SCREENING OF INFECTIOUS HYPODERMAL AND HAEMATOPOIETIC NECROSIS VIRUS (IHHNV) INFECTIONS IN WILD FRESHWATER PRAWN, *MACROBRACHIUM ROSENBERGII*, IN MALAYSIA

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ABSTRACT

Infectious hypodermal and haematopoietic necrosis virus (IHHNV), so called Penaeus stylirostris densovirus (PstDNV) has been detected widely in penaeid culture facilities in Asia and the Americas. A study by Hsieh *et al.* (2006) reported that infection of IHHNV on sub-adult and postlarvae of the giant freshwater prawn, *Macrobrachium rosenbergii* in Southeast Taiwan caused high mortalities of up to 80 %. Eosinophilic intranuclear inclusion bodies were also found on the connective tissues between epithelial cells of the infected samples. In Malaysia, although there is no report of IHHNV infection in *M. rosenbergii*, preliminary works warrant urgent need to setup a screening protocol for IHHNV for both wild and cultured populations of the species. Studies on screening of IHHNV in giant freshwater prawn were conducted in selected hatchery and wild environment throughout 2009/2010. The purpose of this study was to determine the prevalence of IHHNV in giant freshwater prawn broodstocks in Malaysia by standard polymerase chain reaction (PCR), nested PCR, histopathology and *in situ* hybridization.

PCR screening using standard protocol from OIE was carried out in a prawn hatchery in Perak. Six out of thirty samples (20 %) of random sampling wild berried *M. rosenbergii* taken directly from Sungai Perak which used as broodstocks in the hatchery were identified as positive with IHHNV infection. Sequence alignment of these positive isolates (389-bp) (GenBank Acc. No. HM536212) in GenBank revealed close identities (98%) to Taiwanese IHHNV strains isolated from *M. rosenbergii* in Taiwan (GenBank Acc. No. DQ057983). Screening of IHHNV in wild environment were also been conducted where fifty wild-caught giant freshwater prawns were collected monthly (2009/2010) from two selective locations; Sungai Rubana (Perak), and Sungai Timun (Negeri Sembilan). The detection was conducted by nested PCR which based on the

standard PCR suggested by OIE (OIE, 2003) while for the nested step, newly designed primers were used. Through the year 2009/2010, the prawns captured from Sungai Timun and Sungai Rubana showed positive by nested PCR with prevalence of 64.4 % and 48.7 % respectively. Sequence of positive samples were aligned together with available IHHNV sequences obtained from GenBank and phylogenetic tree was generated which revealed that Malaysia isolates of *M. rosenbergii* is clustered together with Taiwanese strain (DQ057983).

Histopathology examination revealed low intensity of eosinophilic intranuclear inclusion bodies in connective tissues of epithelial cells in hepatopancreas and muscle tissue in some of the samples. An additional method, *in situ* hybridization (ISH) was also included in the study in order to confirm the findings of histopathology. However, due to lack of positive control of other prawn viruses, this method is unsuccessful to be optimized and further validation cannot be performed. Further investigation in improving this method is necessary as ISH could provide greater diagnosis sensitivity than other conventional histology methods.

Results obtained in this study revealed that IHHNV infection were common in adult prawn in both Sungai Rubana and Sungai Timun. The economic impact caused by IHHNV in wild-caught *M. rosenbergii* was unknown as all the infected samples did not show any mortality. However, the gross sign such as deformity of rostrum and pleopods were seen in the infected prawn. The present study has successfully identified the partial sequence of ORF1 IHHNV genome which leads to the early documentation on the occurrence of IHHNV infection in giant freshwater prawn in Malaysia. To date, there is no definite treatment for this disease, thus frequent screening program of viral disease in prawn should be conducted in order to control the spread of the disease.

ABSTRAK

"Infectious hypodermal and haematopoietic necrosis virus" (IHHNV), juga dikenali sebagai "penaeus stylirostris densovirus" (PstDNV) telah dikesan secara meluas di kawasan kolam ternakan udang laut di Asia dan Amerika. Kajian yang dijalankan oleh Hsieh *et al*. (2006) melaporkan bahawa jangkitan IHHNV pada induk dan rega udang galah, *Macrobrachium rosenbergii* di Tenggara Taiwan telah mengakibatkan kadar kematian yang tinggi sehingga 80 %. Jasad rangkuman "eosinophilic intranuclear" juga ditemui pada tisu penghubung antara sel-sel epitelium dalam sampel yang dijangkiti. Di Malaysia, meskipun tiada sebarang laporan mengenai jangkitan IHHNV dalam *M. rosenbergii*, kajian awal menyarankan supaya pemeriksaan penyakit khususnya IHHNV dijalankan pada populasi udang galah liar dan juga dalam kolam ternakan. Kajian mengenai pemeriksaan IHHNV dalam udang air tawar ini telah dijalankan di pusat penetasan dan di persekitaran semulajadi yang terpilih sepanjang tahun 2009/2010. Tujuan kajian ini adalah untuk menentukan kadar jangkitan IHHNV dalam induk udang galah di Malaysia dengan menggunakan kaedah tindak balas polimer berantai (PCR), nested PCR, histopatologi dan '*in situ*' hibridisasi.

Penyaringan PCR menggunakan protokol standard dari OIE telah dijalankan di pusat penetasan udang di Perak. Enam daripada 30 ekor udang galah (20 %) yang diambil secara rawak dari Sungai Perak yang digunakan sebagai induk di pusat penetasan tersebut telah dikenalpasti sebagai positif dengan jangkitan IHHNV. Susunan jujukan bagi sampel isolat positif (389-bp) (GenBank Acc No. HM536212) dalam GenBank menunjukkan identiti nukleotida adalah paling hampir (98%) kepada jujukan IHHNV Taiwan yang diperoleh daripada *M. rosenbergii* di Taiwan (GenBank Acc No. DQ057983). Saringan IHHNV di persekitaran semulajadi juga telah dijalankan di mana 50 ekor udang galah telah diambil secara rawak pada setiap bulan (2009/2010) dari dua

lokasi terpilih iaitu Sungai Rubana (Perak), dan Sungai Timun (Negeri Sembilan). Pengesanan IHHNV telah dilakukan secara nested PCR yang mengunakan primer PCR standard OIE (OIE, 2003) sebagai langkah pertama dan primer yang baru dicipta untuk amplifikasi langkah nested. Sepanjang tahun 2009/2010, melalui pengesanan IHHNV nested PCR, udang galah yang ditangkap dari Sungai Timun dan Sungai Rubana menunjukkan positif-IHHNV dengan peratusan jangkitan sebanyak 64.4 % dan 48.7 %. Jujukan positif isolat kemudiannya disusun bersama-sama dengan jujukan IHHNV yang diperoleh daripada GenBank dan 'phylogenetic tree' yang dibina menunjukkan bahawa isolat Malaysia berada dalam kelompok yang sama dengan jujukan IHHNV Taiwan (DQ057983).

Melalui kaedah histopatologi kadar intensiti jasad rangkuman "*eosinophilic intranuclear"* yang ditemui dalam tisu penghubung sel-sel epitelium dalam hepatopancreas dan tisu otot dalam beberapa sampel udang galah adalah rendah. Kaedah '*in situ*' hibridisasi (ISH) juga telah digunakan dalam kajian ini bagi mengesahkan penemuan histopatologi. Walau bagaimanapun, disebabkan oleh kekurangan kawalan positif bagi virus-virus udang yang lain, kaedah ini tidak berjaya untuk dioptimumkan dan pengesahan selanjutnya tidak dapat dilakukan. Kajian lanjutan dalam memperbaiki kaedah ini perlu dijalankan kerana ISH boleh memberikan sinsitiviti diagnosis yang lebih baik berbanding kaedah histologi yang lain.

Hasil keseluruhan kajian ini menunjukkan bahawa jangkitan IHHNV adalah fenomena biasa pada induk liar udang galah di kedua-dua Sungai Rubana dan Sungai Timun. Kesan jangkitan IHHNV pada udang galah ini tidak diketahui kerana semua sampel yang dijangkiti tidak menunjukkan sebarang kematian. Walau bagaimanapun, tanda-tanda awal seperti kecacatan pada 'rostrum' dan kaki renang (pleopod) telah

dikenalpasti dalam udang yang dijangkiti. Kajian ini juga telah berjaya mengenalpasti separa daripada jujukan ORF1 genom IHHNV yang membawa kepada dokumentasi terawal mengenai jangkitan IHHNV dalam udang air tawar di Malaysia. Sehingga kini, tiada rawatan untuk menyembuhkan penyakit udang ini, maka program pemeriksaan penyakit virus dalam udang harus dijalankan sekerap yang mungkin sebagai langkah awal mengawal penyebaran penyakit ini.

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Last but not least, I am deeply grateful to my beloved parents; Zaiton Bt. Abd. Manan and Mohd Khalid Mohd Zen, siblings and sister in-law for giving me support, attention, encouragement and love during the difficult time throughout the study.

Happy working!

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%	Percentage
^{o}C	degree celcius
ml	Milliliter
µl	Microliter
g	Gram
µg	Microgram
ng	Nanogram
µm	Micrometer
nm	Nanometer
M	Molar
mM	Milimolar
µM	Micromolar
pmol	pico-mol
µmol	micro-mol
bp	base pair
kb	kilobase pairs
Da	Dalton
kD	kiloDalton
U	Unit
rpm	rotation per minute
X	time (concentration)
ID	Identity
N	North
E	East
Sg.	Sungai
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
ss	single stranded
ORF	open reading frame
NS	non-structural protein
PCR	polymerase chain reaction
ISH	*in situ* hybridization

DIG	Digoxigenin
Anti-DIG AP	anti-digoxigenin alkaline phosphatase
NBT	nitro blue tetrazolium chloride
Taq	*Thermus aquaticus* YT-1
$MgCl_2$	magnesium chloride
dNTP	deoxynucleotide triphosphate
NaCl	sodium chloride
CsCl	cesium chloride
LB	Luria-Bertani
TE	Tris-EDTA buffer
X-Gal	5-bromo-4-chloro.3-indolyl-β-D-galactopyranoside
UV	Ultraviolet
IHHNV	infectious hypodermal and hematopoietic necrosis virus
MHPV	*Macrobrachium* hepatopancreatic parvo-like virus
WTD	White tail disease
WSBV	White spot syndrome baculovirus
MMV	*Macrobrachium* muscle virus
*Mr*NV	*Macrobrachium rosenbergii* nodavirus
XSV	Extra small virus
HPV	Hepatopancreatic parvo-like virus
MBV	*Monodon* baculovirus
WSSV	White spot syndrome virus
IMNV	Infectious myonecrosis virus
*Pst*DNV	*Penaeus stylirostris* densovirus
*Pm*DNV	*Penaeus monodon* densovirus
*Pemo*NPV	*Penaeus monodon* nucleopolyhedrovirus
SPF	Specific pathogen free
PL	Postlarvae
RDS	runt deformity syndrome
FAO	Food & Agriculture Organization
FRI	Fisheries Research Centre
OIE	World organization for Animal Health
QAAD	Quarterly Aquatic Animal Disease
LIR	Laboratory industrial resource
DS	Davidson solution

H & E	Hematoxylin and Eosin
DPX	Di-N-Butyle Phthalate in xylene; mounting medium
TNE	Tris-NaCl-EDTA buffer
SSC	saline-sodium citrate buffer
TEM	transmission electron microscope
SPA	single particle analysis
CIAs	Cowdry type A inclusion bodies
NCBI	National Center for Biotechnology Information
MEGA	Molecular Evolutionary Genetics Analysis
TCS	The method of Templeton, Crandall and Sing. A computer program to estimate gene genealogies.
ANOVA	Analysis of variance
SPSS	Statistical package for the social sciences
RM	Ringgit Malaysia

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CHAPTER 1

INTRODUCTION

Aquaculture is defined as "*the farming or rearing of aquatic organisms under controlled or semi-controlled conditions"* (Stickney, 2009). The definition can be further elaborated into three major components; (1) *Aquatic* refers to a variety of water environments such as marine, hypersaline and brackish ; (2) *aquatic organisms* refer to any living entity that lives or can live in water and (3) *controlled or semi-controlled* refer to the environment which aquaculturist use to grow one or more types of aquatic organisms that has been altered from the environment in which the species is normally found (Stickney, 2009). Today, the aquaculture industry which is known as the fastest growing sector in the world covers the production of freshwater, brackish and marine including fish farming, prawn farming, and also aquatic plants. In Malaysia, aquaculture industry has expanded rapidly and most of its production such as giant freshwater prawn, tiger shrimp, walking catfish, tilapia and river carp, is marketed locally for domestic consumption.

The giant freshwater prawn, *Macrobrachium rosenbergii* which are native to Southeast Asia, are cultured widely around the world with a global production estimated at 200,000 tonnes per year in 2008 (FAO, 2010). In Malaysia, giant freshwater prawn farming has currently become an important sector in the freshwater aquaculture industry. Although the production of the giant freshwater prawn is relatively small compared with other Asian countries, it is expanding rapidly, and can be a good alternative to tilapia *Oreochromis spp.* and tiger shrimp, *Peneaus monodon* farming as it has big commercial potential and is in good demand in the overseas market. Additionally, this species has the potential of fast growing rate, are large in size and are also tolerant to some diseases (Ranjeet *et al*., 2002).

The current trend in the aquaculture industry development is for the large-scale commercialization of aquatic production. As aquaculture activities expand, this industry has been overwhelmed with problems caused by viruses, bacteria, parasites and other emerging pathogens. In shrimp farming, viral diseases are known to be a limiting factor as they cause serious production losses during acute epizootics. Infectious disease agents can spread rapidly and caused high losses. The disease can spread more efficiently in prawn cultured with high stocking density as stress condition would trigger a disease outbreak.

