CHAPTER 1

INTRODUCTION

Aquaculture farming is an important industry in Asian Coastal Countries including Malaysia. Among all aquatic animals, prawn has found a special important place in comparison with other aquatic animals in Malaysia and among prawn species *Macrobrachium rosenbergii* has been introduced as a high efficiency species to Malaysian Aquaculture Industry. *M.rossenbergii's* farming provides employment for a hundred workers in farming food production and export industry. Unfortunately, during the last decade, viral disease caused severe economic outbreaks in prawn's reproduction farm and affects local and national economies and even threatened the prawn industry in some producing countries. However, during the last decade, Malaysian prawn farmers have earned good progress in *M.rossenbergii's* implementation strategies to combat prawn viral disease.

Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV) is one of the major viral pathogens in Malaysian prawn farms. IHHNV is a virus which was initially reported from most penaeid prawns (Karas and Hillenkamp, 1988; Lightner, 1996a). Since July 2004, it was reported in *M.rosenbergii* because of an unknown disease outbreak with mortalities up to 80–100% than have been frequently encountered in post larvae and juveniles of *M. rosenbergii* in southern Taiwan. Based on studies, IHHNV seems to relate with these outbreaks (Chia *et al.*, 2006) in prawn farm. In order to control virus problems a lot of studies were conducted on antiviral activities of prawn in virus infection and certain proteins have been studied as possible immune therapeutic agents to prevent infections

(Yeh *et al.*, 2009). For instant different protein/DNA vaccinations against White Spot Syndrome Virus (WSSV) infection were reported for the protection of prawn (Kumar *et al.*, 2008).

Despite knowledge of the IHHNV genome, there is little information available about molecular aspects of IHHNV interactions with its host. Since 2-dimensional gel electrophoresis (2-DE) is a known effective tool in protein identification study this technique was applied in this study to do a comparative study of serum from normal prawn and IHHNV infected prawn to identify the proteins which are controlling the immune system during viral infections. For this purpose in this study, attempts were made to identify proteins which are controlling the immune system of prawn during viral disease in terms of what we have. IHHNV viral infection on wild freshwater prawn *M.rosenbergii* was selected as a sample to identify proteins that were involved in *M.rosenbergii*. This allowed us to have a better understanding on the virus effect in wild farm under environmental variation.

The objectives of the present research were to identify proteins involved in immune response under IHHNV infection or host-viral interaction. To achieve this goal, target prawn captured from wild prawn brood stock, and Nested-PCR techniques was used to identify infected and non-infected prawn. Then 2-dimentional gel electrophoresis technique was applied to do comparison between IHHNV infected and non-infected prawn serum. Comparative 2-DE gel analysis was performed using PDQuest analysis software (Biorad) and differential expressed proteins between infected gel and non infected gel are identified. Differentially expressed proteins were subjected to MALDI TOF-TOF Mass spectrometry to identify each individual protein spot profile that might have significant role under IHHNV infection.

Objectives of the study

I. To study the effect of the virus infectious on protein profile and protein content of *M.rosenbergii's* serum.

II. To identify proteins that is involved in the immune system of the prawn against IHHNV infection.

CHAPTER 2

LITERATURE REVIEW

2.1. Biology of *Macrobrachium rosenbergii*

Macrobrachium rosenbergii (Figure 2.1) or giant river prawn, known as the giant freshwater prawn, the Malaysian prawn belongs to the family: Palaemonidae, order: Decapoda, class: Arthropoda, phylum: Animalia. *M. rosenbergii* is an important species in Malaysian economy has been illustrated as a scientifically recognized prawn since 1705 (Sudhakaran *et al.*, 2007). This species as well as other *Macrobrachium spp* (e.g. *M. nipponesis*) is commercially important for its edible value. The basic biology of *M.rosenbergii* was studied in Penang, Malaysia (Ling. and Merican., 1961) and *M. rosenbergii and M. nipponesis* are the only species of freshwater prawn involved in Malaysian culture.

Figure 2.1, *Macrobrachium rosenbergii*

2.2. Aquaculture and Production of *M. rosenbergii*

Major production areas are Asia and the Pacific including Iran, India, Malaysia, Singapore, China, Burma, Thailand, Taiwan, Bangladesh, Indonesia, the Philippines, or the North and Latin America (Brazil, Chile ,Peru ,Honduras ,Mexico) , United States, Europe and Africa. In the last decade, freshwater prawn farming has increased dramatically with total production in India reaching an all-time high of 20,000 mt in 2002, equal to the third rank in the world prawn production (Sudhakaran *et al.*, 2007)(Figure 2.2).

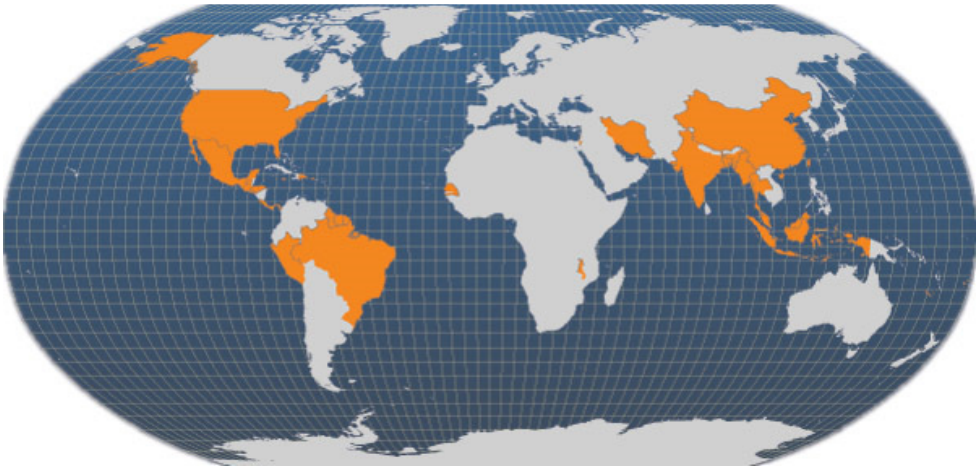

Figure 2.2, Main producer countries of *M. rosenbergii* (FAO, 2006)

The most recently introduced animals in freshwater Chinese aquaculture is Freshwater prawns (Weimin and Xianping, 2002). In many countries, prawn cultivation has economic importance in the aquaculture industry. In Taiwan, production of the giant freshwater prawn, *M. rosenbergii*, reached a peak (16,196 metric/tonnes) in 1991, then decreased to 8200 metric tonnes in 1998. The production increased to over 10,000 metric

tons between 2003 and 2005, but amounted to only 8316 metric tonnes in 2007 (Sung and Ye, 2009)(Figure 2.3).

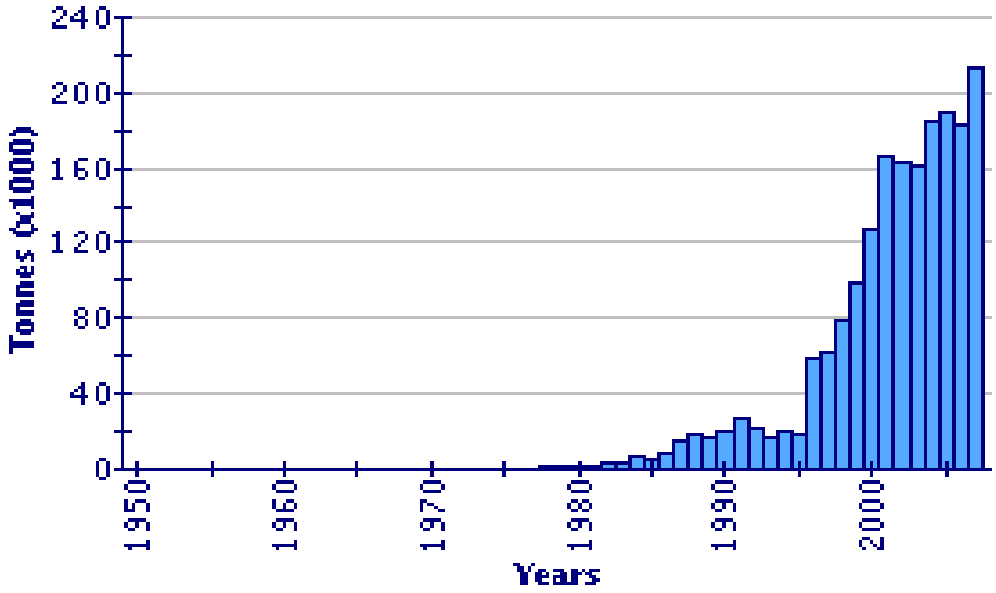

Figure 2.3, Global aquaculture production of *M. rosenbergii* (FAO, 2006)

