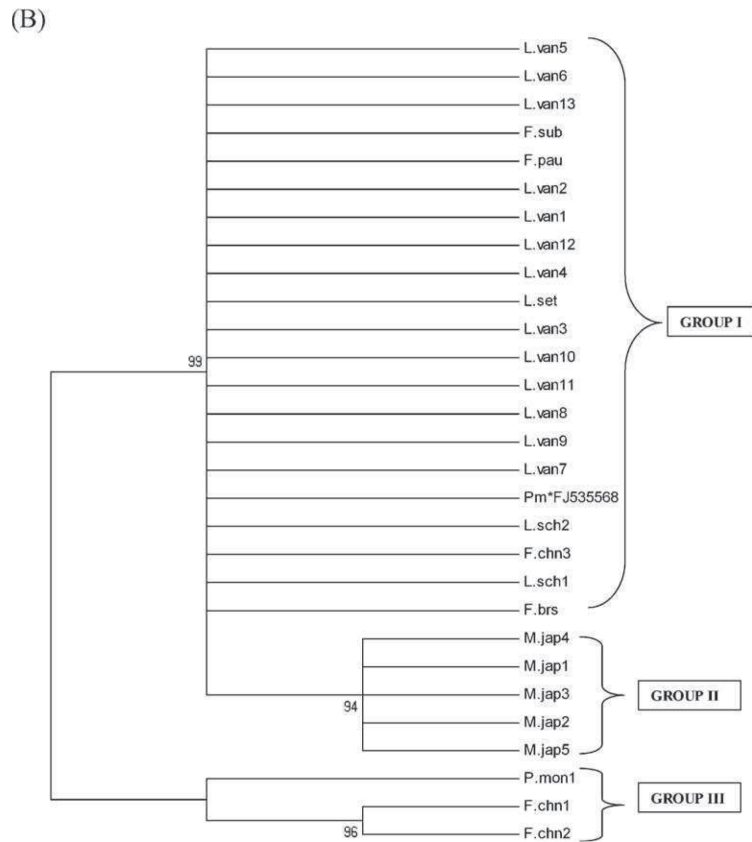




**Fig. 2.** (A) Multiple alignment of deduced amino acid sequence of the *Peneaus monodon* crustin-like AMP (FJ535568) with other shrimp crustins (L. seti – *Litopenaeus setiferus* AF430078, L. van1 – *Litopenaeus vannamei* 1 AF430075, L. van2 – *Litopenaeus vannamei* 2 AF430073, L. van3 – *Litopenaeus vannamei* 3 AF430072, L. van4 – *Litopenaeus vannamei*

*Expression of WSSV genes in the haemocytes of P. monodon in response to various immunostimulants*

All the seven WSSV related genes (DNA polymerase, endonuclease, latency related gene, protein kinase, ribonucleotide reductase, thymidine kinase and VP28) were found to be expressed in the positive (WSSV challenged) control and glucan treated group of shrimps post-challenge WSSV, confirming WSSV infection in these groups (Fig. 5). Intensity of bands showed that the extent of infection was highest in the control group, followed by *C. sake* glucan and *C. haemulonii* glucan diet fed group of shrimps. Moderate amount of transcripts could be noticed in the case of *C. sake* (yeast) fed group for protein kinase. A non-specific amplification of VP28 could also be noticed for the *Micrococcus* fed group. However no viral gene transcripts could be detected in the case of marine yeast and probiotic fed group.

*Expression of crustin-like AMP in various tissues of P. monodon in response to the administration of the marine yeast C. haemulonii and probiotic bacteria bacillus*

Since the performance of *C. haemulonii* and *Bacillus* supplemented diets were found to be the top two post-challenge WSSV, detailed tissue-wise expression was studied for these two treatment groups (Fig. 6). In the control group of animals, crustin gene expression was considerably high in all the tissues pre-challenge and there was a reduction in expression post-challenge. In the case of *C. haemulonii* treated group, the expression was very less pre-challenge and remarkably high post-challenge. However for *Bacillus* treated group, the crustin gene expression was more or less same both pre- and post-challenge. Of the various tissues, the gene expression was found to be maximum in gill and intestine followed by muscle and the least in hepatopancreas.

The expression of WSSV genes were observed only in the control and *C. sake* glucan fed group and no transcripts were noticed in the other immunostimulant/probiotic fed group. Among glucans, the *C. haemuloni* glucan was found to perform better with lesser expression of the WSSV genes.