The most common viruses that threaten *M. rosenbergii* culture are infectious hypodermal and haematopoietic necrosis virus (IHHNV), white tail disease (WTD), *Macrobrachium* hepatopancreatic parvo-like virus (MHPV), *Macrobrachium* muscle virus (MMV) and white spot syndrome baculovirus (WSBV). Bacterial diseases of giant freshwater prawn were commonly caused by Gram-negative bacteria within genera *Aeromonas*, *Vibrio*, and *Pseudomonas* which are associated with black spot lesion (New, 2005). Other than viruses and bacteria, fungal infection by *Lagenidium* have also been responsible for the decimation of larval populations in *M. rosenbergii* hatchery within 24 hours. In addition, the fungus *Fusarium solani* (Burns *et al.*, 1979) and *Debaryomyces hansenii* and *Metschikowia bicuspidata* (Sung *et al.*, 1998) may also invade injured prawns caused high mortality. Most reports of metazoan parasites in cultured *M. rosenbergii* concern the presence of isopods and trematode worms while the most common protozoan found in giant freshwater prawn are *Zoothamnium*, *Epistylis*, *Gregarina*, *Lagenophrys* and *Acineta* (Paul *et al.*, 2010; Kua *et al.*, 2011).

Infectious hypodermal and haematopoietic necrosis virus (IHHNV) is distributed widely in penaeid culture facilities in Asia and America since 1981(Lightner *et al.*, 1983). However, in Malaysia, there have been no reports of IHHNV infection in the giant freshwater prawn, *Macrobrachium rosenbergii*. IHHNV is one of the major causes of shrimp diseases and was listed as a significant disease by the World Organization for Animal Health (OIE). This virus was first discovered in *Penaeus (Litopenaeus) vannamei* and *P. stylirostris* cultured in the America in 1981, starting in Hawaii (Lightner *et al.*, 1983), which caused up to 90 % mortalities. Recent studies have reported the infection of IHHNV on sub-adult and postlarvae *M. rosenbergii* in Southeast Taiwan with high mortalities of up to 80 % (Hsieh *et al.*, 2006). The transmission of IHHNV is known to be rapid and efficient by cannibalism of the weak shrimp. Vertical transmission from broodstock to larvae is common and has been shown that it originate from the ovaries of infected female. IHHNV can cause runt deformity syndrome (RDS) in *P. vannamei*, which will results in cuticular deformities (particularly bent rostrums), slow growth and poor feed conversion.

Over the years, the freshwater prawn industry has relied on wild broodstocks or pond-reared broodstocks. The current need to the industry is to produce good quality domesticated broodstocks and the *M. rosenbergii* production improved exponentially due to market demands. However, there is little documentation or reports on the IHHNV infection status in *M. rosenbergii*. A preliminary screen based on histopathology of wild freshwater prawns from Negeri Sembilan (Kua, unpublished) showed some evidence of viral infection and identified the need to screen the wild giant freshwater prawn broodstock. Therefore, the aim of the study is to screen for IHHNV in giant freshwater prawn wild and cultured broodstock.

Three methods namely; polymerase chain reaction (PCR), histopathology and *in situ* hybridization (ISH) were used in detecting the presence of IHHNV virus particles. The commonly used method for detection of viral nucleic acids is through PCR which is performed based on the IHHNV non-structural protein 1 gene with an amplicon size of 389 bp using published primer sets (OIE, 2003). However in 2009, during a preliminary trial and after several experiments, we showed that there were nonspecific bands amplified in the PCR reactions which indicated that the primers were not specific. This resulted in the development of newly designed nested primers from based on IHHNV from a local *M. rosenbergii* to detect the infection.

In histopathological studies, the procedure has been done according to (Bell *et al.*, 1984). An IHHNV-infected tissue demonstrates Cowdry Type A and intranuclear inclusion bodies in the cells of the ectodermal as well as mesodermal tissues. Viral infection in prawns can be diagnosed histologically by the presence of eosinophilic Cowdry Type A and/or B intranuclear inclusion bodies stained with Haematoxylin and Eosin (H&E). The infected nuclei are enlarged with the eosinophilic inclusion bodies surrounded by marginated chromatin in both ectodermal and endodermal tissues. However, the specificity and sensitivity of this method are low as this characteristic of the inclusion bodies are common for other viral infection such as MHPV. Thus, for further confirmation, selected histology slides of suspected IHHNV-infected samples were chosen for *in situ* hybridization (ISH) using specific DNA probes for IHHNV detection. The ISH method may provide greater diagnostic sensitivity than conventional histology approaches.

The outcome of the study will further confirm the status of IHHNV infection in giant freshwater prawn in Malaysia which is currently hypothesized as endemic where it causes no disease. This information will be useful to enhance breeding programs in the hatchery as the current need for the freshwater industry in Malaysia is to produce good quality domesticated potential broodstocks. Therefore a viral screening program of the broodstocks is needed as a pre-cautionary step to minimize the risk of disease introduction into the culture system particularly at hatchery level in order to achieve mass fry productions which are specifically free from viral pathogens. The information on prevalence of this disease will give the breeders information on the health status of broodstock. With the increasing importance of *M. rosenbergii*, this practice can also be practical in producing potential specific pathogen free (SPF) broodstock generation.

Objectives of the study

The aim of the study was to obtain a baseline data the prevalence of IHHNV infection in wild *M. rosenbergii* which will be used as broodstocks for hatchery fry production and genetic improvement programs. The specific objectives of the study were as follows:-

I. To screen for infectious hypodermal and haematopoietic necrosis virus (IHHNV) infection in cultured giant freshwater prawn, *Macrobrachium rosenbergii* and to develop a specific protocol of IHHNV detection for *M. rosenbergii* (Chapter 3);

II. To screen for infectious hypodermal and haematopoietic necrosis virus (IHHNV) infection in giant freshwater prawn, *Macrobrachium rosenbergii* wild broodstock (Chapter 4).

Thesis organization

This thesis is outlined in a series of five chapters. Chapter 1 presents the general background information, justification and objectives of the study. Chapter 2 presents the overview of the general biology of the studied organism (prawn), prawn diseases, general information of the studies disease and the diagnostic method of the disease. Chapter 3 and 4 correspond to the objectives as mentioned above while Chapter 5 presents a summary of the study and conclusions drawn from the results presented in previous objective chapters. Last but not least, Chapter 6 presents the future planning of the study and recommendation to improve studies. The following are related papers that have been published or submitted for journal publications.

1. **Hazreen Nita, M. K**., B. C. Kua, S. Bhassu and R. Y. Othman.(2011) Detection and genetic profiling of infectious hypodermal and haematopoietic necrosis virus (IHHNV) infections in wild berried freshwater prawn, *Macrobrachium rosenbergii* collected for hatchery production. Molecular Biology Reports.39:3785-3790. *Paper presented as oral presentation in Asian Pacific Aquaculture on 3-6 November 2009, Kuala Lumpur.*

LITERATURE REVIEW

2.1 Giant freshwater prawn

Giant freshwater prawn, *Macrobrachium rosenbergii***,** which are also known as "*udang galah*" locally, is the most favoured crustacean farmed in freshwater environment and are cultured extensively throughout Southeast Asia. The world production of *M. rosenbergii* is reported at over 200,000 tonnes by 2008 (FAO, 2010) (Figure 2.1) and the major freshwater prawn producer countries are China, Thailand, Taiwan, Malaysia, Brazil, India and Vietnam.

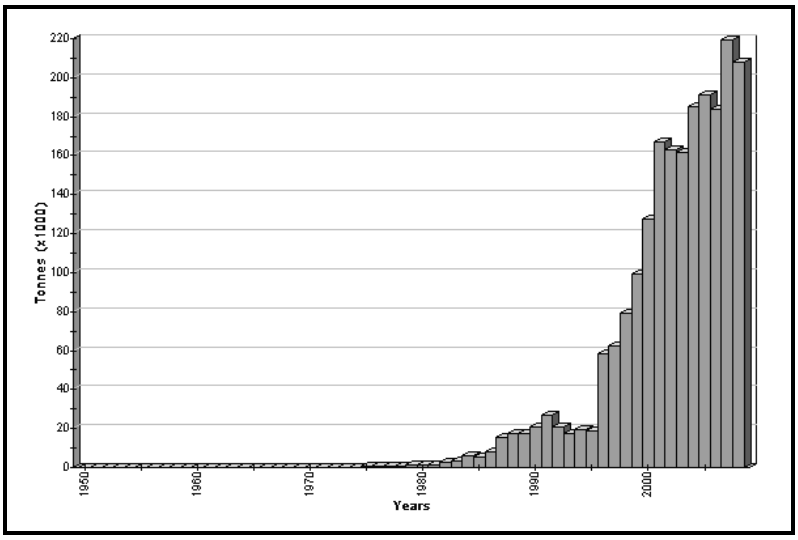


Figure 2.1 Global aquaculture production of *Macrobrachium rosenbergii* (FAO Fishery Statistic) (www.fao.org)

The modern aquaculture of this crustacean began in early 1960's when the Food and Agriculture Organization (FAO) expert, Shao-Wen Ling discovered that the larval of this species required brackish water for survival. Through trial and error of addition of salt, he discovered a key to larval survival and this led to the rearing of larvae through their developmental stages (Ling *et al.*, 1961). In 1965, with this fundamental information, Takuji Fujimura and his research team in Honolulu had successfully

commercialized the culture of giant freshwater prawn with the introduction of broodstock from Malaysia (Ling *et al.*, 1979) . Today, *M. rosenbergii* farming is popular all over the world and has become a major contributor to global aquaculture production.

In their native land of Southeast Asia, this giant freshwater prawn has evolved to survive in the brackish water of the estuaries and the freshwater rivers. This species is often found in extremely turbid conditions. The prawn requires brackish water during its larvae developmental stages and later will adapt to freshwater in the river. Mating occurs throughout the year under natural conditions. The female would migrate downstream into estuaries, where eggs hatch as free-swimming larvae in brackish-water. The plank-tonic larvae pass through several stages before metamorphosis into postlarvae (PL). The larvae usually consume zooplankton, mainly crustaceans, and very small worms. Postlarvae and adults of this species are omnivorous, which consume algae, aquatic plants, mollusks, aquatic insects and worms. In hatchery conditions, the female of the giant freshwater prawn may lay eggs three to four times per year and fully mature female prawns lay between 80,000 and 100,000 eggs during one spawning (New, 2005).

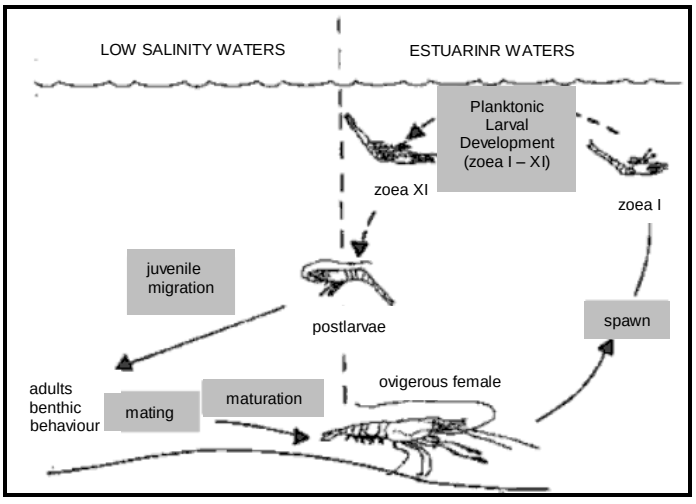


Figure 2.2 Illustration of the life-cycle of giant freshwater prawn, *Macrobrachium rosenbergii* (New, 2005).

Fertilization takes place between a molted female (soft shell) and a hard-shell male where the male deposits sperm contained in gelatinous mass between the walking legs of the female (New, 1990). After a few hours, the fertilized eggs are laid and are incubated at the underside of female abdomen. Once the eggs hatch, the larvae are dispersed by rapid movement of the abdominal appendages of the female prawn. In natural condition, the larvae swim with the tail and ventral side upside down (New, 2005). They eat continuously with variety of feed such as artemia. After 11 molting processes in approximately 10 days, the larvae will complete their larval life into post-larval stage. At this stage, the postlarvae can tolerate a wide range of salinities. After 30 days being cultured in the nursery, the prawns are ready to be released in ponds. The giant freshwater prawns are cultured for between six to 10 months and harvested at different sizes based on the market demands. However, in Malaysia the culture period is generally between three to six months.

2.1.1 Nomenclature and taxonomy

The giant freshwater prawn that has been used in culture belongs to the largest genus of the family Palaemonidae. This crustacean is a member of the phylum Arthropoda and they are decapod crustaceans related to crabs and marine shrimp and are the largest known palaemonid in the world.

M. rosenbergii was one of the first species to become known scientifically in 1705. The first recognizable illustration was published in 'D'Amboinsche Rariteitkamer" by Rumphius (reference in New, 2005). In the past, the nomenclature of this species was in a confusing state both on the generic and species level. The previous names of *M. rosenbergii* have included *Palaemon dacqueti*, *P. carcinus* and

P.rosenbergii and in 1959 its present scientific name which is *Macrobrachium rosenbergii* (De Man, 1879) became accepted.

Class: Crustacea

Order: Decapoda

Family: Palaemonidae

Genus: Macrobrachium

Species: *Macrobrachium rosenbergii*

Currently, *M. rosenbergii* has been described in two forms which are western and eastern sub-species based on their external morphology (De Man, 1879). The taxonomists refer to the western form as *Macrobrachium rosenbergii dacqueti* which are found in the water of the east coast of India, Gulf of Thailand, Malaysia and the Indonesia region of Java and Kalimantan. Another form is called the eastern form which refers as *Macrobrachium rosenbergii rosenbergii* (De Man, 1879) and inhabits the Philippines, northern Australia, and the Indonesian regions of Sulawesi and Irian Jaya.