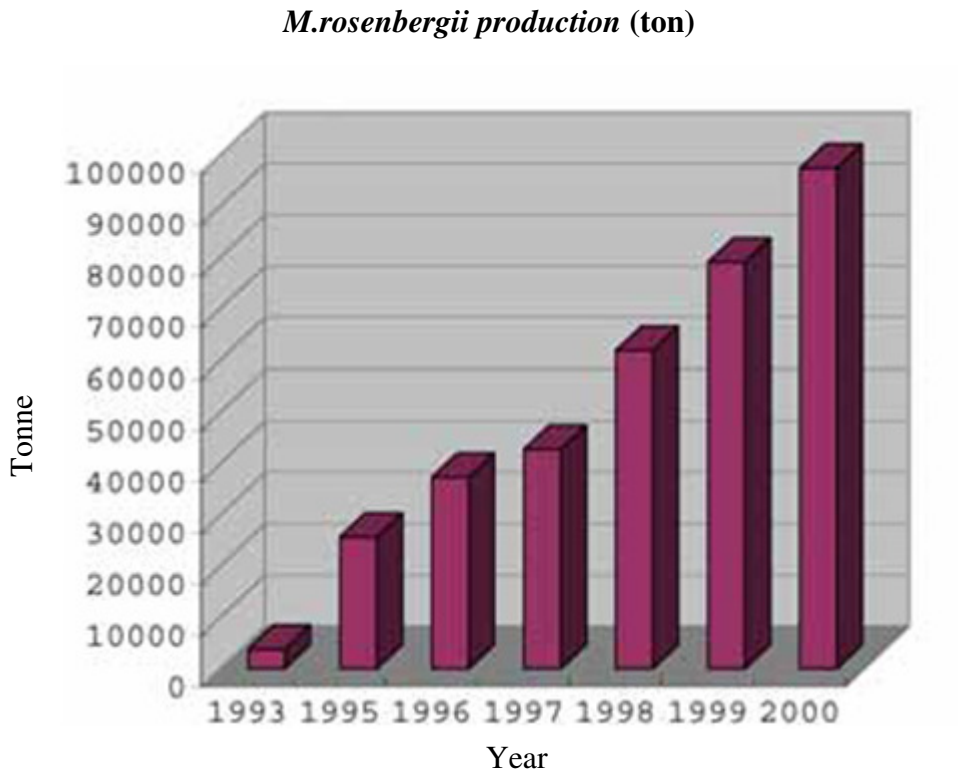

Figure 2.4, Cultured production of *M. rosenbergii* during for China 1993-2000 (Reddy and Sainath, 2009)

At present, one of the major cultured species in the world is tiger prawn but farmers and scientists believe that fresh water giant prawn might be a good option for aquaculture industry. Unfortunately, the epidemic disease problem in the brood stocks of tiger prawn (*Penaeus monodon*) caused a significant decrease in economic yield. Consequently, farming technology for *M. rosenbergii*, is gaining impetus. On the other hand, the success of *M. rosenbergii* culture needs more information in several areas. For example, the reproductive physiology of the prawn is not well understood and obtaining brood stock could become a problem if this species becomes a popular farming choice(Ronnie Tan and Toh, 2011). Some basic knowledge of the hormones controlling the reproductive cycles plus determining the best conditions for their culture is required. Methods for increasing the

production of *Macrobrachium* are necessary because the need for this prawn is progressively greater (Reddy and Sainath, 2009)(Figure 2.4).

Malaysia was the first country which developed the technique of controlled hatching of the giant prawn. Then the other interested countries used this procedure in prawn culture. These countries had successful trials and established hatchery in increasing culture of giant prawn and the progress is proceeding gradually. In Malaysian rivers production fluctuated from 25 to 200 mt per year. Currently two to three million juveniles are being produced in the established hatcheries but during 1981 the production was only 1.6 million. These were both used for culture and scientific experiments(Ronnie Tan and Toh, 2011) (Figure 2.5).

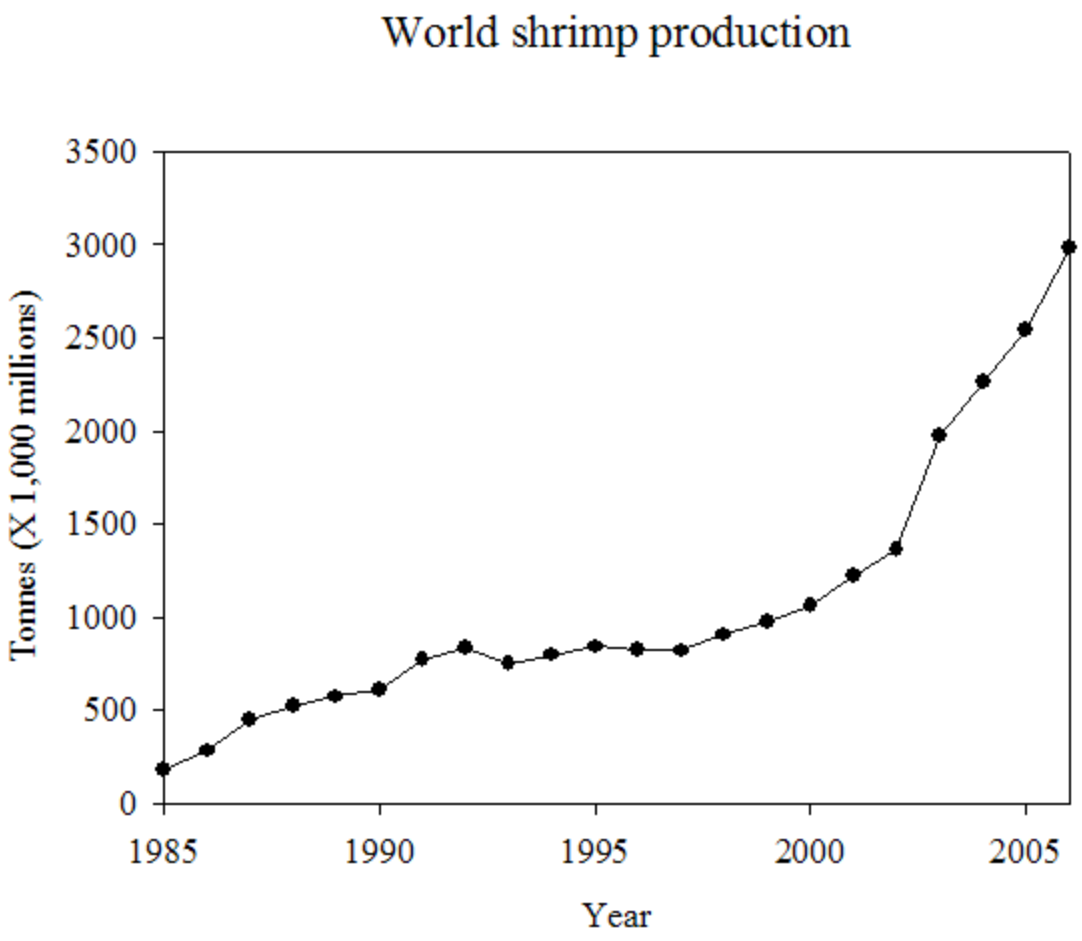

Figure 2.5, Cultured shrimp production in the World (1985-2006) (FAO, 2006)

2.3. Disease outbreaks in *M. rosenbergii*

The main problem in the prawn aquaculture is viral diseases. About 20 prawn viruses have been reported (Gabriel and Felipe, 2000), among them, Infectious hypodermal and hematopoietic necrosis virus (IHHNV), lymphoid parvovirus (LPV)and hepatopancreatic parvovirus (HPV), are related to parvovirus infections in penaeid shrimp (Lightner *et al.*, 1983b) also they were reported in other species. Recently, extra small virus (XSV) and *M. rosenbergii* nodavirus (MrNV) are also found in *M. rosenbergii* with white tail disease. IHHNV is a virus which was initially reported from most penaeid shrimps (Lightner, 1996a). Since July 2004, it was reported in *M.rosenbergii* because an unknown disease outbreak with mortalities up to 80–100% has been frequently encountered in post larvae and juveniles of *M. rosenbergii* in southern Taiwan. Based on studies, IHHNV seems to relate to these outbreaks (Chia *et al.*, 2006) in prawn farm. In order to control virus problem a lot of studies were conducted on antiviral activity of prawn in virus infection and certain proteins have been studied as possible immune therapeutic agents to prevent infections (Yeh *et al.*, 2009). For instant different protein/DNA vaccinations against White Spot Syndrome Virus (WSSV) infection were reported for the protection of prawn/crayfish (Kumar *et al.*, 2008). To use such techniques in viral infections, identifying the proteins which are controlling the immune system are required. For this purpose in this study, we attempted to identify proteins which are controlling the immune system of prawn during viral disease in terms of what we have.