*Post-challenge survival*

Generally, marine yeast diet fed groups showed significantly high survival compared to β-glucan diet fed shrimps and the control group. Among the various treatments, *C. haemulonii* S 27 (CHY) fed group showed maximum survival (93%) followed by *C. sake* S165 (CSY) (75%), *Micrococcus* (M) (73%), *Bacillus* + *Micrococcus* (BM) (56%), *C. haemulonii* glucan (CHG) (42%), *C. sake* glucan (CSG) (38%) and *Bacillus* (B) (42%) fed group (Fig. 8).

## Discussion

The partial coding sequence, denoted as crustin-like AMP had 299 nucleotides and 91 amino acid residues with conserved cysteine residues characteristic of the WAP domain. The C-terminal segment included a high proportion of cysteine-rich region that participate in the formation of disulphide bonds. The partial cDNA fragment had the five conserved cysteine residues ($C_4$–$C_8$ residues). Multiple polyadenylation consensus sequences (AATAAA) were also present at the C-terminus. The partial cDNA fragment lacked the signal peptide region but possessed the cysteine-rich region characteristic to WAP domain. The putative polyadenylation consensus signal (AATAAA) also confirms the 3′ end of the crustin-like AMP. Multiple polyadenylation sites were observed in the fragment.

As predicted by the ScanProsite program, a partial WAP domain signature exists in the C-terminal region and one of the four disulphide core (DSC) domain was found to be located at $Cys^6$–$Cys^{23}$. Since only partial cDNA fragment and only five of the conserved cysteine regions could be retrieved from the sequence, other locations of the DSC could not be located. Searching against the Prosite database, analysis of the crustin-like AMP revealed the existence of WAP type "DSC" domain signature. The expected WAP type "4DSC core" domain signature is:

$$C_1\text{–}(Xn)\text{–}C_2\text{–}(Xn)\text{–}C_3\text{–}(X5)\text{–}C_4\text{–}(X5)\text{–}C_5\text{–}C_6\text{–}(X3\text{–}5)\text{–}C_7\text{–}(X3\text{–}4)\text{–}C_8$$

where X is any amino acid residue and X*n* is a stretch of *n* residues (Bartlett et al. 2002).

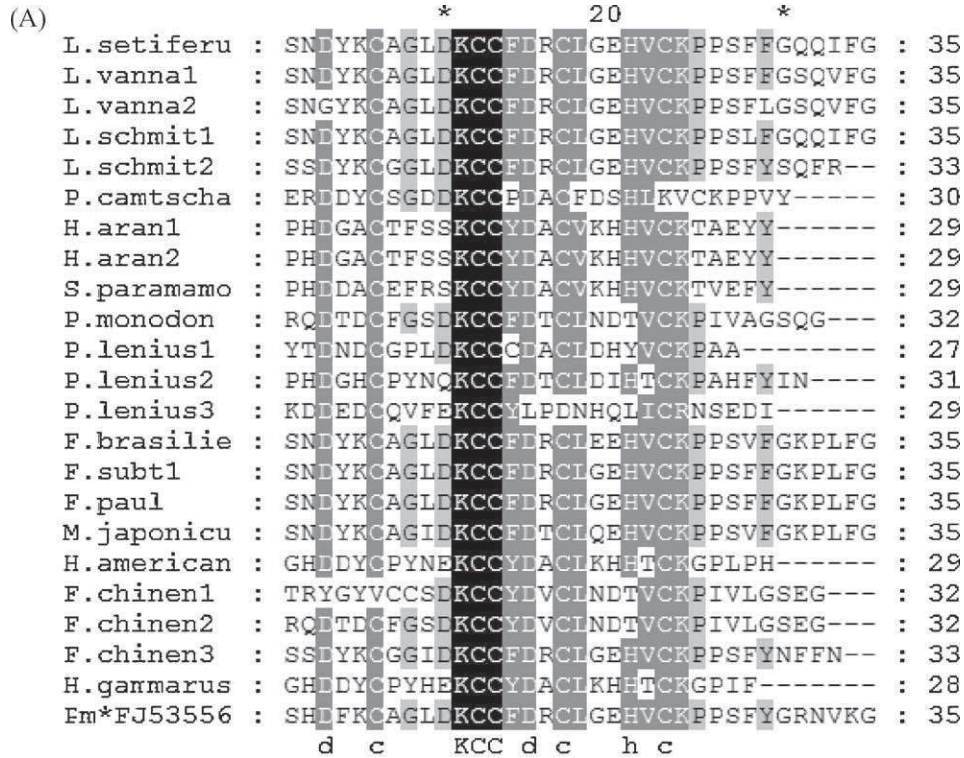
The partial cDNA sequence of the crustin-like AMP showed the presence of $C_4$–$C_8$ and it followed the same pattern as expected in the WAP domain except for one extra residue between $C_7$ and $C_8$ ($C_7$–(X5)–$C_8$). Similar case was reported for the crustins isolated from *F. chinensis*, GenBank no. DQ097703, DQ097704 (Zhang et al. 2007). The WAP domain signature of the present crustin-like AMP is shown in Fig. 7. Several other consensus sequences also appear in the 4DSC domain, i.e. (1) a conserved aspartate (D) residue between C3 and C4. (2) KCC with C5 and C6. (3) CXXP with C8 (Bartlett et al. 2002).