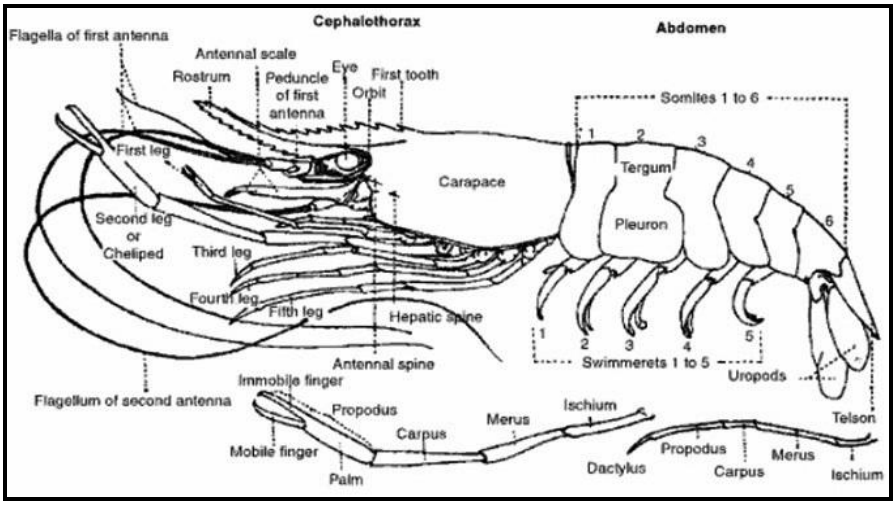


Figure 2.3 The external features of *M. rosenbergii* (De Man). (Source: New 2005)

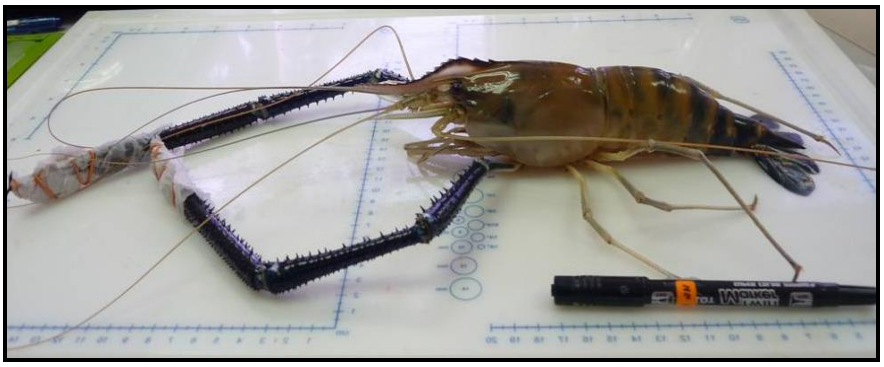


Figure 2.4 The Malaysian giant freshwater prawn, *M. rosenbergii.*

2.2 *Macrobrachium rosenbergii* diseases

Disease is known to be one of the major constraints limiting prawn production all over the world. In the wild environment, there is a natural balance between the organism and disease. However, studies have suggested that this balance is compromised when they are cultured at high densities in enclosed environments and subsequently exposed to repeated population bottlenecks in the farm (Mather *et al.*, 2003). This situation leads to increase the stress level which plays important role in the expression of the diseases. There are several infectious viral and bacterial diseases which affect the production of giant freshwater prawn. Source of the infection in adult prawn is believed to have originated from infected broodstock, eggs, or contaminated water supplies.

Nearly 20 distinct viruses are known to infect penaeid shrimp, but only six viruses pose a threat to the future of giant freshwater prawn culture. Viruses which are known to infect and have been reported in *M. rosenbergii* include infectious hypodermal and hematopoietic necrosis virus (IHHNV) (Hsieh *et al.*, 2006), *Macrobrachium* rosenbergii nodavirus (MrNV) and extra small virus-like particle (XSV) (Arcier, 1999; Sahul Hameed *et al.*, 2000; Yoganandhan *et al.*, 2006), *Macrobrachium* hepatopancreatic parvo-like virus (MHPV) (Anderson *et al.*, 1990; Gangnonngiw *et al.*, 2009), *Macrobrachium* muscle virus (MMV) (Tung *et al.*, 1999),

white spot syndrome baculovirus (WSBV) (Peng *et al.*, 1998; Rajendran *et al.*, 1999; Sahul Hameed *et al.*, 2000; Waikhom *et al.*, 2006).

2.2.1 Infectious hypodermal and hematopoietic necrosis virus (IHHNV)

Infectious hypodermal and hematopoietic necrosis virus (IHHNV) was first discovered in white shrimp *Penaeus (Litopenaeus) vannamei* and blue shrimp *Penaeus stylirostris* in America in 1981 (Lightner *et al.*, 1983; Bell *et al.*, 1984) and was recently classified as *Penaeus stylirostris* densovirus (*Pst*DNV) (Fauquet *et al.*, 2005). However, it was thought to have been introduced with live *Penaeus monodon* from Asia and probably has existed for some time in Asia since it has insignificant effects on the tiger prawn. Tang *et al*. (2002) have suggested that Philippines were the source of the original infection in Hawaii based on their studies of geographic variations in IHHNV isolates (Tang *et al.*, 2002).

Recent studies have reported the infection of IHHNV on sub-adult and postlarvae *M. rosenbergii* in Southeast Taiwan with high mortalities up to 80 % (Hsieh *et al.*, 2006). Based on their studies, the PCR assay for IHHNV showed detection of the expected 389 bp product and eosinophilic intranuclear inclusion bodies (INIs) are found on the hepatopancreatic tubular epithelial cell in the postlarvae of *M. rosenbergii* (Figure 2.5 and Figure 2.6).

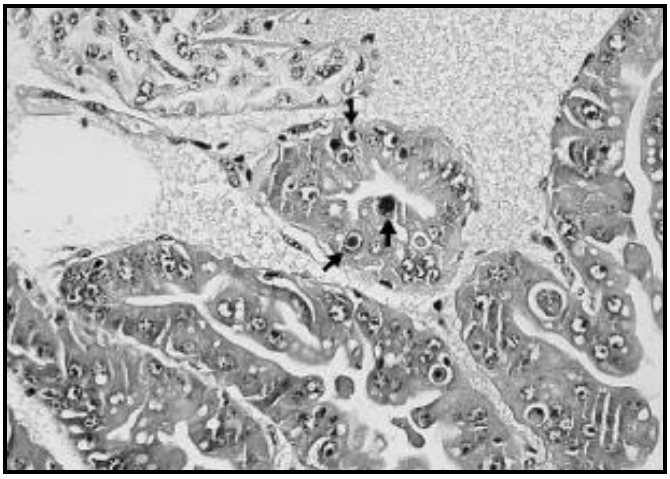


Figure 2.5 Numerous pathognomonic eosinophilic Cowdry type A are observed in tubular epithelial cells of hepatopancreas. **(**H&E histology of IHHNV infected postlarvae of *M. rosenbergii)(*Hsieh *et al.,* 2006*).*

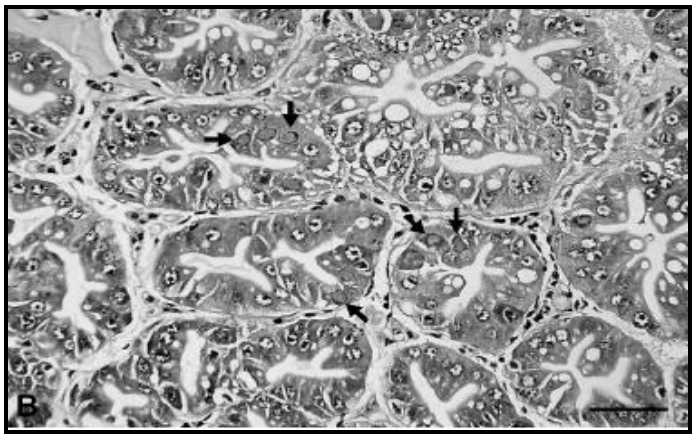


Figure 2.6 The nuclei of epithelial cells were hypertrophied with margination of chromatin and lacked clear halo formation known as Cowdry type B INIs. (H&E histology of IHHNV infected postlarvae of *M. rosenbergii)* (Hsieh *et al.,* 2006).

The previous studies have revealed that IHHNV has an affinity for various tissues ectodermal and mesodermal (Lightner, 1996). However in the studies by Hsieh *et al*. (2006), there is no inclusion being observed in ectodermal and mesodermal origin of the infected *M. rosenbergii*. Based on the sequence analysis, IHHNV strains obtained from *M. rosenbergii* is 99.7 % closely related to Taiwanese strains from *P. monodon* (Hsia *et al.*, 2003) & American strains from *P. vannamei* and *P. stylirostris* (Bonami *et al.*, 1990; Shike, 2000) and it is believed that IHHNV may be introduced to Taiwan from Thailand via imported live shrimp.

2.2.2 White tail Disease (WTD)

Another disease that has been reported which responsible for high mortalities of freshwater prawn is white tail disease (WTD). The causative agent of WTD has been recognized as *Macrobrachium rosenbergii* nodavirus (*Mr*NV) and extra small virus-like particle (XSV) (Figure 2.7). *Mr*NV is a small, icosahedral non-enveloped virus which identified in the cytoplasm of the connective tissue cells (Arcier, 1999; Ravi *et al.*, 2009). XSV is known as virus like particle icosahedral non-enveloped with linear single stranded positive sense RNA of 0.9 kb encoding two overlapping structural proteins of 16 Da and 17 Da (Sri Widada *et al.*, 2003).

The clinical signs of the infection are anorexia, lethargy and opaqueness of the abdominal region of an infected prawn (Sri Widada *et al.*, 2003; Sudhakaran *et al.*, 2006). The presence of basophilic cytoplasmic inclusions in striated muscles of abdomen and cephalotorax and intra-tubular connective tissue of the hepatopancreas of the infected prawn can be observed by histological method (Arcier, 1999). These two viruses may cause 100 % mortality in postlarvae at seven to 12 days post-infection by immersion challenge. The infected PL starts to show whitish muscle at day seven and reached highest proportion at day 10 (Sahul Hameed *et al.*, 2004). The presence of these viruses can be detected by RT-PCR assay of targeted tissue such as gill, head muscle, hemolymph, pleopods and tail muscle (Sri Widada *et al.*, 2003; Sahul Hameed *et al.*, 2004). Studies confirming the infection of nodavirus (*Mr*NV) and XSV have recently been reported in Taiwan (Wang *et al.*, 2008), India (Shekhar *et al.*, 2006; Sahul Hameed *et al.*, 2011) and Malaysia (Goh *et al.*, 2011; Saedi *et al.*, 2012).

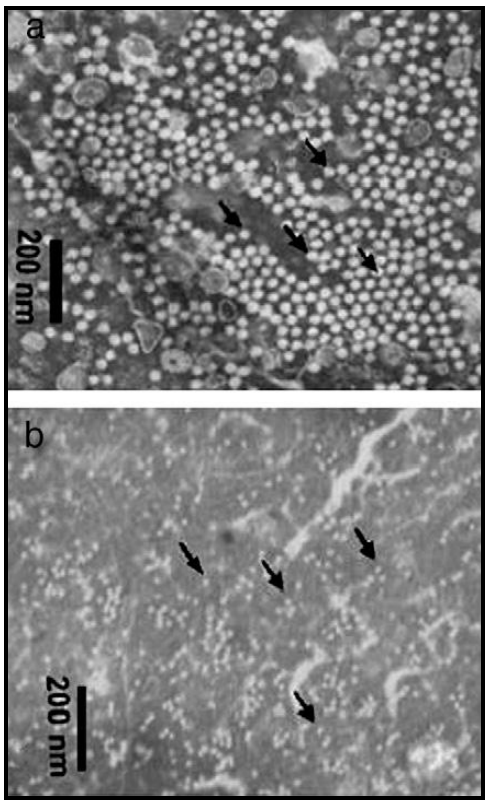


Figure 2.7 Negatively stained micrographs of two viruses which purified from diseased postlarvae of *M. rosenbergii ; Mr*NV (a) and XSV (b) (Sahul Hameed *et al*., 2011).

2.2.3 *Macrobrachium* hepatopancreatic parvo-like virus (MHPV)

Macrobrachium hepatopancreatic parvo-like virus (MHPV) is a viral agents which naturally infects *M. rosenbergii* but the infection has not been associated with prawn mortality (Anderson *et al.,* 1990). This disease requires histological examination of a characteristic inclusion body within hypertrophied nucleus of the affected cells of hepatopancreas epithelia which resemble those observed in hepatopancreatic parvovirus (HPV) infection in shrimp (Lightner *et al.,* 1994). However, the disease can be distinguished from shrimp HPV by *in situ* hybridization test (Lightner, 1996).

Recent studies on a survey of cultivated giant freshwater prawn from Thailand revealed the presence of intranuclear inclusion which similar to the report by Flegel (2006) for other parvoviruses in shrimp (Gangnonngiw *et al.,* 2009). The examination by transmission electron microscopy (TEM) showed that the inclusions contained

tightly packed, unenveloped, viral-like particles of approximately 25 to 30 nm diameter. However, standard PCR for detection of *Pm*DNV and *Pst*DNV from *P. monodon* and *in situ* hybridization tests using PCR-labeled DNA probes from both of these viruses gave negative results with *M. rosenbergii* samples. These results suggests that the genome of the virus differed sufficiently from those in marine shrimp (Gangnonngiw *et al.*, 2009). Based on the TEM and histopathology results, the virus described in the studies is suggested to be closely related to that previously described in *M. rosenbergii* from Malaysia (Anderson *et al.*, 1990). Since there is little knowledge on MHPV, the screening and monitoring program will have to depend on histological analysis.

2.2.4 *Monodon* baculovirus (MBV)

Monodon baculovirus (MBV) is currently listed by the International Committee on Taxonomy of viruses as *Penaeus monodon* nucleopolyhedrovirus (*Pemo*NPV). This virus was first reported in Mexico from a population of laboratory-reared adult *P. monodon* in which the postlarvae were originally came from Taiwan. The clinical signs of MBV infection are anorexia, lethargy and an acute infection may lead to the destruction of the hepatopancreatic and anterior midgut epithelial cells (Natividad, 1991). In late 1987, an occurrence of clinical disease in *Penaeus monodon* juveniles in Malaysia has been reported which involved multiple infections of MBV, a reo-like virus, rikettsial like organism and bacteria (Anderson *et al.*, 1987). In this report, intranuclear MBV occlusions were relatively common in animals from all ponds with overall prevalence, 61 %. However, infected epithelial cells of the stomach or midgut were not observed (Anderson *et al.*, 1987).