2.4. Viral pathogens in *M. rosenbergii*

M. rosenbergii is the species most seriously affected by viral pathogens. The pathogens can be grouped into 5 major categories as viruses, bacteria, fungi, protozoans and unknown agents (Loh *et al.*, 1997). Several diseases are now commonly found in the culture of this species. The major disease problems affecting *M.rosenbergii* generally occur because of poor husbandry, poor intake water treatment, poor sanitation, overcrowding, and non-existing or inadequate quarantine procedures.

Different pathogens specious for prawn were reported from 1997 in different studies. These reports include rotten tail disease, parasitic disease, black gill disease, milky-white body (muscle) disease, and black spot disease. As an example, a large-scale outbreak of milky-white body (muscle) disease was found in Southern China, in 1998. This may cause mortalities as high as 70% of the affected prawns.

Recently, through affected seed purchased, disease spread to other areas like Jiangsu province. Prawns are mostly affected at early stage of culture. Various diseases can affect prawn culture production and quality that might lead to high mass mortality (Weimin and Xianping, 2002).

Between July 2004 and January 2005, in southern Taiwan, high mortalities (up to 80-100%) were encountered in post larvae of *M. rosenbergii*. Pathologically, eosinophilic intranuclear inclusion bodies (INIs) were found only in the hepatopancreatic tubular epithelial cells of the infected postlarvae from hatchery farms. No lesions could be detected in tissue of ectodermal or mesodermal origin (Chia *et al.*, 2006).

There are procedures to overcome disease problems in prawn culture such as husbandry improvement and antibiotic and pharmaceutical. Antibiotics and other

pharmaceuticals have been used in treatment but their addition in (Table 2.1) does not include as Food and Agriculture Organization (FAO) recommendation.

Between 2002-2008 lots of studies have been done based on *M.rossenbergii's* aquaculture outbreaks, and IHHNV was reported as one of the major viruses which causes outbreak in *M.rossenbergii.*

Table 2.1, Disease listed according to FAO (FAO/NACA/UNEP/WB/WWF, 2006)

DISEASE	AGENT	TYPE	SYNDROME	MEASURES
MMV (Macrobrachium Muscle Virus)	Parvo-like virus	Virus	Infected tissue becomes opaque, with progressive necrosis; affects juveniles	IH
WSBV (White spot Syndrome BaculoVirus)	Baculovirus	Virus	White spots; affects juveniles	IH
Unnamed viral disease	Nodavirus	Virus	Whitish tail; affects larvae	IH
Black spot; brown spot; shell disease	Vibrio; Pseudomonas; Aeromonas	Bacteria	Melanised lesions; affects all life stages, but more frequently observed in juveniles & adults	IH; oxolinic acid; nifurpurinol
Bacterial necrosis	Pseudomonas; Leucothrix	Bacteria	Similar to black spot but only affects larvae, especially stages IV & V	IH;nifurpurinol;erythromycin; penicillin-streptomycin; chloramphenicol
Luminescent larval syndrome	Vibrio harveyi	Bacterium	Moribund & dead larvae luminescent	IH; chloramphenicol; furazolidone
White postlarval disease	Rickettsia	Bacterium	White larvae, especially stages IV and V	IH; oxytetracycline; furazolidone; lime, prior to stocking
Unnamed fungal infection	Lagenidium	Fungus	Extensive mythelial network visible through exoskeleton of larvae	IH; trifluralin; merthiolate

Unnamed fungal infection (often associated with IMN - see below)	Fusarium solani	Fungus	Secondary infection; affects adults	IH
Unnamed yeast infections	Debaryomyces hansenii; Metschnikowia bicuspidata	Fungi	Yellowish, greyish or bluish muscle tissues in juveniles	IH
Protozoan infestations	Zoothamnium; Epistylis; Vorticella; Opercularia; Vaginicola; Acineta; Podophyra; etc.	Protozoanse	External parasites that inhibit swimming, feeding and moulting; affect all life stages	IH formalin; merthiolate; copper-based algicides
IMN (Idiopathic Muscle Necrosis)	environmental disease	Unknown	Whitish colour in striated tissue of tail and appendages; when advanced, necrotic areas may become reddish; affects all life stages	IH
MCD (Mid-Cycle Disease)	undetermined aetiology	Unknown	Lethargy; spiralling swimming; reduced feeding and growth; bluish-grey body colour; affects larvae, especially stages VI and VII	IH hatchery disinfection
EED (Exuvia Entrapment Disease), sometimes known as MDS (Moult Death Syndrome)	undetermined aetiology	Unknown but probably multiple causes, including nutritional deficiency	Localised deformities; failure to complete moulting; affects late larval stages; also seen in postlarvae, juveniles & adults	IH dietary enrichment

*Improved Husbandry (IH)

Habitually, stress factors such as low dissolved oxygen or abnormal pH and temperature salinity, high levels of soil fertilizer pollutants; prawn overcrowding and density; larval improper management are considered to trigger shrimps culture outbreak (Praytno and Latchford, 1995).

Moreover, a few possible ways of infectious transmission such as transport by humans and some environmental factors such as birds or other animals including insects and other infected crustaceans; contaminated foods or frozen infected crustaceans used as prawn food (Lightner et al., 1997) and movement of live infected prawns or other aquatic hosts (Kanchanapum *et al.*, 1998) from infected areas to uninfected areas for aquaculture are important factors in prawn farm outbreaks.

Table 2.2 represented some reported viruses in prawn aquaculture through the world (Gabriel and Felipe, 2000)(Gabriel and Felipe, 2000). As it is shown in the table some viruses are unclassified and some are classified and sub-divided into DNA and RNA viruses based on their molecular function (Loh *et al.*, 1997)(Loh et al., 1997).

Seven of the most important viral diseases are now listed as the most important prawn diseases in the Aquatic Animal Health by World Organization for Animal Health (OIE). They are considered to be significantly important for the public health. These viral diseases are: yellow head disease (YHV) caused by yellow head virus, spherical baculovirosis caused by Penaeus monodon-type baculovirus (MBV), spawner-isolated mortality virus (SMV) disease, white spot disease (WSSV) caused by white spot syndrome virus, Taura syndrome caused by Taura syndrome virus (TSV), Baculovirus penaei caused by tetrahedral baculovirosis (BP), and finally infectious hypodermal and haematopoietic necrosis caused by infectious hypodermal and haematopoietic necrosis virus (IHHNV).

Table 2.2, Viruses can be divided as RNA or DNA virus Source: (Gabriel and Felipe, 2000))

Virus	Genome	Virion size (nm)
DNA viruses		
Parvoviruses		
Infectious hypodermal and Hematopoiectic Necrosis virus (IHHNV)	**ssDNA**	22
Hepatopancreatic Parvovirus (HPV)		22-24
Lymphoidal Parvo-like Virus (LPV)		25-30
Baculoviruses		
Baculovirus Penaeid Virus (BP)		55-75 x ~ 300
Baculovirus Mid-gut gland Necrosis Virus (BMNV)	**dsDNA**	~75 x 300
Monodon Baculovirus (MBV)		~75 x 300
Nimaviruses		
White Spot Syndrome Virus (WSSV)		~80 x 270
Iridovirus		
Shrimp Iridovirus (IRDO)		136
Large Baculo-like Virus Hemocyte Infecting Nonoccluded Baculovirus (PHRV)		90 x 640
RNA Viruses		
Picornavirus		30-32
Taura Syndrome Virus (TSV)		
Nidovirus	**ssRNA**	30-50 x 150-170
Yellow Head Virus (YHV)		
Gill-Associated Virus (GAV)		
Toga-like virus		30-55
Lymphoid Organ Vacuolization Virus (LOVV)		70 x 125
Rhabdovirus		
Rhabdovirus of Penaeid Shrimp (RSP)		
Reovirus	**dsRNA**	50-70
Reo-like Virus (REO) type III-IV		

2.5. Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV)

Infectious hypodermal and hematopoietic necrosis virus (IHHNV) is the smallest of known penaeid shrimp viruses which can infect many species of penaeid shrimp including *P. vannamei, P. styliristris, P. japonica and P. monodon*. It causes financially viable losses mostly in *P. styliristris, P. vannamei* farm and is derived from the icosahedral and un-enveloped and 19 to 22 nm in diameter (Lightner *et al.*, 1983b)(Figure 2.6) viron characteristics, IHHNV is classified as a member of the Family: Parvoviridae, subfamily Densovirinae, Genus Brevidensovirus or Contravirus (Shike *et al.*, 2000).