Cysteine residues present in the WAP domain of the crustin were reported to have functions in maintaining the tertiary structure of crustins (Gross et al. 2001). According to the previous reports on the crustin-like proteins, the 4DSC domain played important roles in the biological function (Zhang et al. 2007).

Although much research has been done to investigate various AMP classes and their structure and function, the enhanced production of AMPs using immunostimulants has rarely been evaluated. It is very important to find suitable immunomodulators that could suppress or completely eliminate WSSV by up-regulating the expression of immune genes.

As one of the important AMPs in crustaceans, crustins have gained the attention of many researchers. Until recently, a few sequences of crustins have been described in penaeid shrimps (Gross et al. 2001; Bartlett et al. 2002; Vargas-Albores et al. 2004; Rattanachai et al. 2004; Zhang et al. 2007). As AMPs play an important role in shrimp defense, the expression levels of these molecules are possible indicators of the immune state of shrimps. Haemocytes have been proved to be the site of production and storage of crustins at very high levels (Soderhall and Cerenius 1998; Hauton et al. 2006; Supungul et al. 2007; Amparyup et al. 2008). Hence haemolymph is the best tissue to study the expression of AMPs in relation to various conditions.

4 AF430071, L. van5 – *Litopenaeus vannamei* 5 AF430076, L. van6 – *Litopenaeus vannamei* 6 AF430074, L. van7 – *Litopenaeus vannamei* 7 AY486426, L. van8 – *Litopenaeus vannamei* 8 AY488497, L. van9 – *Litopenaeus vannamei* 9 AY488496, L. van 10 –*Litopenaeus vannamei* 10 AY488495, L. van11 – *Litopenaeus vannamei* 11 AY488494, L. van12 – *Litopenaeus vannamei* 12 AY488493, L. van13 – *Litopenaeus vannamei* 13 AY488492, L. sch1 – *Litopenaeus schmitti* 1 EF182748, L. sch2 – *Litopenaeus schmitti* 2 EF601056, F. brs – *Farfantapenaeus brasiliensis* 1 EF601055, F. sub – *Farfantapenaeus subtilis* 1 EF450744, F. pau – *Farfantapenaeus paulensis* EF182747, M. jap1 – *Marsupenaeus japonicus* 1 AB121740, M. jap 2 – *Marsupenaeus japonicus* 2 AB121744, M. jap3 – *Marsupenaeus japonicus* 3 AB121743, M. jap4 – *Marsupenaeus japonicus* 4 AB121742, M. jap 5 – *Marsupenaeus japonicus* 5 AB121741, F. ch 1 – *Fenneropenaeus chinensis* 1 DQ097704, F. ch2 – *Fenneropenaeus chinensis* 2 DQ097703, F. ch 3 – *Fenneropenaeus chinensis* 3 AY871268, P. mon 1 – *Penaeus monodon* 1 EF654658) obtained using GenDoc programme Version 2.7.0. Black and grey indicates conserved sequences. (B) A bootstrapped neighbour-joining tree obtained using MEGA version 4.0 illustrating relationships between the deduced amino acid sequences of the *Peneaus monodon* crustin-like AMP (FJ535568) with other shrimp crustins (GenBank accession number details as given above). Values at the node indicate the percentage of times that the particular node occurred in 1000 trees generated by bootstrapping the original deduced protein sequences.