The principal diagnosis of MBV infection is the presence of multiple spherical occlusion bodies in hepatopancreatic epithelial cells and midgut epithelial cells. In the later stages of MBV infection, histology and TEM studies showed that hepatopancreas cells possess hypertrophied nucleus which contain randomly occluded enveloped virions (Lightner, 1992). Previous studies reported that MBV's widespread distribution has been documented in shrimp culture facilities in Philippine and Indonesia (Natividad, 1991). Recently, MBV infection in *Macrobrachium rosenbergii* was reported in Thailand (Gangnonngiw *et al.*, 2010). The field specimens of giant freshwater prawn postlarvae showed intranuclear inclusions in hepatopancreatic tubule epithelial cells which resembled those produced by baculoviruses. By using standard PCR methods for detection of MBV, four out of six samples that showed intranuclear inclusions, gave positive results (Figure 2.8). In their study, they suggested that MBV can infect giant freshwater prawn and produce lesions in hepatopancreas which is similar to shrimp species prior to the formation of polyhedral occlusion bodies (Gangnonngiw *et al.*, 2010).

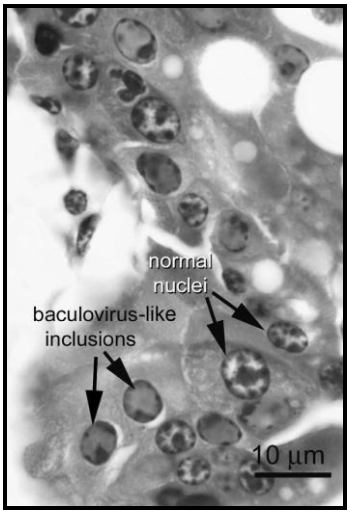


Figure 2.8 Photomicrograph of hepatopancreas tissue of a post-larval specimen of *M. rosenbergii* stained with H&E and showing eosinophilic intranuclear inclusions (Gangnonngiw *et al.*, 2010).

2.2.5 *Macrobrachium* muscle virus (MMV)

Macrobrachium muscle virus is an agent that infects muscle tissue only which caused the infected tissue becomes opaque with progressive necrosis. The first report of this disease is from southern Taiwan by Tung in 1999. Histologically, there are basophilic inclusion bodies observed in the cytoplasm of striated muscle cells (Tung *et al.*, 1999). The outbreak of this disease occurs within 10 days of transfer of PL to the rearing pond which the mortality may exceed 50 % within 2 weeks (New, 2005).

2.2.6 White spot syndrome baculovirus (WSBV)

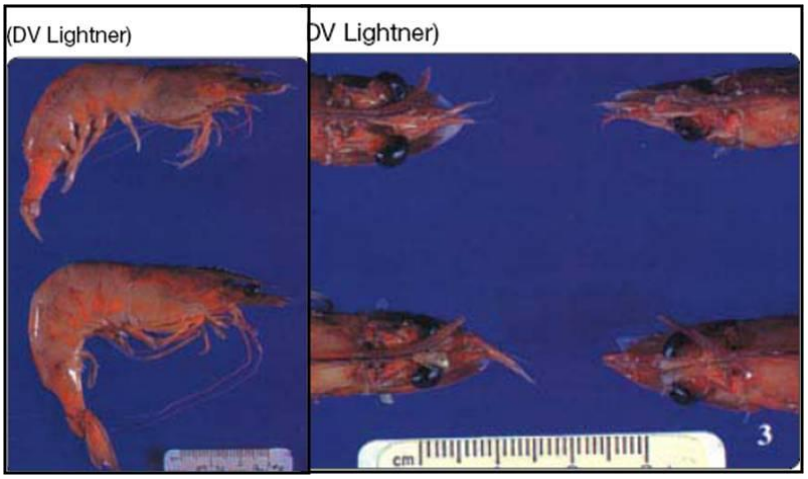
M. rosenbergii is also reported to be infected with a member of the white spot virus complex known as white spot syndrome baculovirus (WSBV). The infection can be detected by polymerase chain reaction (PCR), *in situ* hybridization method on the target tissue such as cuticular epidermis, stomach, gills and hepatopancreas (Chang *et al.*, 1996). In 1999, Rajendran and teams had performed experimental studies where the WSSV obtained from *P. monodon* from the Southeast coast of India was used to challenge *M. rosenbergii* and other prawn species (Rajendran *et al.*, 1999).

The experiment which was conducted using viral injection and feeding with the infected tissue resulted in 100 % survival up to 70 days. Another study (Sahul Hameed *et al.*, 2000) had used a viral inoculums obtained from *P. monodon* with WSSV to test the relative susceptibility of two shrimp species and three freshwater prawn species. The output of the experiment showed that all species tested had mortality except for *M. rosenbergii*, even when they gave multiple dosages. The results show that sub-adult and adult of *M. rosenbergii* were found to be tolerant to WSBV.

2.3 Infectious hypodermal and hematopoietic necrosis virus (IHHNV)

IHHNV is known as enzootic in Taiwan, Malaysia, Thailand, Indonesia, Australia and Philippine. Natural infections of IHHNV have been reported to occur in cultured and wild population of *Penaeus stylirostris, P. vannamei, P. monodon,* and *P. japonicus* (Bonami *et al.*, 1990; Brock *et al.*, 1990; Nunan *et al.*, 2000; Nunan *et al.*, 2001; Kathy, 2002; Tang *et al.*, 2003).

IHHNV caused acute disease in juvenile *P. stylirostris* with high mortalities more that 90 %. In this species, the juvenile and sub-adult stages shrimp are the most severely affected (Bell *et al.*, 1984). The gross sign of IHHNV infection is not specific and depends on the host species, but infected *P. stylirostris* will become weak and lose their appetite for food (Lightner *et al.*, 1983). Then it followed by changes in behavior and appearance. The shrimp exhibit erratic swimming behavior where they will rise slowly to the water surface, become motionless, rolling over until the ventral side is up and then it will sinks back to the bottom. They repeat the process until they die which usually within four to 12 hours or until they are cannibalized by other shrimp. The presence of the virus can cause death of the cells of the cuticle, blood forming tissue and connective tissue of the shrimp. In *P. vannamei,* runt deformed syndrome (RDS) often occurs as a result of IHHNV infection (Bondad-Reantaso *et al.*, 2001). This chronic form of IHHNV disease has also been observed in cultured stock of *P. stylirostris* and *P.monodon*. The infected shrimp with RDS may display a bent or deformed rostrum, deformed sixth abdominal segment, wrinkled antennal flagella, cuticular roughness and other cuticular deformities (Bondad-Reantaso *et al.*, 2001) (Figure 2.9). However, infected adults seldom show signs of the disease. There is no documentation on the clinical sign of the infected *M. rosenbergii*.



(a) (b)

Figure 2.9 Gross sign of IHHNV infection **(**a) from lateral view of juvenile *P. vannamei* with RDS showing cuticular abnormalities of the sixth abdominal segment and tail fan. (b) dorsal view of juvenile *P. vannamei* showing a bent or deformed rostrum (Bondad-Reantaso *et al*., 2001).

2.3.1 Genome and classification of IHHNV

Infectious hypodermal and hematopoietic necrosis (IHHN) disease is a chronic penaeid shrimp disease caused by the smallest penaeid DNA virus known as IHHNV. This virus is a typical densovirus which belongs to family Parvoviridae with the size of the virion between 20 to 22 nm in diameter, with a density of 1.40 g/mL in CsCl (Figure 2.10) (Bonami *et al.*, 1990; Bondad-Reantaso *et al.*, 2001). It is a non-enveloped virus, icosahedral in shape which contains linear single-stranded DNA with an estimated size of 4.1 kb and a capsid with 74 kD, 47 kD, 39kD and 37.5 kD polypeptides (Bonami *et al.*, 1990).

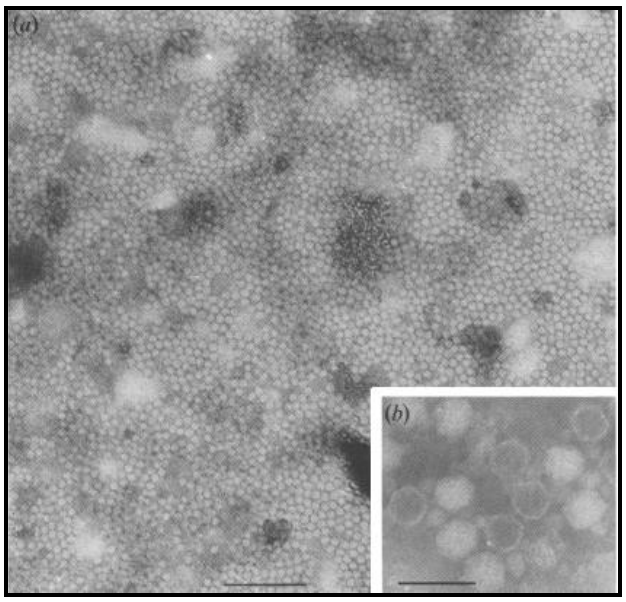


Figure 2.10 Purified IHHNV. (a) Large amount of full particles; marker represents 200 nm. (b) Full and empty particles at higher magnification from a non-fully purified virus suspension; bar marker represents 40 nm. Negative stain (SPA) (Bonami *et al.*, 1990).

The genome of IHHNV consists of three open reading frames (ORF) (Figure 2.11). The left ORF encodes the non-structural proteins (NS), while the right ORF encodes the capsid protein. The function of the middle ORF is still remaining unknown due to lack of homology with any other viral proteins from GenBank database (OIE, 2003; Dhar *et al.*, 2007). The non-structural protein 1 (NS1) has a role in the trans-activation of the viral promoter and required for viral replication. (Dhar *et al.*, 2007).

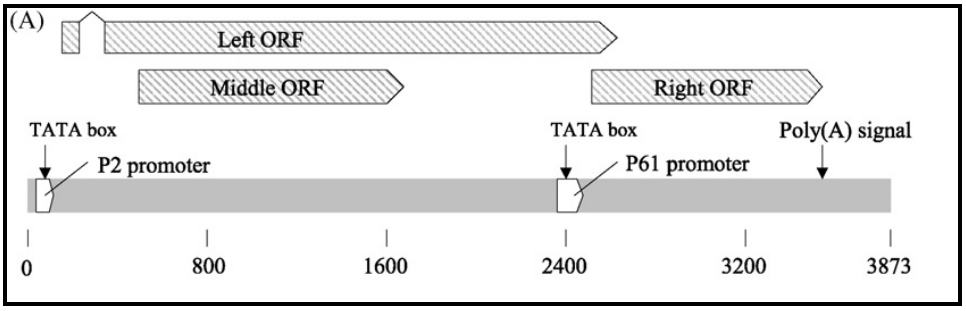


Figure 2.11 A schematic representation of the genome organization of IHHNV (based on GenBank Acc: AF273615). The numbers along the bottom scale correspond to the nucleotide numbers of the sequence ORF (Dhar *et al.*, 2007).

There are three distinct genotypes of IHHNV that have been identified (Tang *et al.*, 2002; OIE, 2003). The two genotypes which are infectious are known as IHHNV Type 1 and IHHNV Type 2. IHHNV Type 1 is derived from the Americas and East Asia while IHHNV Type 2 is from South-East Asia. IHHNV Type 3 is divided into two generic variants known as Type 3A and Type 3B. The Type 3 genotype is reported not infectious to *P. vannamei* and *P. monodon* and its related sequences have been found inserted into the genome of *P. monodon* in Africa, Australia and the western Indo-Pacific (Tang *et al.*, 2006; Tang *et al.*, 2007).

2.3.2 Transmission mode of IHHNV

IHHNV has been reported to occur in all stages of penaeid life cycle including eggs, larvae, sub-adult and juvenile (Bondad-Reantaso *et al.*, 2001; OIE, 2003). There are two mechanisms of the transmission of IHHNV which are horizontal and vertical routes.

Horizontal transmission of this virus is known to be occurred by cannibalism of infected individuals or by indirect contact of contaminated water (Lightner, 1996; Bondad-Reantaso *et al.*, 2001; OIE, 2003). Cannibalism is one of the effective mechanisms and is also known as the basis of the bioassay test for asymptomic carriers in shrimp population. The IHHNV- resistance species or survival prawn may carry the virus (carrier) and pass the virus to their progeny via vertical transmission (Lightner *et al.*, 1983).

Lightner (1983) has stated that vertical transmission is a very efficient transmission mode with high infection rates typically measured in batches of PLs from infected broodstock. However, there is no studies been published to confirm this. In another studies, Pantoja (1999) suggested that this mode of transmission may have

contributed significantly to the rapid spread of IHHNV in aquaculture operation in Mexico (Pantoja *et al.*, 1999). Thus, they suggested that there is a need to determine the effect of IHHNV infection on the reproductive organs of infected broodstock in order to confirm the epidemiological significant of vertical transmission. In the studies, IHHNV Cowdry Type A inclusion bodies (CIAs) was found in apparently healthy, fully functional gonads of both sexes which suggested the potential of vertical transmission. Vertically infected larvae may not become diseased but at approximately PL35 or older, the gross signs of the disease may be observed and then followed by mass mortalities.

Other potential sources for IHHNV transmission that can be considered are seabirds migration (Vanpatten *et al.*, 2004), human activities, infected pond sediments and untreated infected shrimp by-products such as liquid and solid wastes from processing plants.

2.3.3 Incidence of IHHNV infection

In 2002, Owens *et al*. reported on the occurrence of chronic low-grade mortalities in a batch of *Peneaus esculentus* hybridized with *P. monodon* in Australia. Based on histopathological examination, infected cells displayed Cowdry type A inclusions which is pathognomonic for IHHNV. This study revealed that the pattern of mortalities was similar in *P. stylirostris* cases, but separated by age and size (Owens *et al.*, 1992). A study by Morales-Covarrubias *et al*. (1999) revealed that wild adult *P. stylirostris* female (86 % and 89 %) and males (56 % and 57 %) from two stations at Gulf of California, Mexico were positive for IHHNV by dot blot technique. Histopathology results showed the presence of Cowdry type A inclusion (CAI) in hematopoietic tissue, ovary and gill, with prevalence of 80 % and 100 % at both stations (Morales-Covarrubias *et al.*, 1999).