IHHNV is an ssDNA virus with a genome of approximately 4 kb. It is produced in the host cell nucleus and total concentration is often found in the cytoplasm of cells of infected shrimp also membrane-bound inclusions, possibly shows phagosomes existence (Bonami *et al.*, 1990).

Almost 100% of the IHHNV genome was sequenced and its 3 large open reading frames (Mid ORF, Left ORF and Right ORF ORF1, 2, 3) have been determined (Nunan *et al.*, 2000),(Shike *et al.*, 2000). Mid ORF cover 1,092 nucleotides, which is equivalent to nt 760-1,891 encoding an unknown protein(s). Left ORF covers 2001 nucleotides, which is equivalent to nucleotides 816-2,816 encoding non-structural protein. Right ORF covers 990 nucleotides, which is equivalent to nucleotides 2,758-3,747 encoding structural protein (GenBank AF218266) or viral capsid polypeptides (VP). The left ORF or ORF1 transcribes non-structural protein-1 (NS-1), which is known to have pleiotropic function, including an essential role in parvoviral DNA replication (Bell *et al.*, 1984)(Figure 2.7).

Non-structural protein-1 was found to be involved in DNA packing in vertebrate parvovirus (Susan and attersall, 1989). It also enhances capsid gene expression and is

responsible for cytotoxic activity, oncolysis and stimulation of apoptosis (Cotmore and Tattersall, 1987). The position of the highly conserved amino acid sequences in NS-1 homologues varies significantly among parvoviruses. ORF1 represents approximately 50% of the genome and may encode a 666 amino acid polypeptide (Vanacker and Rommelaere, 1995). The mid ORF or ORF2 starts downstream from the beginning of the left ORF and terminates with a TAG, which is upstream of the termination codon of the left ORF. Thus, the mid ORF is located completely within the left ORF but in a different reading frame. The potential function of this protein is unknown (Shike *et al.*, 2000).

The function of mid ORF which may encode the non-structural protein-2 is unidentified. It could encode a 434 amino acid polypeptide. The right ORF or ORF3 which is in the same reading frame as the mid ORF starts before the stop codon of the left ORF and the right ORF overlaps in their 3'and 5'extremities, respectively. By analogy of other parvoviruses, the right ORF of IHHNV is presumed to encode the capsid protein which could encode a 329 amino acid polypeptide. Computer analysis of amino acid sequence of IHHNV right ORF and database entries was not able to reveal any significant homology. Given the lack of homology in capsid protein among the diverse densoviruses is not surprising. The base composition of viral strand of the IHHNV genome is 20.68% A, 19.00% C, 24.04% G and 36.28% T. Thus, the A+T content are 56.96% and the G+C content is 43.04%. Using SDS-PAGE analysis, the structural proteins of the virus are composed of four polypeptides of 74, 47, 39, and 37.5 kDa, that make up its nucleocapsid. Isolated viral DNA from purified IHHNV is composed of 85% negative and 15% positive polarity (Regenmortel, 2000). After DNA extraction, the complementary strands may hybridize in vitro to form double stranded DNA.

IHHNV belongs to the Genus Brevidensovirus from family of Parvoviridae and Brevidensovirus encapsidate Infectious particles, positive and negative strands, a majority of which are of negative polarity (85%) (Regenmortel, 2000). The virion contains no viral or cellular enzymes. These DNAs also carry complementary palindromic sequences favoring Y-shaped hairpins at both ends (Fig. 6). IHHNV may have both a 5' and 3' hairpin(Levy *et al.*, 2004). The 3'-terminal hairpin is 115-116 nucleotides (ntss) in length, the 5' structure is 200-242 nucleotides long (Figure 2.6).

The parvovirus genome serves as cis-acting sequences necessary for replication of the viral genome (Tattersall and Ward, 1976; Samulski, 1983). Members of virus in Subfamily Densovirinae are usually highly pathogenic to their insect hosts and cause a disease known as "densonucleoses" (Vago *et al.*, 1964), which refers to histopathological and ultrastructural characteristics of hypertrophied and densely stained nuclei of sensitive cells in infected insect larvae.

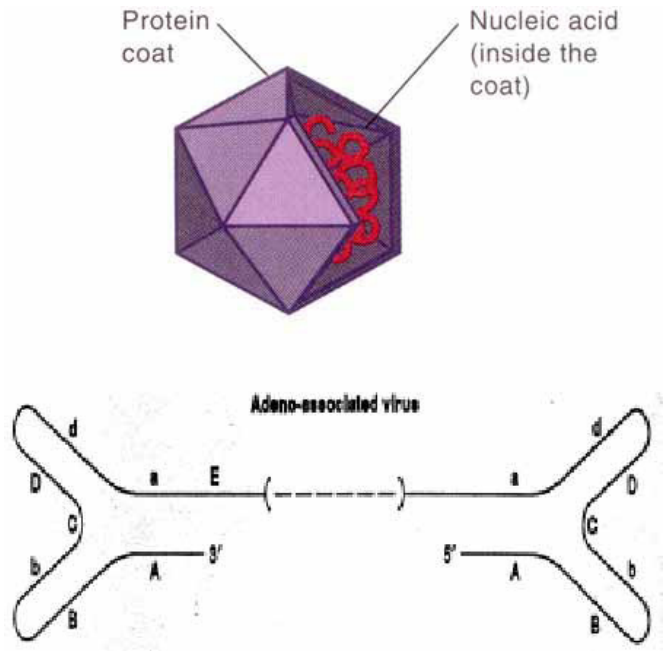

Figure 2.6, Based on virus classification IHHNV is a member of the Family Parvoviridae, Subfamily Densovirinae, and Genus Brevidensovirus Complementary palindromic sequences favoring Y-shaped hairpins at both ends are the specific structural characteristic of its DNA(Lightner, 1996a).

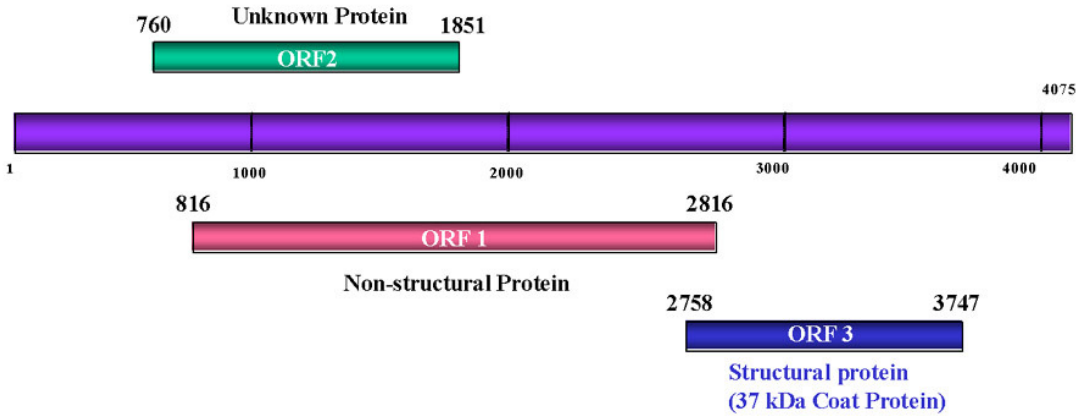

Figure 2.7, Entire IHHNV genome (GenBank, AF218266) contains 4075 bp, which is composed of ORF1 encoding non-structural proteins (816-2816 nts), OFR2 encodes unknown proteins (760-1851 nts) and ORF3 encoding structural proteins (2758-3747 nts)(Lightner, 1996a).

Previous studies proved that IHHNV can infect many species of penaeid shrimp including *P. vannamei, P. japonicas, P. stylirostris, , P. monodon, P. duorarum P. aztecus and* (Lightner, 1996a) but it causes impressive economic losses only in *P. stylirostris, P. vannamei* and mortality in postlarvae of *M. rosenbergii*.