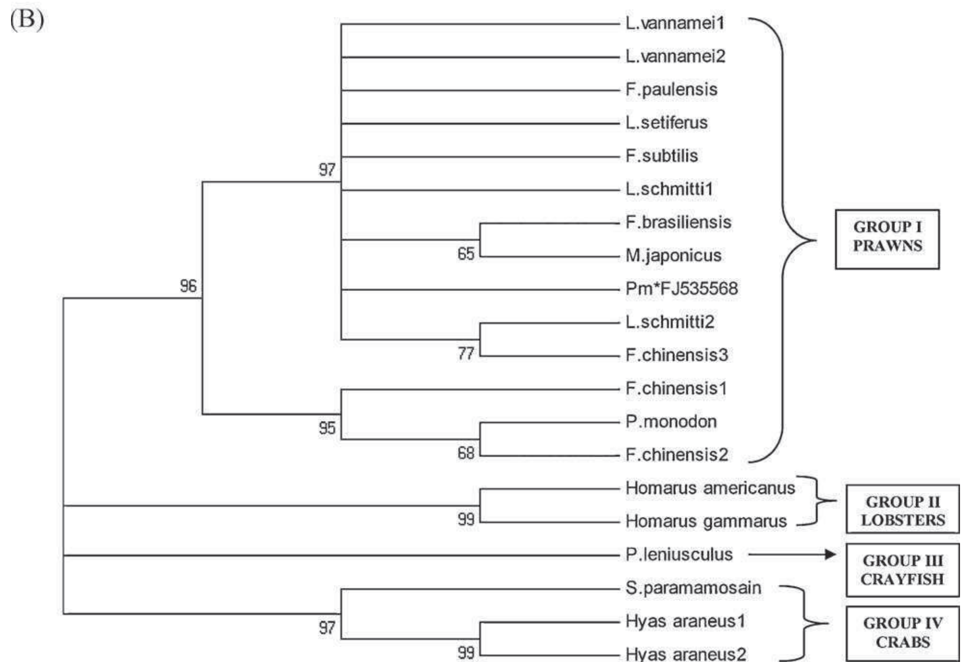




**Fig. 3.** (A) Multiple alignment of deduced amino acid sequence of the *Peneaus monodon* crustin-like AMP(FJ535568*) with all known crustins (*Litopenaeus setiferus* AF430078, *Litopenaeus vannamei* 1 AF430075, *Litopenaeus vannamei* 2 AY486426, *Litopenaeus schmitti* 1 EF182748, *Litopenaeus schmitti* 2 EF601056, *Paralithodes camtschaticus* EU921643, *Hyas araneus* 1 EU921642, *Hyas araneus* 2 EU921641, *Scylla paramamosain* 1 EU161287, *Penaeus monodon* 1 EF654658, *Pacifastacus leniusculus* 1 EF523614, *Pacifastacus leniusculus* 2 EF523613, *Pacifastacus leniusculus* 3 EF523612, *Farfantapenaeus brasiliensis* 1 EF601055, *Farfantapenaeus subtilis* 1 EF450744, *Farfantapenaeus subtilis* 2 EF182747, *Marsupenaeus japonicus* AB121740, *Homarus americanus* EF193003, *Fenneropenaeus chinensis* 1 DQ097704, *Fenneropenaeus chinensis* 2 DQ097703, *Fenneropenaeus chinensis* 3 AY871268, *Homarus gammarus* AJ786653) obtained using GenDoc programme Version 2.7.0. Black and grey indicates conserved sequences. (B) A bootstrapped neighbour-joining tree obtained using MEGA version 4.0 illustrating relationships between the deduced amino acid sequence of the *Peneaus monodon* crustin-like AMP (FJ535568) with all known crustins (GenBank Accession Number details as given above). Values at the node indicate the percentage of times that the particular node occurred in 1000 trees generated by bootstrapping the original deduced protein sequences.