In previous studies, Tang *et al.* (2003) reported on the sequence variations among isolates of IHHNV. All sequence variations which isolated from samples of *P. monodon* were compared with an isolate from Hawaii by histology, *in situ* hybridization and laboratory challenge studies with *L. vannamei* (Tang *et al.*, 2003). The Philippine isolates (99.8 %) had a very high identity to Hawaiian isolate while Thailand isolate showed slightly lower nucleotide sequence identity (96.2 %). This study revealed that the putative IHHNV sequences collected from Tanzania and Madagascar showed greater divergence, which 8.2 % and 14.1 % difference for both sequence respectively from Hawaii isolate sequence. The infection characteristics of Philippine and Thailand were examined by laboratory challenge studies of *L. vannamei* but the infected shrimp did not show any mortality (Tang *et al.*, 2003). A study of rediscovery of Australian strain of IHHNV was done by Krabsetsve *et al*. (2004), in order to verify the presence of the virus amongst *P. monodon* culture in Australia by using PCR analysis with IHHNV specific primers. Seven published primers for IHHNV detection were tested on Australian shrimp but only one elicited a positive IHHNV with 16 out of 20 samples examined (Krabsetsve *et al.*, 2004). The results of the studies suggested that the strain of IHHNV found in *P. monodon* in Australia is endemic in the Australian environment.

IHHNV was also reported to infect giant freshwater prawn, *M. rosenbergii*. A study by Hsieh *et al*. (2006) reported that high mortalities (80 - 100 %) occurred in postlarvae of *M. rosenbergii* in southern Taiwan and eosinophilic intranuclear inclusion bodies were found in the epithelial cells of hepatopancreatic of the infected samples (Hsieh *et al.*, 2006). A study by Tang *et al*. (2006) revealed that infectious hypodermal and hematopoietic necrosis virus (IHHNV)-related sequence were found within the shrimp genome of *P. monodon* collected from Africa and Australia. Laboratory bioassays were carried out but none of the indicator shrimp showed sign of disease

(Tang *et al.*, 2006). Based on the negative results of both PCR and histopathology examination, they suggested that the Africa type IHHNV-related sequence are not infectious. In 2007, a modified method was developed for discriminating between infectious form of IHHNV and virus-related sequence in the genome of *P. monodon* (Tang *et al.*, 2007).

In a study by Braz *et al* (2009), prevalence of IHHNV infection from 9.4 % to 81 % was determined by using PCR on samples collected from 26 rearing ponds in Northeastern Brazil. The findings suggested that infection of IHHNV in rearing ponds was common and could have the non-infectious IHHNV form in *P. vannamei (Braz et al., 2009)*. A study by Saksmerprome *et al*. (2010) has described an infectious type of IHHNV in *P. monodon* from Australia which identified by using PCR amplification from two regions of IHHNV genome. They managed to amplify all the ORFs of the IHHNV genome by using overlapping primers and the results revealed the presence of infectious IHHNV from *P. monodon* in Australia (Saksmerprome *et al.*, 2010) .

Kim *et al*. (2011) has described the first report of IHHNV infection in *L. vannamei* in South Korea cultured shrimp. In this study, the results of phylogenetic tree showed that sequence of Korean strains was closely related to the Type 1 infectious IHHNV strains from Ecudor, China and Taiwan (Kim *et al.*, 2011a; Kim *et al.*, 2011b). A current study by Teixeira-Lopez *et al*. (2011) revealed the occurrence of a natural infection with IHHNV and infectious myonecrosis virus (IMNV) in *L. vannamei* in Northeast Brazil. Disease monitoring was done using molecular techniques and the results showed that most of positive samples were simultaneously co-infected with both IHHNV and IMNV.

A preliminary survey of IHHN infection to study the disease status of wild giant freshwater prawn, *M. rosenbergii* was done in Sarawak by Kua *et al*. (2011). However, the samples were tested negative by using the OIE suggested primer (Kua *et al.*, 2011). Another monitoring program was carried out at the same location and the results were also negative (unpublished data). This suggested that the wild *M. rosenbergii* in the areas tested in Sarawak was IHHNV-free.

2.3.4 Diagnostic method

Viral infections are the most common threat for prawn farming production. In the past, the detection methods have been lacking but today, the increased use of viral diagnostic and new technology in viral detection help us to detect and control aquatic viral diseases. IHHNV can be detected by several methods including histopathology, *in situ* hybridization (ISH) using an IHHNV-specific gene probe, and molecular techniques such as conventional polymerase chain reaction (PCR) (Lightner *et al.*, 1998). Confirmation of this disease can be determined with any combination of at least two methods which are PCR positive results, sequencing of PCR specific product in order to determine the genotype of IHHNV or ISH positive histological signal to IHHNV-type lesion (OIE, 2003).

(a) Polymerase chain reaction (PCR)

When samples contain low copy numbers of virus, the detection efficiency can potentially be increased by using PCR which is capable of amplifying DNA sequences million-fold (Carter *et al.*, 2007). In this approach, primer design plays an extremely important role for effective amplification. The primer must be very specific to the target region for specific amplification. In addition, the primer must not be capable of self-priming which might caused the amplification of short nonsense DNAs. The

disadvantage of the protocol is that, the Taq polymerase enzymes can cause frequent mismatch mistakes during the elongation step. Thus elongation time can be shorten for mismatching event to be minimized.

Direct detection using polymerase chain reaction (PCR) is the most commonly used method to detect IHHNV genome. There are several primers that have been described for IHHNV detection (Nunan *et al.*, 2000; Hsia *et al.*, 2003; OIE, 2003; Hsieh *et al.*, 2006; Tang *et al.*, 2007; Braz *et al.*, 2009; Rai *et al.*, 2009; Saksmerprome *et al.*, 2010; Kim *et al.*, 2011a). This method is a sensitive and specific tool for IHHNV detection. However, most of the published and commercialized IHHNV detection primers are design based on the Hawaiian strains. This difference may be the reason why the use of these primers has not been able to detect IHHNV freshwater prawn in Malaysian isolates (unpublished data). Thus, specific primers need to be designed from local strains of IHHNV obtained from Malaysian freshwater prawn in order to detect IHHNV infection in Malaysian giant freshwater prawn without performing any false positive and false negative.

Various PCR-based strategies have also been employed for molecular characterisation and for epidemiological studies of the disease (Abbas et al., 1996). However, detection of this virus is complicated by the fact of the presence of virus-related sequence which are integrated into the genome of *P. monodon* (Tang *et al.*, 2006). Teixeira-Lopes (2010) has demonstrated that PCR diagnostic of IHHNV based on OIE recommended primers (77012F/77353R), was not sufficiently strong to discriminate between infectious IHHNV and integrated IHHNV genome (Teixeira *et al.*, 2010). There are another sets of primer that suggested by OIE are IHHNV389F/R and

IHHNV392F/R which both are commonly used to detect the infectious IHHNV based on the non-structural protein 1 coding region (Tang *et al.*, 2003).

(b) **Histopathology**

Histopathology is one of the approaches used to examine tissues microscopically to study the manifestation of disease. Generally, biopsy samples of abnormal tissue are tested with this approach to determine the presence of the pathogen, type and also the severity of the disease. There are several steps involved in normal histopathology method which begins with the collection of the tissues and fixation with a suitable fixative solution. For prawn samples, the fixative used is Davidson's solution. This is followed by embedding tissue in the paraffin block and sectioning of the embedded tissue. The final step of this procedure is staining of the processed histology slide for subsequent microscopic examination.

In the histopathological observation, IHHNV forms Cowdry Type A and B intranuclear inclusion bodies associated with widespread cytopathological changes including hypertrophy of the nucleus and margination of the chromatin (Figure 2.12) (Lightner *et al.*, 1983; Mari *et al.*, 1993). Cowdry Type A or B were also observed in cells of ectodermal origin (epidermis, gills, antennal gland and neurons) and mesodermal origin (haematopoietic tissue, haemocytes, heart, lymphoid organ and connective tissues). The nuclei of epithelial cells which are hypertrophied with margination of chromatin and lacked clear halo formation is known as Cowdry type B INIs. In some IHHNV infected prawns, melanised nodules were observed in the connective tissues (Owens *et al.*, 1992; Mari *et al.*, 1993).

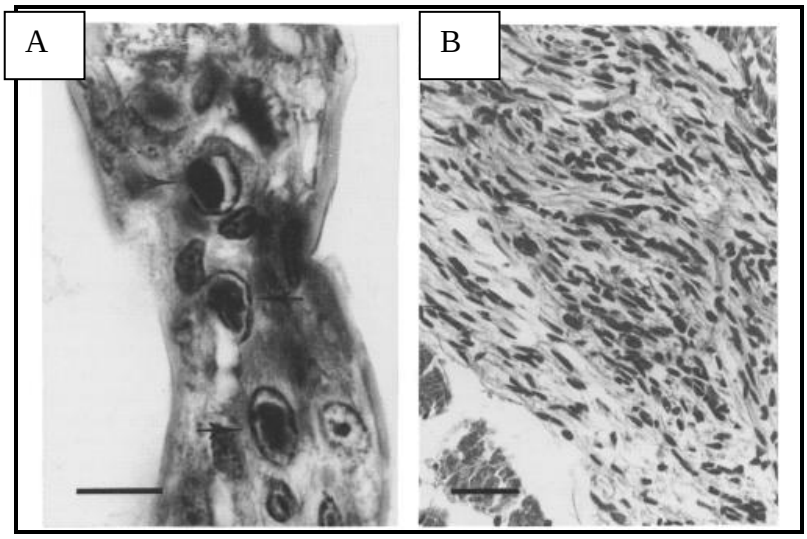


Figure 2.12 Histopathology examinations (H&E stain). (*A*) Gill section. Heavily infected animal; Cowdry type A inclusion bodies are notice clearly (*arrows*). Bar represents 10 µm. (*B*) Nerve cord. Section from the same infected animal. Bar represents 20 µm. (Mari *et al.*, 1993).

(c) *In situ* Hybridisation (ISH)

Current methods for identification of prawn viral infections have been relied on histopathology approaches and PCR based methods which have several practical limitations for routine use. *In situ* hybridization of IHHNV with DNA probes is one of the detection methods that may also provide greater diagnostic sensitivity than other conventional histology approaches. This method which is also referred to as hybridization histochemistry, was first described in 1969 in localizing specific DNA sequence on chromosomes in cytological preparations of Xenopus oocytes (Gall *et al.*, 1969).

The isolated tissue is processed by embedding it in paraformaldehyde to preserve the transcripts in place (Overturf, 2009). The nucleic acid target sequence of interest can be detected using the selective probes. The probe is labeled with an antigen which will hybridize directly to the target virus transcript in the embedded tissues. The hybridization between these probes and tissue sections will allow the detection of virus DNA within tissue regions and cell types. For IHHNV infection confirmation by ISH, dark-blue or black precipitation can be observed on the slides which mark sites where IHHNV DNA is present. The pathodiagnostic intranulear Cowdry Type A inclusions are well marked with the probe (Mari *et al.*, 1993).

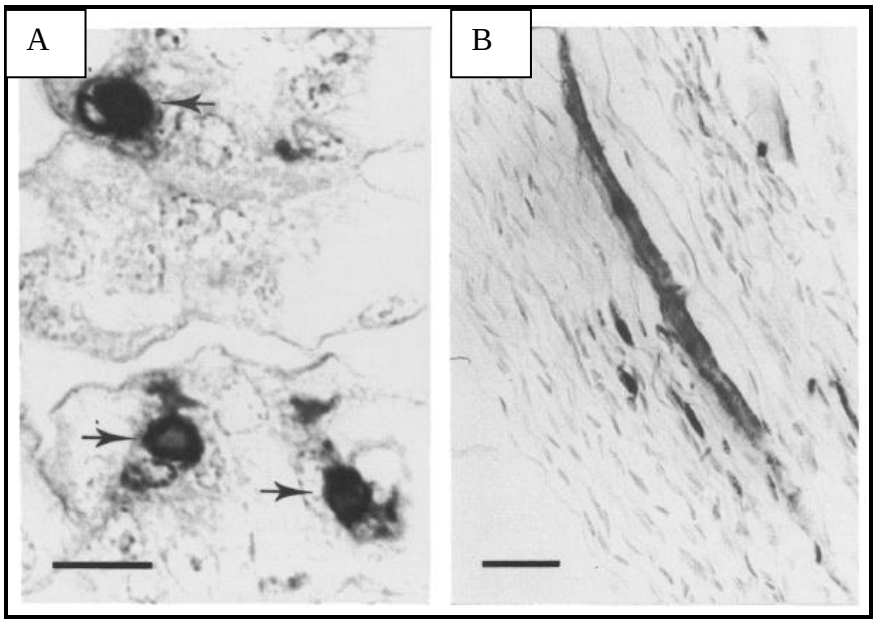


Figure 2.13 *In situ* hybridization (ISH) by using BS4.5 DIG-labelled probe. (*A*) Gills section with infected nuclei, a part of the cytoplasm and Cowdry type A inclusion bodies (*arrows*) react intensely with the probe. Bar represents 10 µm. (*B*) nerve cord showed light IHHNV infection of the nervous tissue; some nuclei react positively and one nerve cell exhibits a strong positive reaction in all the cytoplasm. Bar represents 20 µm.(Mari *et al.*, 1993).

CHAPTER 3

SCREENING OF IHHNV IN CULTURED GIANT FRESHWATER PRAWN, *Macrobrachium rosenbergii* AND DEVELOPMENT OF NESTED PCR FOR IHHNV DETECTION

3.1 INTRODUCTION

Freshwater prawn, *Macrobrachium rosenbergii*, is commercially important for their value as a local food source as well as a valuable export product. Viral diseases are known to be a limiting factor in prawn farming as they cause serious production losses during acute epizootics. IHHNV is one of the major causes of shrimp diseases and has been listed as one of the significant diseases by the World Organization for Animal Health (OIE).