Disease susceptibility comparison in *P. vannamei* (0% to 100% incidence) was infected at remarkably lower levels than *P. vannamei, P. stylirostris* and *P. stylirostris* (67% to 100% incidence) after 6.5 weeks (Bell *et al.*, 1984). In order to develop shrimp virus-resistant lines, Super Shrimp INC (Hartland, Michigan, US), has developed a line of *P. stylirostris* shrimp which is resistant to IHHNV. The population from Panama wild larvae was survived under IHHNV epidemic, and has undergone more than 20 generations of captive breeding. This line of prawn shows greater than 80% survival in an IHHNV-positive culture system (Califord, 1998). Super Shrimp, *P. stylirostris* postlarvae and juveniles have been shown to be resistant to IHHNV by in situ hybridization using an IHHNV-specific probe, and PCR, (Tang *et al.*, 2000). Except for, *P. monodon*, information is limited about other possible native carriers of IHHNV in other species. However, according to experience with multiple species infectivity of other prawn viruses (Lightner, 1996a), it is likely that other potential carriers do exist and may create a threat to Thai prawn farmers who rear *P. vannamei*. There is even some possibility that aquatic life stages of insects could be carriers (Roeking *et al.*, 2002). The availability of cheap and easy to use diagnostic tests will help in screening for such potential carriers.

2.6. Signs of disease in infected prawn

The reaction to IHHNV infection depends on the species and the variety of infected prawns. *P. stylirostis*, the most seriously affected species; infected prawn shows changes in behavior, off feed and outer shell. The prawn becomes restless and goes up gradually to the water surface, spin over and sink with the ventral side up. The ceases activity of the pleopods and peripods causes the animal to sink to the pool bottom. In laboratory tanks, prawn on the tank bottom continues to be inactive and stops feeding, and repeats the process of going up to the surface and sinking till it dies, usually during 4 to 12 hours. The prawn regularly has white spots in the cuticular epidermis particularly at the junction of the targal plates of the abdomen in this sensitive stage. Although, mottled appearance and strongly thick musculature seems normal in the infected prawn's body. During or following molt (in stage A, B or D4), most affected prawns die. Some prawns that survive the acute phase recover slowly. These animals are inactive, show incompatible stress resistance, do not molt frequently and do not grow properly as normal for their stage. (Lightner *et al.*, 1983a).

Having a very soft cuticle in general body, appendages and gills due to multifocal melanization in this stage is one of the special characteristics of the prawn. These signs are summarized in Table 1. However, resistance to IHHNV was demonstrated in lines of *L. stylirostris* as marked Super Shrimp TM in the 1990's (Tang *et al.*, 2000). The juvenile prawn with runt deformity syndrome (RDS) display markedly reduced growth rates, deformed rostrums, wrinkled antennal flagella, cuticular roughness, and other cuticular deformities. However, gross signs of IHHNV infection in this species are usually nonspecific. Only severely stressed juvenile *L. vannamei* displays clinical IHHNV disease, and under most aquaculture conditions this species seems to carry the virus without any

apparent ill effect (Bell *et al.*, 1984). However, in farms some subpopulations of IHHNV-infected *L. vannamei* appear to grow normally while others remain small. In *M.rosenbergii,* atrophic changes in abdominal muscles from the fourth to sixth segment and tail fan, associated with a reddish discoloration, were prominent features in this prawn, but there was no unusual mortality or intranuclear inclusion bodies formation in IHHNV infections (Chia *et al.*, 2006).

Comparison done about IHHNV infection severity between small and large prawns showed that small prawns had a higher IHHNV prevalence (100%) than their larger cohorts (70%). Often, ranted prawns were shorter with laterally or ventrally deviated rostra, with deformed ("crinkled") antennal flagella of reduced-length and/or nonsymmetrical, misshapen areas of the cuticle (Kalagayan *et al.*, 1991; Lightner, 1996b). The mean IHHNV severity indices for the six organ systems examined histologicaly were significantly different ($P<0.01$) between small and large cohorts. Cow dry A inclusions (CAIs) were found in the ventral nerve cords, antennal glands, epidermis (cuticle and gills) and hematopoietic organs of prawn in both size groups. CAIs were not observed in the lymphoid organ or the epicardium of the heart (Kalagayan *et al.*, 1991).

IHHNV was originally reported as a highly lethal disease of juvenile *P. stylirostris* (mortality rates of 90%), with impaired growth and cuticle deformities (Bell *et al.*, 1984; Kalagayan *et al.*, 1991). However, an epidemiological study of wild *P. stylirostris* in 1998 in the northern Gulf of California, using histopathology and dot-blot hybridization, revealed the prevalence rate of 80-100% in female and 60% in male. Such studies have not been done on *M.rosenberhii* yet, but according to 2006 report (Chia *et al.*, 2006) ,80% mortality in *M.rosenbergii* postlarve drew researchers attention to study about it.

The prevalence of IHHNV infection in unfertilized eggs was 100% and in sperm was 60% in *P. stylirostris* and 100% in swimming leg in *M.rosenbergii* (Hazreen Nita *et al*., 2011).

However, wild adult *P. stylirostris* that were infected with IHHNV did not show clinical signs of the disease although they contained 6 x 10^8 single-stranded genome equivalents per gram of tissue (Morales-Covarrubias *et al.*, 1999), infected *M.rosenbergii*, also didn't show significant clinical sign of the disease. It is not known whether the reduced virulence is due to mutations in the viral genome, introduction of a new but less virulent strain, or an increased resistance of the host.

In IHHNV-infected *P. monodon*, it was reported that the prawns are often distinctly bluish in color, with opaque abdominal musculature (Lightner *et al.*, 1983b) and, as reported in the Philippines, also being stunted and deformed, with defective sperm quality (Primavera and Quinitio, 2000). Different responses to IHHNV in *P.monodon* and *P. vannamei* (Withyachumnarnkul *et al.*, 2006) and M.rosenbergii (Chia *et al.*, 2006) proves the lack of our knowledge to study IHHNV's affect on prawn immune response and much more studies are needed.

Histopathology cells that are infected by IHHNV usually display eosinophilic inclusions inside hypertrophied nuclei that contain marginated chromatin. Such inclusions are termed Cowdry Type-A Inclusions (CAI). The trophic tissues are of ectodermal origin, e.g., gills, sub-cuticular epidermis (including that of the fore-gut and hind-gut), nerve cord, nerve ganglia, and antenules; and of mesodermal origin, e.g., hematopoietic tissue, gonads, lymphoid organ, connective tissue and striated muscle, heart, mandibular organ, hemocytes

(Bell *et al.*, 1984). These ectodermally and mesodermally derived tissues might undergo necrosis. (Figure 2.8)

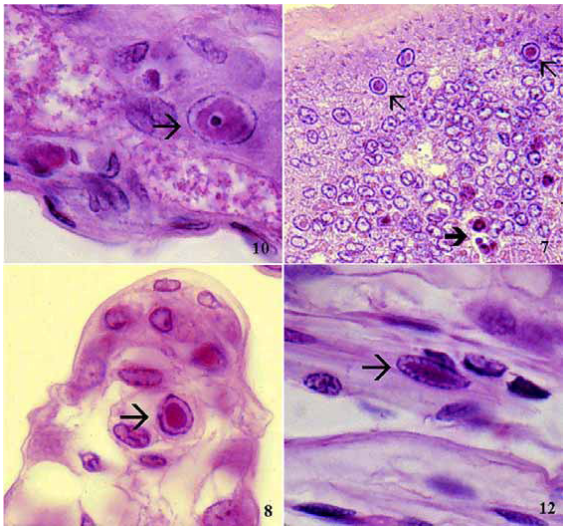

Figure 2.8, Characteristic of Cowdry Type-A Inclusions (CAI) observed in IHHNVinfected cells of the prawn(Lightner, 1996a)

2.7. Effects of IHHNV on Prawn Culture

IHHNV is very pathogenic for *P. stylirostris* while infection of *P. vannamei* results in development and growth abnormalities (Kalagayan *et al.*, 1991) and causes mortality in *M.rosenbergii* (Chia *et al.*, 2006). Size reduction and disparity have a significant impact on *P. vannamei* production with economic losses that range between 10% and 50% by comparison with IHHNV-free crops (Lightner *et al.*, 1983a). There is little information concerning the effect of virus infection on prawn immunology and physiology, in particular related to reproduction and embryo development (Withyachumnarnkul *et al.*, 2006). Transmission of IHHNV has been performed experimentally by the injection of viral suspension, by ingestion of infected material and by immersion in contaminated water (Bell *et al.*, 1984; Lotz, 1997).

Vertical transmission may be a crucial factor for the increase of IHHNV prevalence in domesticated prawns from generation to generation. It is believed that broodstock give rise to larvae that have been infected through vertical transmission and that there is a subsequent increase of prevalence through horizontal transmission in hatcheries and ponds. Since the majority of infected prawn survive (except *P. stylirostris*) without clinical signs, many prawns selected for broodstock are effectively healthy carriers of IHHNV that can transmit it vertically (Lotz, 1997).