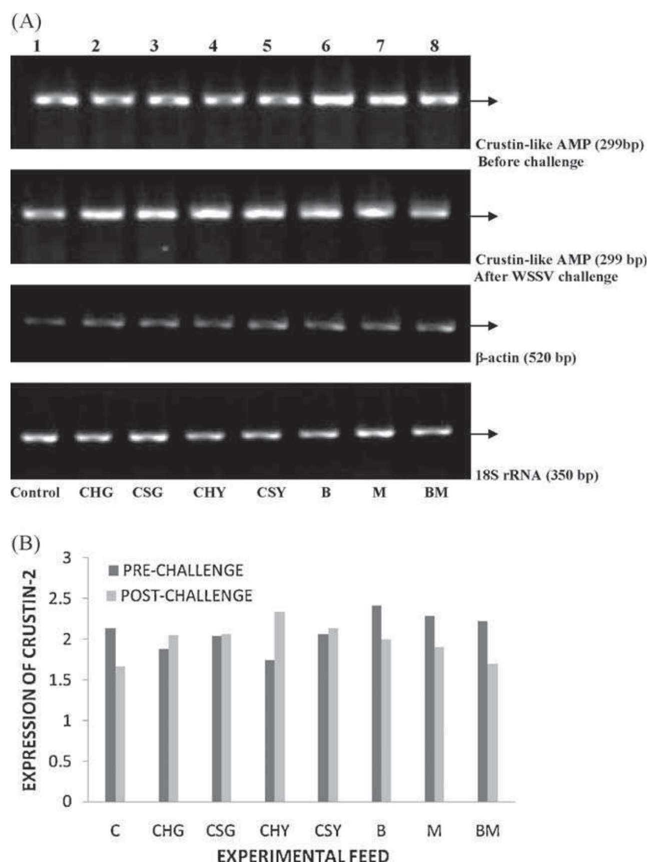



**Fig. 4.** Expression profile of crustin-like AMP (FJ535568) gene in the haemocytes of *Penaeus monodon* in response to various immunostimulants before and after challenge with WSSV (CHY = *Candida haemulonii* S27 yeast, CSY = *Candida sake* S165 yeast, CHG = *Candida haemulonii* S27 glucan, CSG = *Candida sake* S165 glucan, B = *Bacillus* MCCB101, M = *Micrococcus* MCCB104, BM = combination of *Bacillus* MCCB101 and *Micrococcus* MCCB104). (A) Gel photograph, (B) ratio of target gene/β-actin (*x*-axis = immunostimulants used, *y*-axis = ratio of target gene/β-actin).

The probiotic gram-positive bacteria incorporated diet up-regulated the crustin gene to significant levels proving its immunostimulatory property. The yeasts and glucans caused a down-regulation of crustin gene expression pre-challenge WSSV. This is in agreement with the earlier work in *L. vannamei*, where no significant variations in the expression of crustins have been reported in β-glucan supplemented diet fed animals (Wang et al. 2007). Significant down-regulation of crustin was noticed with the viral challenge in the control group of organisms. However, all treatment groups showed better crustin expression post-challenge with the best performance displayed by yeast and glucan fed group.

The down-regulation of crustin in the control group 48 h post-challenge showed that the animal had already become weak and succumbed to infection earlier than that of the experimental groups. This observation is in agreement with the earlier work of Sun et al. (2008) where a down-regulation of lectins (another group of AMPs) has been reported 24 h post-challenge WSSV in *F. chinensis*. Vargas-Albores et al. (2004) and Okumura (2007) have also reported a decrease in the number of crustin transcripts when infected with *Vibrio* in *L. vannamei*. Chiou et al. (2005) have reported no up-regulation of AMPs in the haemocytes of tiger shrimps when challenged with *Vibrio*. But contrary observations have been reported in the white shrimps in which AMPs have been up-regulated with bacterial challenge (Jiravanichpaisal et al. 2007; Amparyup et al. 2008).

In the present work, glucans and different yeast strains exhibited almost similar efficacy in terms of crustin gene expression. Whole yeast cells especially *C. haemulonii* were found to perform better under WSSV challenge. It was interesting to note that *C. haemulonii* did not induce the crustin gene pre-challenge, but effected significant up-regulation post-challenge WSSV, proving it to be a good immunostimulant conferring protection to shrimps against WSSV infection.

The response of crustin expression to bacterial challenge is supposed to be enigmatic and often does not follow the pattern expected for immune genes and other AMPs (Gross et al. 2001; Rojtinnakorn et al. 2002; Lorgeril et al. 2005). The present results are consistent with the earlier works where up-regulation of crustin gene has been reported. Application of PG in *M. japonicus* (Rattanachai et al. 2005) resulted in the up-regulation of crustin gene in unchallenged shrimps. In *P. monodon* also a five-fold up-regulation of the crustin transcripts has been reported following challenge with *V. harveyii* (Amparyup et al. 2008). On the contrary a few authors have observed an unexpected down-regulation of crustin transcripts after bacterial challenge with gram-negative bacteria. Administration of LPS in *L. vannamei* (Okumura 2007) resulted in the down-regulation of the same. A down-regulation in the crustin transcripts has also been reported after 24 h of challenge by *Vibrio alginolyticus* in *L. vannamei* (Vargas-Albores et al. 2004; Jiménez-Vega et al. 2004). Also in *P. monodon* crustin transcripts were found to be down-regulated after infection with gram-negative bacteria (Supungul et al. 2007).