According to the Quarterly Aquatic Animal Disease (QAAD) Report (Asia-Pacific Region) referring to Malaysia, frozen samples of black tiger shrimp from Pekan, Pahang were found infected with IHHNV in August 2010. Recently, QAAD reports also highlighted cases with IHHNV infection where one out of 28 juvenile-adult samples from Perak (February 2011) and two of 81 *Penaeus monodon* samples in the state of Terengganu (April 2011) that were tested positive IHHNV by Laboratory Industrial Resource (LIR). This disease has never been reported in giant freshwater prawn in Malaysia. However, in this study, IHHNV has been shown to infect giant freshwater prawn, *M. rosenbergii*.

In this study, the screening of IHHNV focused on the giant freshwater prawn from a hatchery in Perak, Peninsular Malaysia. The hatchery was selected based on the preliminary study which was done in 2008 (See Chapter 1). From the preliminary

results, a sample of giant freshwater prawn collected from a hatchery in Kampung Acheh were tested positive using PCR screening IQ2000 Detection Kit for IHHNV (unpublished data). However, due to limited amount of samples, the DNA could not be sequenced.

In current studies reported, IHHNV infection can be detected using the standard primer suggested by OIE which was based on a 389 bp fragment (OIE, 2003). However, the use of this suggested primer along with the suggested experimental procedure revealed nonspecific bands in the polymerase chain reaction (PCR) result. It may be caused by the mutations in the IHHNV viral genome, or that the viral loads were too low in the samples. Therefore, a more specific primer and a more effective method has to be developed to obtain a more accurate and reliable detection result for IHHNV. As the currently suggested primers for PCR detection of IHHNV are not capable of giving a specific and reliable result, it is hence desirable to provide a specifically-designed primer set, coupled with a simple yet specific method for detecting IHHNV to overcome the detection problem.

3.2 MATERIALS AND METHODS

To achieve the objectives above a series of polymerase chain reaction (PCR) experiments were carried out.

3.2.1 Study area and sample collection

Currently the hatcheries used gravid prawn caught from the wild for broodstocks. The wild adult broodstocks were captured from Sungai Rubana, Teluk Intan Perak (latitude: 3^o 58' 60 N; longitude 100^o 55' 0 E) by the local fishermen. A total of 30 wild berried female broodstocks were sampled randomly in the hatchery. Pleopods were removed from adult broodstock of giant freshwater prawns before and after spawning. The pleopods were fixed in absolute ethanol. The postlarvae from the broodstocks were raised in larvae culturing tanks. After performing a necropsy procedure on the larvae, all samples were kept in -80^{o}C freezer. The DNA of the samples was extracted before proceeding with downstream applications.

3.2.2 DNA extraction

Genomic DNA was extracted using IQ2000^{TM} IHHNV (Nested) Detection and Prevention system lysis buffer according to the manufacturer's instructions. Approximately 25 mg tissue was cut and placed in 1.5 ml microcentrifuge tube. 500 µl of lysis buffer was added into the tube. The sample was homogenized and incubated at 95 ^{o}C for 10 minutes. The sample was centrifuged at 12,000 rpm for 10 minutes. Approximately 200 µl of upper clear solution was transferred into a new tube. Then 400 µl of 95 % ethanol was added into the tube. The mixture was mixed thoroughly by vortexing and the tube was centrifuged at 12,000 for five minutes. The ethanol was decanted and the pellet was dried. The DNA pellet was dissolved with 50 µl TE and stored at 4 ^{o}C until used. The quality of the extracted DNA was analyzed by gel electrophoresis and quantify using a NanoDrop spectrophotometer (NanoDrop Technologies, Inc.).

3.2.3 Polymerase chain reaction (PCR) of IHHNV infection

The protocol of IHHNV detection using PCR approach was performed using the method obtained in the OIE manual (OIE, 2003) and the primer used in the screening of infection was IHHNV389. DNA extracted from all samples was subjected to polymerase chain reaction (PCR) amplification using OIE forward primer (5'- CGG AAC ACA ACC CGA CTT TA -3') and reverse primer (5'- GGC CAA GAC CAA AAT ACG AA -3') which amplifies a 389 bp fragments (OIE, 2003). The DNA template (40 ng), primers (0.2 µM) and Maxime PCR Premix, *i*-taq (iNtRON) were combined in 20 µl reaction volume.

The PCR reactions was subjected to initial denaturation at 94 ^{o}C for two minutes in a thermal cycler (MyCycler Personal Thermal Cycler BioRad) followed by 35 cycles of denaturation at 94 ^{o}C for 20 seconds, annealing at 55 ^{o}C for 10 seconds, and extension at 72 ^{o}C for 30 seconds before the final extension at 72 ^{o}C for five minutes. The PCR products were electrophoresed through a 1.5 % agarose gel before staining with ethidium bromide and viewed under UV light. The amplified bands within the expected size were purified using the QIAquick Gel Extraction Kit (QIAGEN) and sequenced based on Sanger's dideoxy sequencing method.

3.2.4 Sequence analysis

The partial non-structural protein 1 gene sequences (389 bp) of IHHNV obtained from this study and submitted to GenBank (GenBank Acc. No.HM 536212) were compared with related sequences obtained from GenBank in NCBI using NCBI-BLAST. Multiple alignments were performed with Clustal X (Jeanmougin *et al.*, 1998) and the maximum parsimony phylogenetic tree (1,000 bootstraps) was constructed using MEGA software (Tamura *et al.*, 2007). Genealogical network was constructed using TCS software

(Clement *et al.*, 2000) to estimate the genealogical relationship among the generated haplotypes. TCS calculated a 90 % parsimony connection limit of 12 steps and resulted in a haplotype network of the sequences of partial non-structural protein 1 (NS1) gene for IHHNV (Figure 3.4). There are 12 different haplotypes and all identical sequences were grouped into the same haplotypes in the network.

3.2.5 Primers designing from Malaysian IHHNV isolate for nested PCR

A standardized nested PCR method that amplifies a region in nonstructural protein of IHHNV has been developed for the diagnosis of IHHNV infection in *M. rosenbergii*. To isolate the gene of interest, primers for nested amplification was designed based on the published sequence of IHHNV Malaysian isolate (HM536212). This non-structural protein IHHNV sequence was obtained from giant freshwater prawn during screening of IHHNV using the OIE primer. The designated forward and reverse primer sequences are GF1_F (5'- CGG AAC ACA ACC CGA CTT TA -3') and GF1_R (5'- CAG CTT CGG CTC TGG CAG CA -3'). The size of the amplicon for this new primer set for nested amplification is 247 bp (Table 3.1).

Table 3.1 List of primer used in the nested protocol

Reaction	Primer Name	Primer Sequence
1st PCR Reaction (OIE, 2003)	389F	5'- CGG AAC ACA ACC CGA CTT TA-3'
Size : 389 bp	389R	5'- GGC CAA GAC CAA AAT ACG AA-3'
*Nested PCR Reaction	GF1 F	5'- ATG GAC GGA AGG CGA CTG GA -3'
Expected size:247 bp	GF1 R	5'- CAG CTT CGG CTC TGG CAG CA -3'

* PI No.: PI 2011002235 (UM)

3.2.6 Construction of positive control plasmid

The PCR product amplified by OIE suggested primers was cloned using the yT&A vector (Yeastern Biotech, Taiwan). The ligation process was done according to the manufacturer's instructions (Table 3.2). The components for ligation are as follows:

Table 3.2 The component for ligation of PCR amplicons with yT&A cloning vector

Component	Volume (µl)
yT&A cloning vector	2
Buffer A	1
Buffer B	1
PCR amplicon	5
yT&A ligase	1
TOTAL	**10**

The above reaction was prepared in 0.2 ml PCR tubes and the ligation mixture was incubated at 4 ^{o}C overnight. For transformation, the tube containing competent cells was thawed on ice (The cells are kept in the -80 ^{o}C freezer). Each tube contains 100 µl of competent cells and once cells are thawed, aliquot 50 µl of cells into a new 1.5 ml centrifuge tube. Then, 5 µl of ligation mixture was pipetted into the tube containing 50 µl ECOS^{TM} 101 competent cells. The tube was tapped gently to mix and placed on ice for 30 minutes. For the heat shock, after the 30 minutes incubation, the tube was incubated at 42 ^{o}C for approximately 45 seconds. Then, the tube was removed from 42 ^{o}C bath and was placed on ice for 2 minutes.

Immediately, 50 µl of transformed cells were spread on the LB agar medium containing 100 µg/ml ampicilin and X-gal (50 µg/ml) using sterile beads. Then the plate was inverted and incubated at 37 ^{o}C overnight. The next day, there should be many colonies which evenly distributed on the agar. The white colonies are the one that will be used for the sub-cloning and PCR colony. The purified plasmid was sequenced to

ensure the correct sequence and then was used as positive control in IHHNV PCR screening for this study (Figure 3.1).

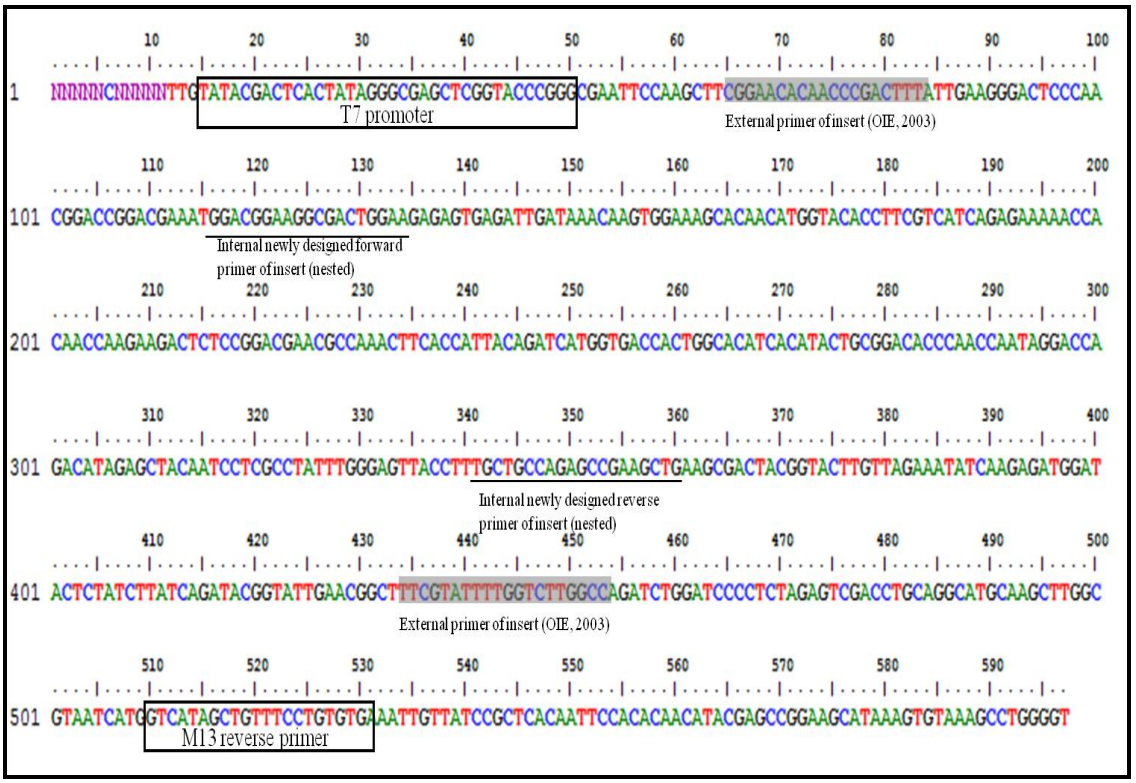


Figure 3.1 Partial DNA sequence of IHHNV isolated from Malaysian giant freshwater prawn, Genbank acc. no HM536212, in yT&A® cloning vector. The size of the recombinant plasmid is 3117 bp.

3.2.7 PCR optimization of primer designed for nested PCR

The optimization of nested PCR was done by optimization of temperature and concentration of $MgCl_2$. The optimization step was performed in C1000 Thermal Cycler (BioRad) and used the plasmid containing IHHNV389 region as DNA template.

The detection of IHHNV by nested PCR involves two set of primers and the first PCR reaction was based on 389 bp using OIE primers (OIE, 2003) (Table 3.1). The first PCR reaction was performed using the DNA extracted from pleopods as template. The optimized PCR conditions were as follows: primers (5 µmol each), dNTPs (50 µmol), Go Taq®DNA Polymerase (1.25 U/25 µl) and 2 mM $MgCl_2$ in GoTaq®Flexi Buffer. A

volume of 1 µl of template was used per 25 µl reaction volume. Double stranded amplifications were performed using forward primer (5'- CGG AAC ACA ACC CGA CTT TA -3') and reverse primer (5'- GGC CAA GAC CAA AAT ACG AA -3'). The DNA template, primers and PCR ingredients were combined and denatured at 95 ^{o}C for two minutes in a thermal cycler with the following condition: denaturing 95 ^{o}C for 30 seconds, annealing 55 ^{o}C for 30 seconds, extension 72 ^{o}C for a minute. The samples were run for 15 cycles before final extension at 72 ^{o}C for five minutes, then held at 4 ^{o}C before continuing with the nested PCR.

The nested PCR reaction was performed using a pair of specific primers which were designed from local IHHNV Malaysia isolate strain, Genbank acc. no HM536212. The amplifications were continued using another set of forward primer (5'- ATG GAC GGA AGG CGA CTG GA -3') and reverse primer (5'- CAG CTT CGG CTC TGG CAG CA -3'). The optimized conditions for nested PCR were as follows: primers (5 µmol each), dNTPs (50 µmol), Go Taq®DNA Polymerase (1.25 U/25 µl) and 1 mM $MgCl_2$ in GoTaq®Flexi Buffer. A volume of 1 µl of PCR product was used as template in 25 µl reaction volume. The DNA template, primers and PCR ingredients were combined and denatured at 95 ^{o}C for two minutes in a thermal cycler with the following condition: denaturing 95 ^{o}C for 30 seconds, annealing 58 ^{o}C for 30 seconds, extension 72 ^{o}C for a minute. The samples were run for 35 cycles before final extension at 72 ^{o}C for five minutes, and held at 4 ^{o}C.