Because of the negative impact of IHHNV on prawn production of *P. vannamei*, prevention of this viral disease was a chief priority for prawn farming in Latin America. Consequently, it was important, first, to demonstrate experimentally the vertical transmission of IHHNV through the egg and, later, to establish if the identification of virus-free females through nested PCR would prevent the vertical transmission of IHHNV.

IHHNV-free nauplii and larvae were produced from females and males that were found to be free of virus on the basis of nested PCR performed on females after eyestalk ablation and after the first spawning. The reliability of this testing process was shown to be very high since about 87% of females were confirmed IHHNV-free through a second nested-PCR analysis performed after some additional spawning.

The nauplius productivity was higher for IHHNV-free females than for infected ones. Mass mortality of *P. stylirostris* in the acute infection phase can reach approximately 90% within 20 days. Sub-acute infections do not cause mass mortality but inhibit feeding and lead to poor growth rate. Consequently, there is a negatively effect on size and yield of farmed prawn and this can lead to economic loss (Carpenter and Brock, 1992). IHHNV can be transferred to healthy prawn by direct contact with contaminated water or from parents (Bell *et al.*, 1984). Therefore, prevention is the top priority in control strategies. In addition, stocking at low density in nurseries and ponds can help in slowing the spread of the virus, if it is present (Jimenez *et al.*, 1999).

In short, IHHNV may be a serious disease for wild prawn in addition to aquaculture prawn. Formerly, IHHNV was not a virus of concern in Thailand because of its lack of negative effects on the culture of *P. monodon* (Flegel, 1997; Chayaburakul *et al.*, 2005; Withyachumnarnkul *et al.*, 2006). However, the recent introduction and dominant production of the susceptible exotic prawn species *P. vannamei* means that the disease has become an issue of major concern for Thai prawn farmers. The reports from Malaysia by (Hazreen Nita *et al.*, 2011) shows that this virus is becoming important in Malaysiasince, a lot of mortality happened with IHHNV affect on *M.rosenbergii's* culturs.In order to assist in implementation of control measures, cheap, rapid and effective diagnostic tools are needed.

2.8. Antiviral Immune Responses in Crustacean

Viral components like genomic DNA and RNA or dsRNA generated in virally infected cells can be sensed by host pattern-recognition receptors (PRRs). After recognition, PRRs trigger effective and appropriate antiviral responses, including production of various cytokines and induction of inflammatory and adaptive immune reactions (Kawai and Akira, 2006).

The molecular mechanisms that underlie the majority of crustacean antiviral immune responses are still unknown and are only starting to be addressed (Yeh *et al.*, 2009). So far, few host cellular genes/proteins involved in TSV and YHV pathogenesis have been studied. An increasing number of immune responsive genes/ proteins involved in WSSV pathogenesis have been described in prawn and crayfish. also the major progresses made in anti-WSSV response in prawn, crayfish, and other crustaceans is summarized (Zhao *et al.*, 2007) but still no studies have been done on anti-viruses responses in *M. rosenbergii* specially in anti-IHHNV in this species.

Recent studies have demonstrated that the viral disease progression can be blocked by injecting prawn with dsRNA/siRNA specific to viral genes. This strategy is effective against three unrelated viruses: WSSV, TSV and YHV (Obalino *et al.*, 2004). The mechanism for this phenomenon is still not clear. In these studies, positive strand RNA virus (e.g. TSV and YHV) and DNA virus (e.g.WSSV) often induce the formation of dsRNAs during their infectious cycles such as genomic replicative intermediates, intramolecular interactions within viral transcripts, and bi-directional transcription (Weber *et al.*, 2006) (Figure 2.9).

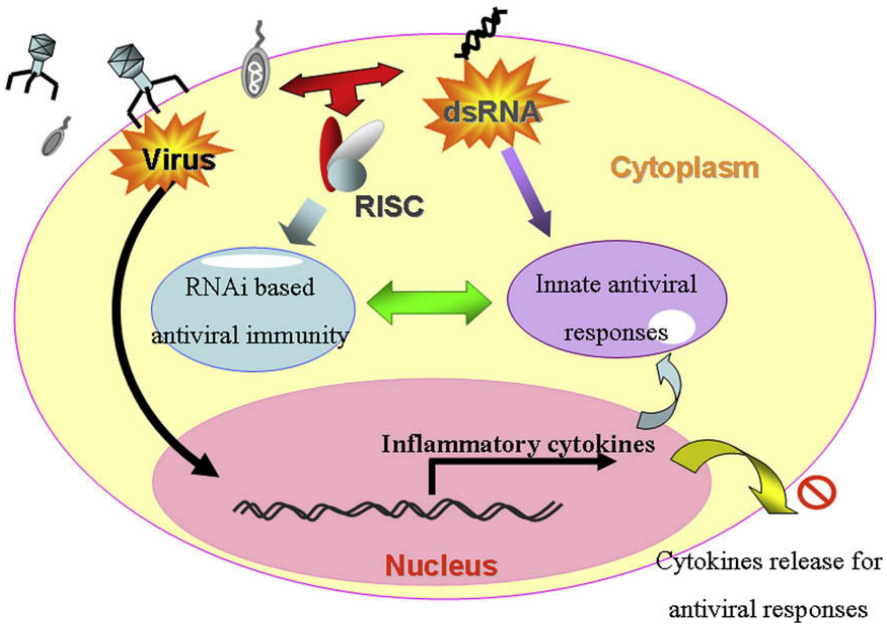

Figure 2.9, A hypothetical scheme for dsRNA-induced antiviral immunity in crustacean. Viral dsRNA or extracellular dsRNA enters the RNAi pathway, and/or activates innate antiviral responses. Signal transduction triggered by dsRNA detection may control transcriptomic expression leading to antiviral reactions. These pathways may function separately and interact together to process the antiviral responses(Liu *et al.*, 2009).

2.9. Antiviral-related Proteins/Genes in Crustaceans

To find antiviral substances, the substances have been isolated from tissue extracts of prawn. These extracts can bind to various DNA and RNA viruses such as Sindbis virus, vaccinia virus, vesicular stomatitis virus, mengovirus, Banzi virus, and poliomyelitis virus (Lan *et al.*, 2006), However there is not any particular information available for IHHNV under the mechanism of this inhibitory activity remains unclear (Pan *et al.*, 2000). Recently, many studies using different techniques have been carried out on host-WSSV interactions in crustaceans (Liu *et al.*, 2006).

It has been reported that virus-inhibiting proteins could be produced and some genes were up-regulated upon viral infection in crustaceans (Dhar *et al.*, 2003). Genes induced by viral infections and genes whose expression are associated with the ability of prawn to survive from viral infections have been reported but their importance to produce antiviral substances is little known (Rojtinnakorn *et al.*, 2002).

Most of the studies were conducted on antiviral activity of prawn in WSSV and its proteins have been studied as possible immune therapeutic agents to prevent infections (Yeh *et al.*, 2009). Different protein/DNA vaccinations against WSSV infection were reported for the protection of prawn/crayfish (Kumar *et al.*, 2008)(Figure 2.10).

Unfortunately, no studies have been done on antiviral activity of *M.rosenbergii* under IHHNV infections and no proteins have been reported as immune therapeutic agent to prevent infections.

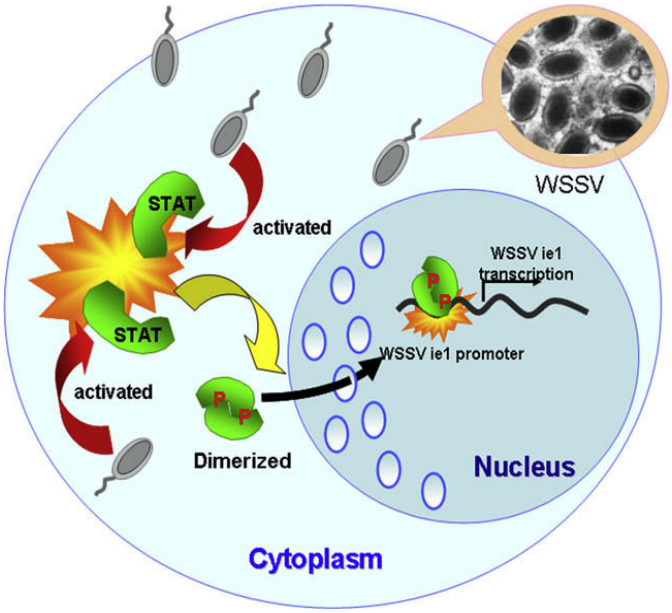

Figure 2.10, White Spot Syndrome Virus interaction model suggests that the host STAT can be activated after WSSV attack (Liu *et al.*, 2009; Liu *et al.*, 2007). Activated STAT will dimerize and be phosphorylated, which then enters the nucleus and activates the promoter of WSSV for viral transcription. (Arbouzova *et al.*, 2006)

Invertebrates only have an innate immune system which is the first line of defense against infections. It is activated by host proteins that recognize conserved surface determinants of pathogens., such as lipopolysaccharide (LPS), lipoteichoic acid (LTA) and peptidoglaycan (PGN) from bacteria and ß-1,3-glucans from fungi (Medzhitov and Janeway 2002) but still not found from other invertebrates. Both cellular and humoral immune responses are activated to eliminate pathogens (Imler and Hoffmann, 2000).