The up-regulation by the combination of two strains (*Bacillus* MCCB101 and *Micrococcus* MCCB104) was not up to the level effected individually. Even though a combination of the two strains induced the gene pre-challenge a significant up-regulation was not found under viral challenge. This indicates that these two strains are potent when applied individually and not in combination. *Bacillus* MCCB101 and *Micrococcus* MCCB104 are widely used as probionts in shrimp culture systems, and the present study proved them to be better immunostimulants when administered individually.

Expression of WSSV related genes post-challenge showed that *C. haemulonii*, *Bacillus* and combination of *Bacillus* and *Micrococcus* treated groups were less infected by WSSV (Fig. 5). This is in agreement with the up-regulation noted for crustin gene except for the combination of *Bacillus* and *Micrococcus*. When the expression of WSSV genes are taken into account, yeasts especially *C. haemulonii* proved to provide better protection to WSSV than glucans. Minor expression of some WSSV genes was noted for *C. sake* yeast and *Micrococcus*. Also *Micrococcus* treated group showed non-specific amplification for VP28. Such non-specific amplification has been reported earlier by Marks et al. (2003). These WSSV gene expressions showed that the extent of protection was lower in *C. sake* whole cell and *Micrococcus* treated group of shrimps compared to C. *haemulonii* and *Bacillus* + *Micrococcus* combination treated groups (Fig. 8).

Tissue-wise expression profile of crustin was in conformity to those found in other arthropods (Iwanaga and Kawabata 1998). Since *C. haemulonii* and probiotic *Bacillus* were found to be the best among the eight experimental groups, tissue-wise analysis of the crustin gene expression was carried out for these two treated group of animals and the control group.

Tissue expression profile of crustins in unchallenged shrimps revealed highest expression in gill followed by intestine, muscle, heart and the lowest in hepatopancreas. Earlier works in *P. monodon* have reported the absence of any crustin transcripts in heptopancreas (Supungul et al. 2004). In *M. japonicus*, however, crustin mRNA was only detected in haemocyte, not in any other tissue (Rattanachai et al. 2004). Whereas, reports in *L. vannamei* is in agreement with the present result where mRNA transcripts could be observed in all tissues including hepatopancreas (Wang et al. 2007).

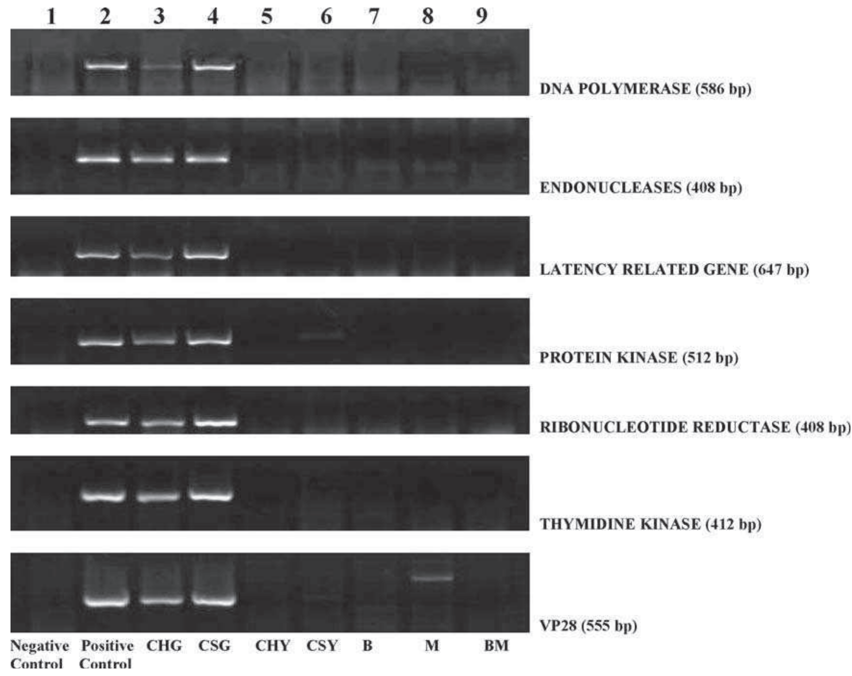



**Fig. 5.** Expression of WSSV genes in the haemocytes of *P. monodon* in response to the application of various immunostimulants (negative control = pre-challenged control shrimp, positive control = WSSV challenged control, CHY = *Candida haemulonii* S27 yeast, CSY = *Candida sake* S165 yeast, CHG = *Candida haemulonii* S27 glucan, CSG = *Candida sake* S165 glucan, B = *Bacillus* MCCB101, M = *Micrococcus* MCCB104, BM = combination of *Bacillus* MCCB101 and *Micrococcus* MCCB104).