At the completion of PCR, four microliters of the amplified products were electrophoresed through a 1.5 % agarose gel before staining with ethidium bromide and viewed under UV light.

3.2.8 Nested PCR sensitivity and specificity

The forward primer GF1_F (5'- ATG GAC GGA AGG CGA CTG GA -3') and the reverse primer GF1_R (5'- CAG CTT CGG CTC TGG CAG CA -3') designed using the primer design program Primer_BLAST at NCBI website and were synthesized by Bioneer, Inc. (Alameda, CA). The primers pair of GF1_F/GF1_R gives a 247 bp fragment product.

Sensitivity of the nested PCR assay was determined using serial dilutions of the positive control cloned in the T&A cloning vector, pTA-IHHNV. The purified plasmid containing 1.01 X 10^9 copies of IHHNV plasmids were serially diluted 10-fold to 1 X 10^2 copies of IHHNV plasmid and further diluted to 10 copies of IHHNV plasmids in sterile distilled water (Table 3.4). Two microliters of each dilution were subjected to nested PCR as describe in the previous section. In this test, the number of DNA copy is calculated using the following formula:

$$\text{DNA(copy)} = \frac{[6.022 \times 10^{23}\ \text{(copy/mol)} \times \text{DNA amount(ng)}]}{[\text{DNA length(bp)} \times 650\text{(g/mol/bp)}]}$$

The specificity of nested PCR was tested on the DNA samples extracted from HPV-infected *M. rosenbergii* and plasmid clones (pET) containing *M. rosenbergii* nodavirus (MrNV) gene fragments. Ten nanograms of each DNA and plasmid clones were subjected for nested PCR to determine the sensitivity of the newly designed nested set primers.

3.3 RESULTS

3.3.1 Screening of IHHNV in cultured *M. rosenbergii*.

In this study, the wild-caught adult broodstocks were captured from Sungai Rubana, Teluk Intan Perak by local fishermen. A total of 30 wild berried female broodstocks were sampled randomly in the hatchery for screening of IHHNV infection. The pleopods of the berried prawns were sampled before and after the hatching of eggs (Table 3.3).

Table 3.3 PCR amplification results of IHHNV based on primer 389

Samples	Number of samples collected (randomly)	Positive with IHHNV (Prevalence)
Berried female broodstock	30	6/30 (20 %)
Spawned broodstock	12	2/12 (16 %)

For confirmation of the identity of the amplified 389 bp products, the PCR products were purified using the QIAquick Gel Extraction Kit (QIAGEN) and sequenced (Figure 3.2). The sequences derived from positive 389 bp products were analyzed and comparison with IHHNV sequences that are available in the GenBank (Figure 3.3). The sequence alignment and analysis were carried out using the Clustal X (Figure 3.4), MEGA version 4 software (Tamura *et al.*, 2007) and TCS software (Figure 3.5) (Clement *et al.*, 2000).

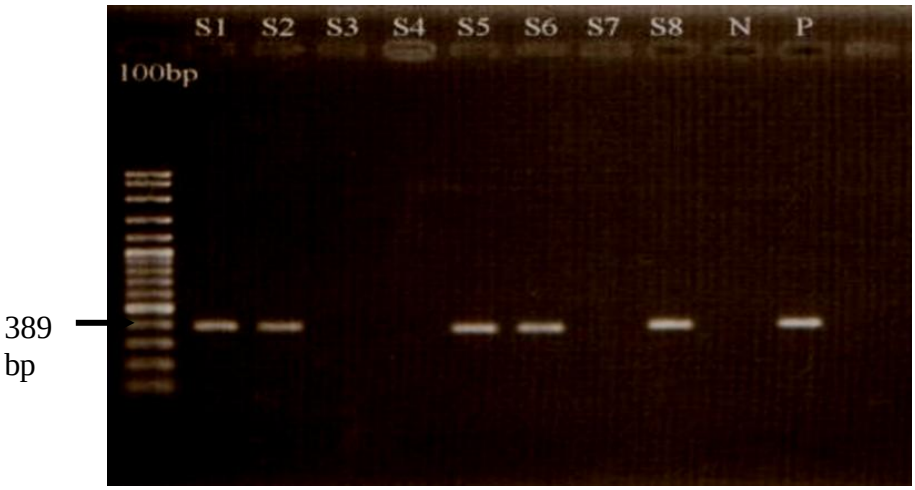


Figure 3.2 Agarose gel electrophoresis of PCR products amplified from extracted DNA for detection of IHHNV in *M. rosenbergii*. **Lane 1:** 100 bp DNA ladder (Fermentas); **Lane P:** Positive control with IHHNV genomic DNA. **Lane N:** Negative control. **Lane S1-S8:** sample adult broodstocks *M. rosenbergii* from Sungai Rubana, S1- S8. Samples S1, S2, S5, S6 and S8 showed the 389 bp amplification.

The Malaysian isolates showed 98 % identity to six other IHHNV sequences available in the GenBank isolated from marine shrimp strains; GenBank Acc. No. AF218266 (Bonami *et al.*, 1990), Taiwan C strain (AY355308), Taiwan A strain (AY355306), Ecuador strain (AY362548) (Hsia *et al.*, 2003), AF273215 (Shike, 2000), and Fujian strain (EF633688). Multiple alignments were performed with the maximum parsimony phylogenetic tree (1,000 bootstraps) was constructed using MEGA software (Tamura *et al.*, 2007)(Figure 3.3) and Clustal X (Jeanmougin *et al.*, 1998)(Figure 3.4). Identical sequences were grouped into haplotypes. Genealogical network was constructed using TCS software (Clement *et al.*, 2000) to estimate the genealogical relationship among the generated haplotypes.

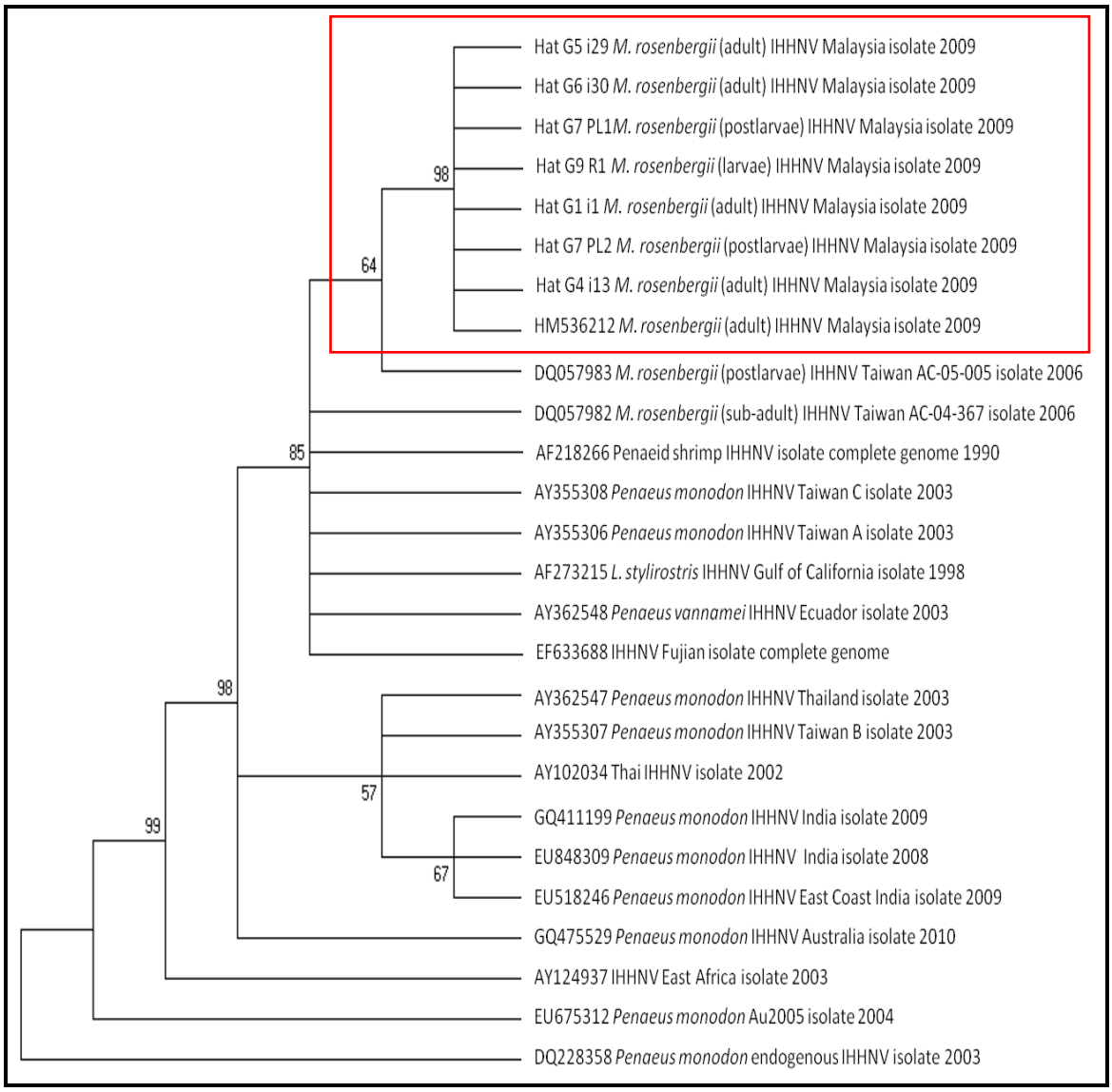
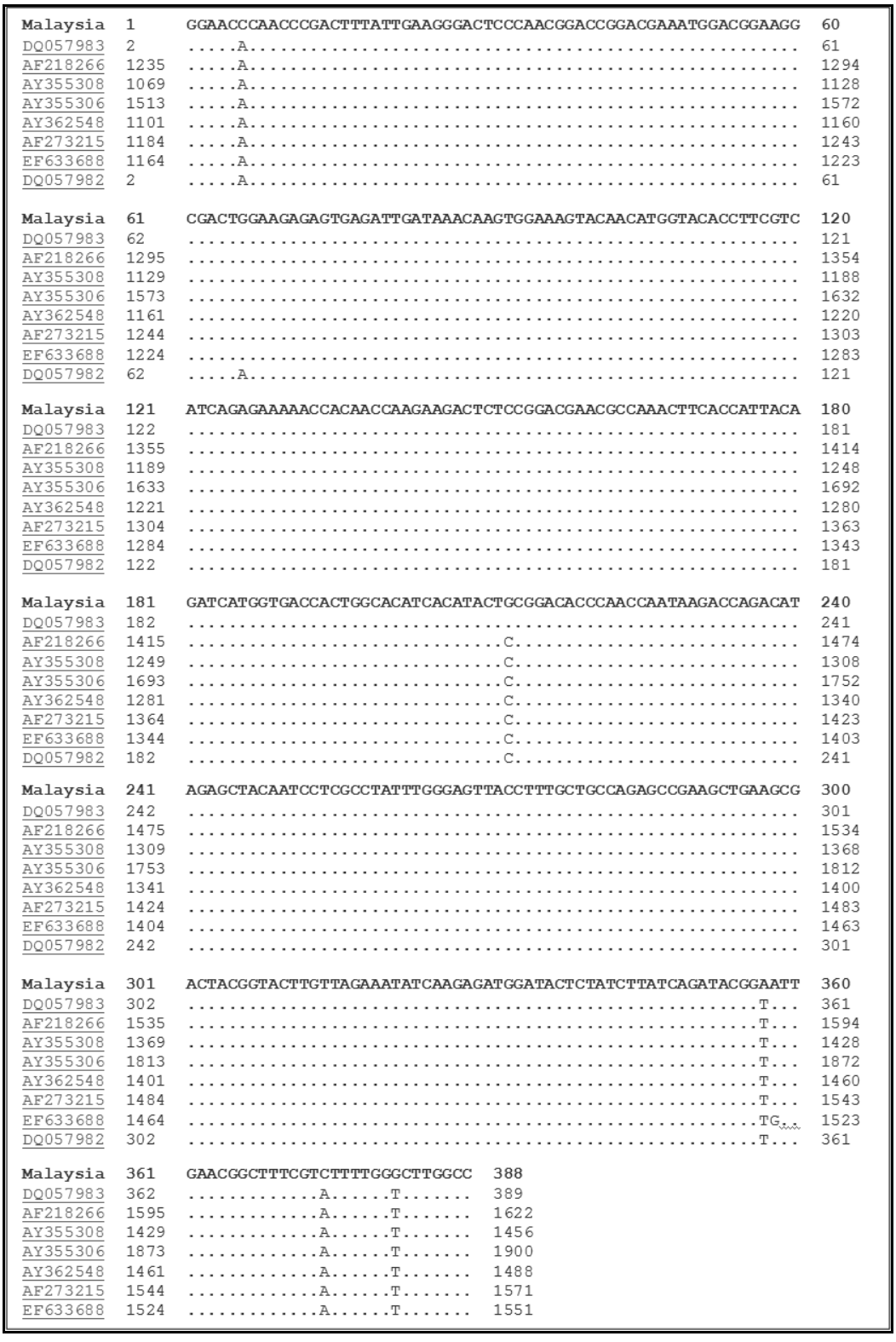