Antimicrobial peptides and other immune-related proteins are synthesized within the hemocytes and secreted into the hemolynph. Both mRNA-based and proteomic approaches should be combined to decipher the complexity of innate immunity (Levy *et al.*, 2004). However proteomic analysis allows the investigation of innate immunity at the protein level, the real mediators of physiological function. Moreover, genomic approaches cannot

be used for fluids such as hymolymph. As a global reference for proteomic approaches, proteins in the hemolymph of Drosophila Melangaster have been extensively researched. The second dimension (2D) map of hemolymph proteins of Drosophila was constructed to serve as reference database for researchers investigating the changes that occur at protein level in different developmental stages, physiological conditions or after infection (Vierstraete. *et al.*, 2003).

Between July 2004 and January 2005, high mortalities (up to 80-100%) were frequently encountered in postlarvae of *M. rosenbergii* in southern Taiwan. Pathologically, eosinophilic intranuclear inclusion bodies (INIs) were found only in the hepatopancreatic tubular epithelial cells of the infected postlarvae from hatchery farms. No lesions could be detected in tissue of ectodermal or mesodermal origin.

Interestingly, different lesions were found in sub-adults collected from a grow-out farm. Atrophic changes in abdominal muscles from the fourth to sixth segment and tail fan, associated with a reddish discoloration, were prominent features in this prawn, but there was no unusual mortality or intranuclear inclusion bodies formation (Chia *et al.*, 2006). A report on observed mortality in postlarve of freshwater prawn by(Hazreen Nita *et al.*, 2011), prompted the study on proteomics for *M.rosenbergii's* IHHNV infected and non-infected hemolymph.

In PCR assays for the detection of IHHNV, positive reactions using specific primers, confirmed IHHNV as the causative agent. Based on some studies, IHHNV seems to be related to outbreaks in Malaysia. To the best of our knowledge, indications of IHHNV have not been described in *M.rosenbergii* yet. The purpose of this study was to report on natural

infections of IHHNV in the postlarvae *M.rosenbergii* by comparing protein profiling of IHHNV infected and non- infected wild prawns.

2.10. Proteomics Analysis

The word “proteome” was firstly proposed by Marc Wilkins as the term resulted from protein and genome words combination. It was relevant to the total proteins expressed by a genome (Wilkins *et al.*, 1996). Proteomics, is a new field of research of the total proteins derived from the genome and is oriented towards analysis of the presence of each protein and its relative abundance in the cells, organisms, or tissues by measuring the expression level (Wilkins *et al.*, 1996). Consequently, it was more particularly able to describ that which proteins would be expressed at a certain time and under certain cell circumstances from entire genome (Nägele *et al.*, 2004).

By contrast, the inherited information would be describes through genome analysis (static state) and it’s totally different from proteome analysis that elucidate according to cells and tissues in different situations at defined times and under specific conditions (dynamic states). The important perspectives of this new technology is to offer opportunities in biomedical science to investigate the cells or tissues entire protein content at different physiological states, e.g. during regulation, differentiation, or at tumor development. The wide range of protemomics facilities paved the way to study the posttranslational modifications, subcellullar localization, protein-protein interaction and protein function (Panicker RC and Chattopadhaya S, 2006). It also has been used to gain a better understanding of the molecular mechanisms of disease for diagnostic purposes. Being able to do the analysis and identification of proteins produced during a particular state of disease in order to find diagnostic biomarkers is a wonderful facility in molecular science that was made possible by proteomics (Macarthur and Jacques, 2003).

The most important techniques in protein study is two-dimensional gel electrophoresis (2-DE) which is used to separate and visualize proteins, this technique is accomplished by mass spectrometry (MS) for protein identification. These powerful techniques have been continuously developed to deal with the challenges in proteomics research field. The typical flow chart of proteomic analysis is illustrated in (Figure 2.11).

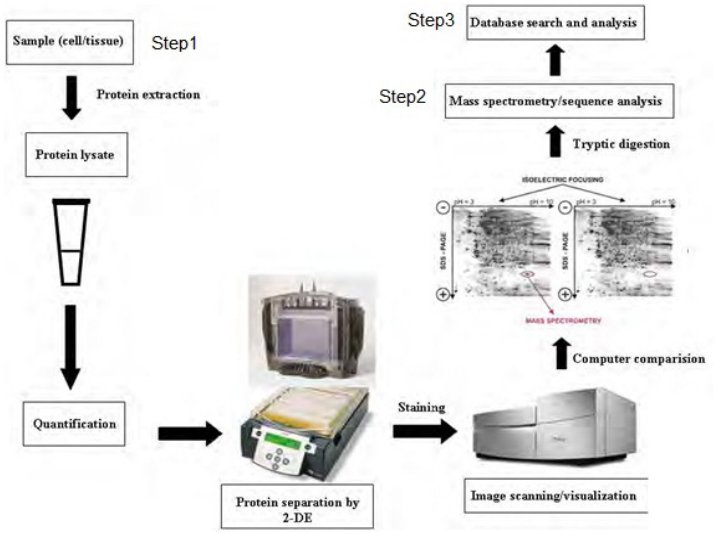

Figure 2.11, The proteomic schematic analysis from step1: sample preparation to 2- DE, step 2: mass spectrometry and step3: database searching (Walker, 2002)

2.11. Two-Dimensional Gel Electrophoresis (2-DE)

Proteomic analysis relies on the separation and visualization of the complex protein extracted mixtures from tissues, cells, or other biological samples in proteomic studies. Two-dimensional gel electrophoresis (2-DE) technique which was firstly introduced worldwide in the mid 1970's by two pioneers, O' Farrell (O'Farrell PH, 1975) and Klose (Klose, 1975) contains two steps; the first step is the first-dimension isoelectric focusing (IEF) that separates proteins according to their isoelectric points (pI) and is accomplished by second-dimension SDS-polyacrylamide gel electrophoresis (SDS-PAGE) that separates proteins according to their molecular weights (MW). Therefore, 2-DE has the ability to resolve protein mixtures according to two independent factors (MW and pI) by mixing two different steps.

To prepare samples for 2-DE the proteins are solubilized in a high concentration of urea buffer, a reducing agent (proportional to sample) and a proper no-interferring detergent. These chemicals help with sample denaturation, protein reduction and prevention of protein aggregation or precipitation.

During IEF stage, protein mixtures must be solubilized in high concentrated urea solution as denaturing buffer [(8M Urea or 7M urea with 2M Thiourea)]. This buffer contains chaotrophs(CHAPS) as surfactants and (DTT) as reducing agents. To gain better result this buffer does not contain non-ionic detergents.

Immobiline Drystrip with pH gradient is used to absorb and immobilize the solubilized sample on a gel strip. An electrical potential divergence is applied across two electrodes of the gel strip. Each individual protein will migrate under the applied electrical field until it arrives at the point which is equal to its pI. At this stage, the protein will have

no net charge and will stop migration and hence accumulate into a specific IEF band (Garfin, 2005). Since different types of samples have different ion content, adjusting the IEF buffer, electrical profile and the length of the suitable dry strip gel is necessary as well as optimizing isoelectric focusing pH gradients to each type of sample (Görg *et al.*, 2002) to obtain high quality data. Since any problems at this stage will influence all stages of the experiment, IEF step is considered as the most critical step of the 2-DE process.

After first-dimension step and complete separation base on pH by IEF, the IPG strip needs to be prepared for transferring the protein to SDS-PAGE. To provide the best transferring condition, IPG strip needs to be saturated with the SDS buffer using equilibration step.

After equilibration system, the strip is subjectd to the second dimension SDS-PAGE system that separates proteins in accordance to their molecular weight (MW).

The SDS-PAGE gel must be prepared flat on top to have good contact between the IPG strip and the SDS-PAGE gel. The secondary separation resolution can be optimized by varying of the acrylamide gel percentage according to the protein sample concentration.