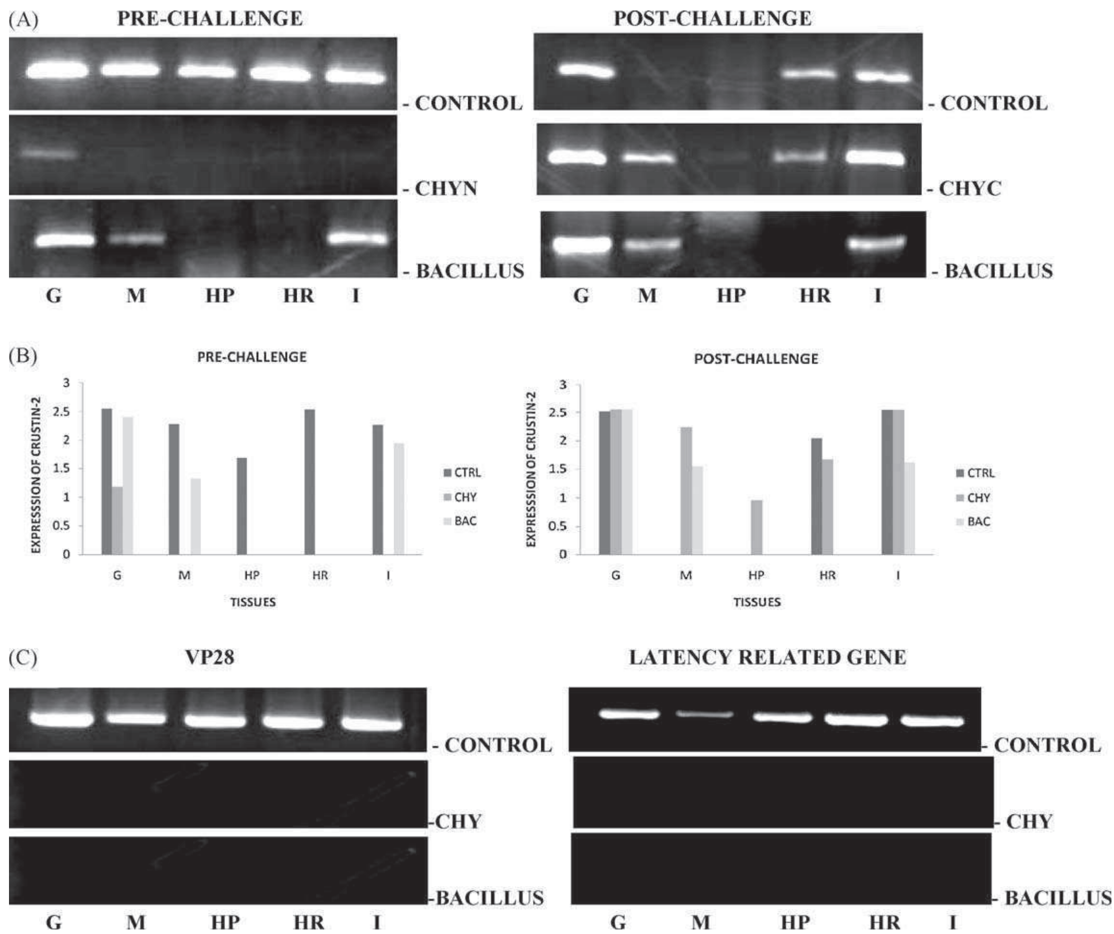



**Fig. 6.** Expression of crustin in various tissues of tiger shrimp *Penaeus monodon* in response to the application of immunostimulant/probiotic before and after WSSV challenge. G: gill, M: muscle, HP: hepatopancreas, HR: heart, and I: intestine. (A) Gel photograph. (B) Expression of crustin gene in target tissues. (C) Expression of WSSV related gene in target tissues.