Combination of first-dimension IEF and second dimension SDS-PAGE techniques in 2-DE allows efficient separation of protein mixtures into individual proteins. Nowadays highly technical improvements are available to achieve high quality of reproducible 2- DE gels to prepare experimental gels for profile comparison. To compare 2-DE gels, protein spots cannot be visualized by eye, therefore accurate spot excision is not possible.

To have accurate comparison between experimental 2-DE gels, it is necessary to visualize proteins, so staining or labeling methods are applied (chemical or radioactive) to protein detection. Several methods for protein detection, quantification and visualization

on 2-DE gel have been developed, each of these methods have its advantages and technical problems (Switzer *et al.*, 1979). The two main conventional and classical staining methods are Silver and Coomassie Brilliant Blue. In Coomassie Brilliant Blue staining, CBB G-250 is used for protein detection in conventional SDS-PAGE. It is generally used in the experiments that about 30-50 ng protein sensitivity detection is required. (Candiano *et al.*, 2004). Silver staining is more sensitive and compatible than CBB G-250 that makes it a better choice for further mass spectrometry analysis (Shevchenko *et al.*, 1996; Görg *et al.*, 2002). Difficultly in spot matching and quantification is the main problem with these two conventional methods, so, high sensitivity fluorescent staining has been developed as an alternative choice of staining but fluorescent staining has also some difficulties such as; it is more expensive than conventional CBB and silver staining and requires specific gel documentation instrumentation (Shevchenko *et al.*, 1996). To reduce expenses of staining some Commercial dyes are available that include 5- hexadecanoylamino-fluorescein and SyproRuby™, deep Purple™ (Steinberg *et al.*, 2000).

After staining the image scanning showed protein spots in 2-DE gels that revealed different thousands spots which shows complex mixtures of protein. This image also can determine protein spots concentration and expression level.

The resulting "protein profile maps image" can be compared between experimental and control samples to identify differentially expressed proteins between two samples using image processing software. Some proteins would be chosen according to the project purpose.

The next step is to identify the excision cut protein spot from SDS-PAGE gel through in-gel digestion and mass spectrometry techniques. Recently, mass spectrometry (MS) has

been introduced as an capable tool which facilitates for sensitive protein identification and proteomic analysis (Yates, 1998). In this method proteolytically digested proteins are used to achieve higher protein identification analysis accuracy (Rosenfield *et al.*, 1992). Proteins proteolytic digestion is often carried out in the gel plaque so, it is called 'in-gel digestion'.

Proteolysis is achieved using general enzymes such as trypsin, proteinase K and pepsin. Also some chemical digestion reagents are available for protein digestion such as cyanogenbromide (CNBr).

The identification procedure begins with cutting out the protein spot from the 2- DE gel then the staining chemical would be removed by using a suitable de-staining method, shrivel up the gel pieces and apply protease treatment (Rosenfield *et al.*, 1992). Among the proteolytic enzyme trypsin is the most common one in-gel digestion. Because of its special characteristic, it is able to hydrolyze peptide bonds on arginine residues and on the C terminal side of lysine while other enzyms such as proteinase K, pepsin and even CNBr do not have that much accuracy. Also the use of CNBr cause high yields of large peptide fragments which cause cleaves proteins at methionine residue, these chemical phenomena make it useless for peptide sequencing by Mass spectrometry.

The obtained eluted peptides from the gel are subjected to MS analysis. The basic modules of mass spectrometers are composed of three important parts; Ion production, resolving the ion, ion detection. Ion would be produced from the samples through an ionization source, resolving ions is based on their mass-over-charge (m/z) ratio by mass analyzer, and ion detection is based on the ion passing from the analyzer by ion sensitive detector.

Peptides and proteins ionization is a very important step of MS analysis therefore two different techniques have been developed. One is electro spray ionization (ESI) (Fenn *et al.*, 1989). and the other is matrix-assisted laser desorption/ionization (MALDI) (Karas and Hillenkamp, 1988).

For MALDI, peptides or proteins are mixed with a matrix, deposited on a special plate and irradiated with a pulsed laser. The mixture is heated and expands then the matrix absorbs energy and the result is the ionization of the analytes and transfering into the gas phase.

For ESI, a high voltage current applied between a slim duct tube that delivers the inlet and the analytic stream of the mass spectrometer to construct the charged droplets. The charged droplets are finally released from the tip of the capillary. The formation of analytic ions in the gas phase occurs by rapid solvent evaporation and subsequent droplet fission.

Two techniques are developed for protein identification. One is Peptide mass fingerprinting (PMF). The peptide masses obtained from mass spectrometry or the tandem masses from intact proteins peptide fragmentation are then compared against theoretical tryptic peptide masses of each individual protein sequence in a protein database and the most matched sequence would confirm protein result. PMF studies by MALDI-TOF MS has been used widely in proteomics. The other one is time of flight (TOF) mass analyzer which measures the m/z ratio of a peptide ion by the time it takes to progress through a vacuum and filed-free tube to a detector. It allows for inexpensive and rapid high throughput identification. The m/z ratio of the peptide ion progresses through a vacuum tube to a detector measured by the time of flight (TOF) mass analyzer. However, there are several limitations (McDonald and Yates, 2000) for this technique.

Limitation by comparing experimental PMF data with the databases is that there is insufficient number of protein sequence. Sometimes splice edited transcripts, variants, unaccounted isoforms or other differences cause incorrect identification.

Another important limitation for MALDI-TOF is that it cannot correctly identify protein in a complex experimental sample which contains more than one protein. Whenever MALDI-TOF is failed to identify a protein, Tandem MS/MS instrument can overcome the problem by selecting a precursor parent ion or a particular ion from the mixture of ions and fragment it within a collision module or fragmentation, and therefore identify the resulting ion fragments.

In addition, peptide MS/MS spectrum contains information that can explain peptide sequence (Patterson and RH, 2003) also using MS/MS data in database search gives higher accuracy and confidence in comparison with PMF data. Additionally, MS/MS data is capable enough to search against coding sequence databases or expressed sequence tag (EST) databases.

The extracted MS/MS or MS (PMF) data can be used as the input data for Computer software analysis and Database searching to match input data to protein sequence in databases, later on the software matches the input data to a particular protein sequence and reports the most matched queries as output results. It also can determine the protein modification which is not possible to identify from the nucleotide sequence.

Several software's are well- known to analyze MS/MS or MS (PMF) data such as MASCOT, PHENYX, SEQUEST, Profound and Protein Prospector (Patricia Palagi *et al.*, 2006). They have been developed using advanced MS technology. Currently more and more internet websites are offering advice on these techniques. They also provide open

access to various software's which allow searching of the databases such as MASCOT and Protein Profound.

2.12. Proteomics Application to Study Crustacean Disease

Knowledge of the interactions between virus and host is critical to understand the pathogenesis of disease. These interactions may result in immune responses against the invader or may cause changes in the genes expression levels of favor virus replication in the host genes. Studies on prawn's antiviral response, shows that prawns responses to viral infections is not at the translational level but is highly observed at the transcriptional level. Therefore, proteomic analysis has been done on prawn's differential organ such as lymphoid organ, stomach and hemocytes to identify differentially expressed proteins in immune response to viral and bacterial pathogens.

Previously, some studies have been done on the transcription level using molecular methods such as expressed RT-PCR; sequence tags (ESTs), microarray chips, differential hybridization and suppression subtractive hybridization.

These studies focused on the most important immune organs (hemocytes and lymphoid organ). The results of these studies have provided good insights into biological defense mechanisms. The latest studies on prawn immune response have indicated that proteomic based techniques are a useful method to study immune response and prawn's immune-related proteins identification.

So far, two-dimensional gel electrophoresis (2-DE) was used in studies related to protein expression upon *Vibrio harveyi* infection in *Penaeus monodon* hemocytes (Somboonwiwat *et al.*, 2010), white spot syndrome virus (WSSV) *Litopenaeus vannamei* stomachs (Wang *et al.*, 2007), in hemocytes of *Penaeus vannamei* during Taura syndrome virus (TSV) infection (Chongsatja *et al.*, 2007), and also studies involving functional

lumphooid organ of Chinese shrimp *Fenneropenaeus chinensis* (F.chinensis) (Jinkang Zhang *et al.*, 2010).

Proteomic approach is a useful method to study prawn immunity; which involves the cellular events associated with IHHNV infection in *M.rosenbergii*. In the present study comparative proteomics approach is due to identify differentially expressed proteins in the hemocytes of *M.rosenbergii* during IHHNV infection using a gel-based proteomics approach. Identifying these proteins is an important first step toward improving our understanding of the cellular pathways that are necessary for IHHNV infection.

Proteomic analysis of prawn hemocytes is to elucidate the *M.rosenbergii* immune responses at the translational level upon IHHNV infection.