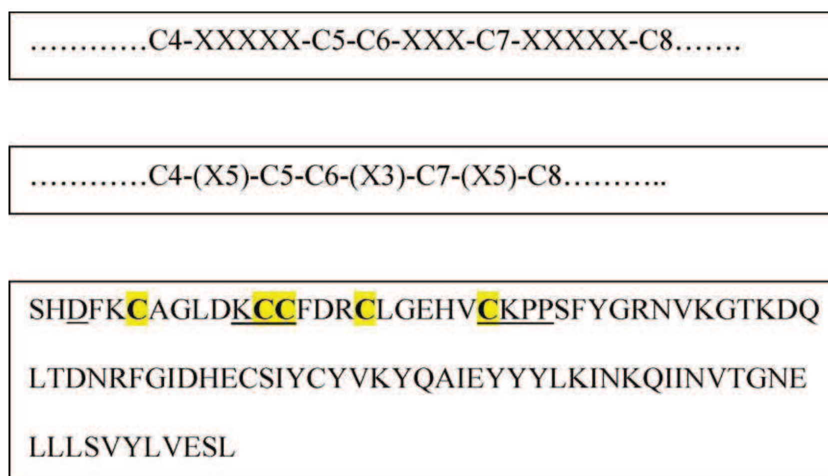



**Fig. 7.** The WAP domain signature of the crustin-like AMP (FJ535568). Consensus sequences that appear in the 4DSC domain are underlined and the $C_4$–$C_8$ cysteine residues are being highlighted.

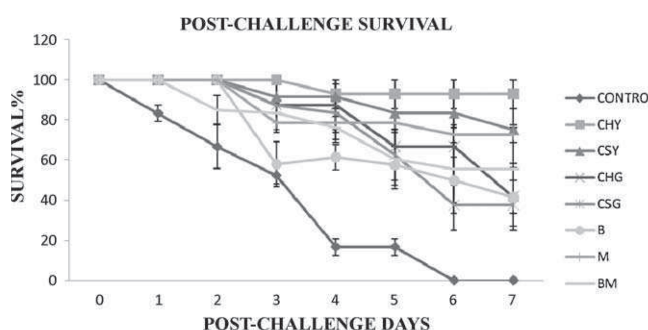



**Fig. 8.** Post-challenge survival of *Penaeus monodon* fed with different immunostimulant/probiotic bacteria incorporated diets and challenged with WSSV (CHY – *Candida haemulonii* yeast fed group, CSY – *Candida sake* yeast fed group, CHG – *Candida haemulonii* glucan fed group, CSG – *Candida sake* glucan fed group, B – *Bacillus* fed group, M – *Micrococcus* fed group, and BM – *Bacillus* + *Micrococcus* fed group).

Shrimps possess an open circulatory system that allows haemocytes to infiltrate and adhere to many tissues. All gene expressions are from the infiltrating haemocytes and the relative expression levels of these genes would reflect the amount of haemocytes infiltrating or fixed in tissues. Since haemocyte is the site of synthesis of AMPs, there is no point in comparing haemolymph with other tissues. Variation in the expression of crustin genes in various tissues might have been contributed by the differential infiltration of haemocytes into various tissues which in turn is due to the varying levels of virions in the tissues. This tissue-wise variation in crustin expression point to the tissue specificity of viruses for its multiplication.

Secondary expression sites such as intestine or gonads have been reported for AMPs in insects (Hoffmann and Reichart 1997; Manetti et al. 1998). Abundance of mRNA transcripts of AMPs in shrimp intestine suggests intestine to be a possible expression site, besides the haemocytes.

Yeast, *C. haemulonii* and probiotic bacteria, *Bacillus* treated group of animals did not show the presence of any WSSV gene transcripts in any of the tissues tested confirming the absence of WSSV infection.

Yeast, *C. haemulonii* was proved to be the best in terms of AMP gene expression. The constitutive production of these AMPs ensures that animals are able to protect themselves from low-level assaults by pathogens present in the environment. As these molecules play an important role in the shrimp immune system, the expression levels of these AMPs are possible indicators of the immune state of shrimps. Generally, expression of crustin in the haemocytes was higher on WSSV challenge, suggesting their role in antiviral defense. The up-regulation of the crustin gene by yeasts, glucans and gram-positive bacteria proved that these compounds have potent immunostimulating activity against WSSV infection. Further investigations on the range of activity as well as the regulation of the gene expression of the tiger shrimp crustin during various stages of growth and development would shed light in developing strategies to protect tiger shrimp from infection by WSSV.

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