Figure 3.3 Maximum parsimony tree based on nucleotide sequence (389 bp) of the partial non-structural protein 1 gene for IHHNV were conducted using MEGA version 4 (Tamura *et al.*, 2007). Malaysian isolates from *M. rosenbergii* is clustered with Taiwanese strains isolated from *M .rosenbergii* postlarvae (red box).



```
Malaysia  1     GGAACCCAACCCGACTTTATTGAAGGGACTCCCAACGGACCGGACGAAATGGACGGAAGG  60
DQ057983  2     .....A.....................................................  61
AF218266  1235  .....A.....................................................  1294
AY355308  1069  .....A.....................................................  1128
AY355306  1513  .....A.....................................................  1572
AY362548  1101  .....A.....................................................  1160
AF273215  1184  .....A.....................................................  1243
EF633688  1164  .....A.....................................................  1223
DQ057982  2     .....A.....................................................  61

Malaysia  61    CGACTGGAAGAGAGTGAGATTGATAAACAAGTGGAAAGTACAACATGGTACACCTTCGTC  120
DQ057983  62    .....................................................  121
AF218266  1295  .....................................................  1354
AY355308  1129  .....................................................  1188
AY355306  1573  .....................................................  1632
AY362548  1161  .....................................................  1220
AF273215  1244  .....................................................  1303
EF633688  1224  .....................................................  1283
DQ057982  62    .....A...............................................  121

Malaysia  121   ATCAGAGAAAAACCACAACCAAGAAGACTCTCCGGACGAACGCCAAACTTCACCATTACA  180
DQ057983  122   .....................................................  181
AF218266  1355  .....................................................  1414
AY355308  1189  .....................................................  1248
AY355306  1633  .....................................................  1692
AY362548  1221  .....................................................  1280
AF273215  1304  .....................................................  1363
EF633688  1284  .....................................................  1343
DQ057982  122   .....................................................  181

Malaysia  181   GATCATGGTGACCACTGGCACATCACATACTGCGGACACCCAACCAATAAGACCAGACAT  240
DQ057983  182   .....................................................  241
AF218266  1415  ............................C............................  1474
AY355308  1249  ............................C............................  1308
AY355306  1693  ............................C............................  1752
AY362548  1281  ............................C............................  1340
AF273215  1364  ............................C............................  1423
EF633688  1344  ............................C............................  1403
DQ057982  182   ............................C............................  241

Malaysia  241   AGAGCTACAATCCTCGCCTATTTGGGAGTTACCTTTGCTGCCAGAGCCGAAGCTGAAGCG  300
DQ057983  242   .....................................................  301
AF218266  1475  .....................................................  1534
AY355308  1309  .....................................................  1368
AY355306  1753  .....................................................  1812
AY362548  1341  .....................................................  1400
AF273215  1424  .....................................................  1483
EF633688  1404  .....................................................  1463
DQ057982  242   .....................................................  301

Malaysia  301   ACTACGGTACTTGTTAGAAATATCAAGAGATGGATACTCTATCTTATCAGATACGGAATT  360
DQ057983  302   .....................................................T...  361
AF218266  1535  .....................................................T...  1594
AY355308  1369  .....................................................T...  1428
AY355306  1813  .....................................................T...  1872
AY362548  1401  .....................................................T...  1460
AF273215  1484  .....................................................T...  1543
EF633688  1464  .....................................................TG..  1523
DQ057982  302   .....................................................T...  361

Malaysia  361   GAACGGCTTTCGTCTTTTGGGCTTGGCC  388
DQ057983  362   ............A......T.......  389
AF218266  1595  ............A......T.......  1622
AY355308  1429  ............A......T.......  1456
AY355306  1873  ............A......T.......  1900
AY362548  1461  ............A......T.......  1488
AF273215  1544  ............A......T.......  1571
EF633688  1524  ............A......T.......  1551
```

Figure 3.4 Nucleotides of IHHNV Malaysian isolate (GenBank Acc. No. HM536212) of *M. rosenbergii* (broodstock) compared to sequences obtained from GenBank with 98 % similarities. The Malaysian sequence isolate shows only four nucleotide substitutions compared to Taiwanese strain GenBank Acc. No. DQ057982 and six nucleotide substitutions compared to DQ057983 with no deletion or insertion.

3.3.2 Sequence analysis using TCS

In this analysis, TCS calculated a 90 % parsimony connection limit of 12 steps and resulted in a haplotype network of the sequences of partial non-structural protein 1 gene for IHHNV.

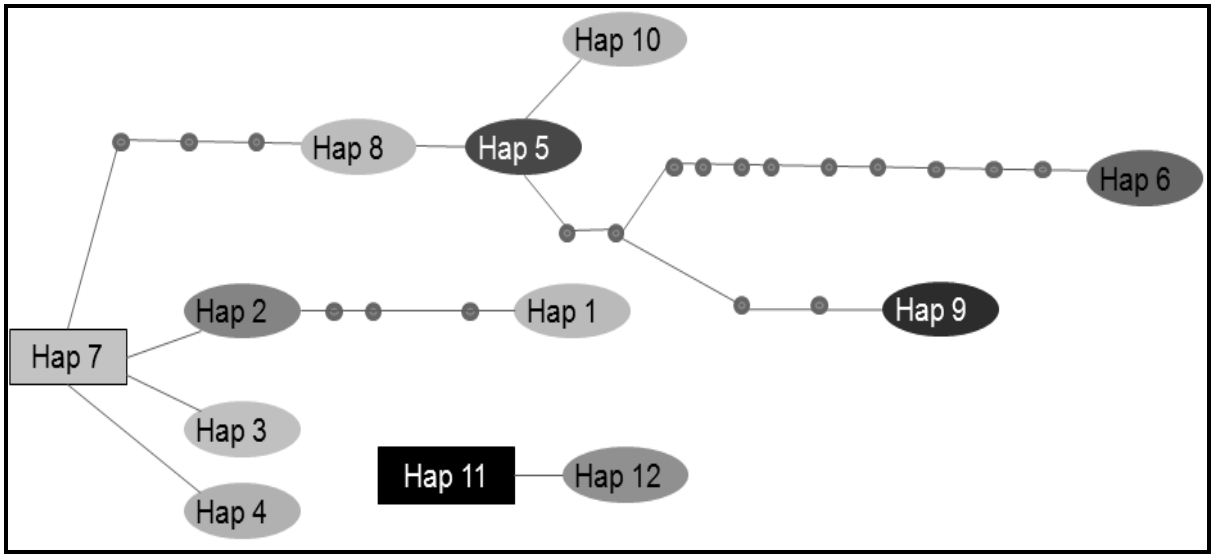


Figure 3.5 TCS haplotype networks showing genealogical relationships estimated among the partial non-structural protein 1 gene for IHHNV (Figure 3.3). The dots depict missing intermediate haplotypes that were not found in the analyzed individuals. The haplotype group can be referred in Figure 3.6.

Post.	6	34	35	38	41	61	64	66	73	75	78	98	99	100	118	131	153	157	162	175	180	186	202	207	209	212	213	222	228	232	244	245	255	256	261	262	305	313	317	319	357	358	374	381	Individual number	Genbank Acc. no
Hap	C	A	A	G	C	C	C	G	A	T	G	G	T	A	G	A	C	C	G	A	A	T	A	A	A	G	C	A	T	A	G	C	C	G	T	T	C	G	G	A	A	A	C	G		
1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	HM536212 (Malaysian isolate)
2	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	A	T	1	DQ057983
3	A	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	A	T	1	DQ057982
4	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	G	A	T	1	EF633688
5	A	G	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	A	.	.	.	.	.	C	.	C	.	.	.	.	.	.	C	.	.	.	G	T	.	A	T	2	AY102034, AY355307
6	A	G	.	.	.	.	.	.	.	A	A	.	C	.	.	.	.	.	A	G	.	C	G	G	.	C	.	.	.	T	.	.	.	C	.	A	A	G	T	.	A	T	1	AY124937
7	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	A	T	5	AF273215, AY355308, AY355306, AY362548, AF218266
8	A	G	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	C	.	.	.	.	T	.	A	T	1	AY362547
9	A	G	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	C	.	.	C	.	.	.	.	.	C	.	.	.	.	T	.	A	T	1	GQ475529
10	A	G	.	A	.	.	.	.	.	.	.	.	.	G	.	.	.	.	A	.	.	.	.	.	C	.	.	.	.	.	.	.	.	C	.	.	.	G	T	.	A	T	3	GQ411199, EU848309, EU518246
11	A	.	G	.	.	A	T	.	G	.	A	A	C	.	A	G	G	A	.	G	G	C	.	G	T	C	A	C	.	T	A	A	T	A	C	C	.	A	A	G	T	.	A	T	1	EU675312
12	A	.	G	.	.	A	T	.	G	.	A	A	C	.	A	G	G	A	.	G	G	C	.	G	T	C	A	C	.	T	A	A	T	A	C	C	T	A	A	G	T	.	A	T	1	DQ228358

Figure 3.6 Variable sites of the haplotypes of partial non-structural protein 1 gene for IHHNV.

3.3.3 Sensitivity analysis of the nested PCR primers

The lowest limit of nested PCR detection was determined by using a serial dilution of the purified plasmid, pTA-IHHNV. The primer combination GF1_F/GF1_R gave a DNA amplification product of 247 bp (Figure 3.7). A single and specific amplification product of 247 bp was observed with the limit detection of 10 copies of IHHNV plasmid. However, further analysis still need to be performed in order to confirm the sensitivity of this primer in different organs of the infected prawn.

Table 3.4 The serial dilution of the positive IHHNV plasmid stock used for the sensitivity test.

Tube No.	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Copy of IHHNV plasmid (copies/ul)	10	10^2	10^3	10^4	10^5	10^6	10^7	10^8	10^9

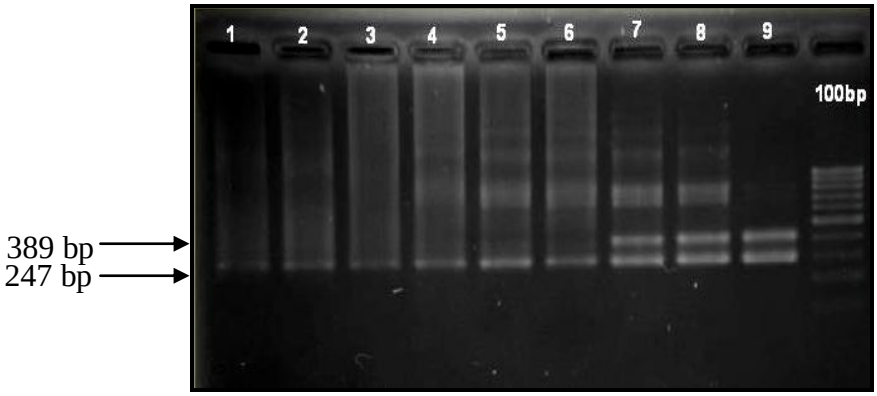


Figure 3.7 Agarose gel electrophoresis of the nested PCR reaction with the amplified product at approximately 389 bp and 247 bp (arrows). Lane 1: purified plasmid stock containing 10 copies of IHHNV plasmid per µl; Lane 2: containing IHHNV plasmid of 1 X 10^2 copies/µl; Lane 3: containing IHHNV plasmid of 1 X 10^3 copies/µl; Lane 4: containing IHHNV plasmid of 1 X 10^4 copies/µl; Lane 5: containing IHHNV plasmid of 1 X 10^5 copies/µl; Lane 6: containing IHHNV plasmid of 1 X 10^6 copies/µl; Lane 7: containing IHHNV plasmid of 1 X 10^7 copies/µl; Lane 8: containing IHHNV plasmid of 1 X 10^8 copies/µl; Lane 9: containing IHHNV plasmid of 1.01 X 10^9 copies/µl; Lane 100 bp: DNA Ladder (Fermentas).

3.3.5 Specificity analysis of the nested PCR primers

The specificity of the nested primers were tested on DNA samples extracted from HPV-infected prawn (source: Lab-Ind Resource, Selangor) and plasmid clones containing *M. rosenbergii* nodavirus (*Mr*NV)(Source: *Prof. Dr. S.G. Tan, University of Putra Malaysia (UPM)*). When the nested primers were tested with these two available prawn viruses, an amplification of 247 bp product was only observed with IHHNV purified plasmid (Figure 3.8).

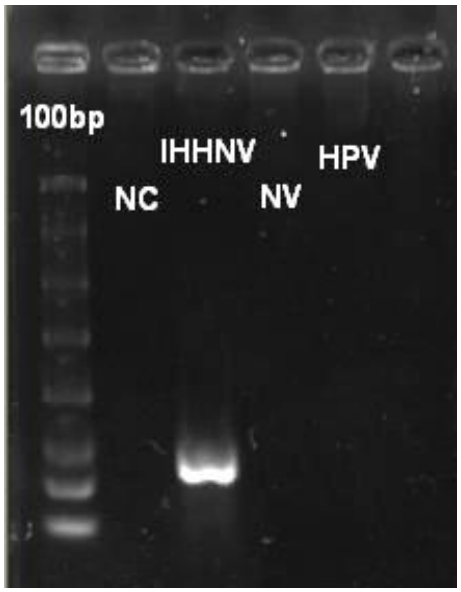


Figure 3.8 Agarose gel electrophoresis of the results of the specificity test of the nested primer. Arrow shows amplified 247 bp band corresponding to positive PCR result with IHHNV. 10 ng of each DNA and plasmid containing the available prawn viruses was used for PCR reaction. Lane 1: DNA Ladder 100bp (Fermentas); Lane 2: Negative control (NC); Lane 3: purified plasmid containing IHHNV plasmid of 389 bp ; Lane 4: purified plasmid clones containing *Mr*NV (NV) gene fragment; Lane 5: DNA extracted from HPV-infected prawn (HPV) as template.

3.4 DISCUSSION

The PCR assay for detection of IHHNV was optimized using DNA extracted from purified DNA containing IHHNV virus (*L. vannamei*) as the template. Optimal conditions were applied to screen for the infection of IHHNV in adult broodstocks samples obtained from the hatchery.

There were no clinical symptoms being observed on the prawn samples. Under the optimized condition a single 389 bp product was amplified and detected in six out of 30 berried female broodstocks collected from Sungai Rubana, Perak. In this study, the primer set IHHNV389F/R was used for screening purpose on wild berried broodstocks and postlarvae in the hatchery as it is commonly used to detect the non-structural protein of the virus. The results showed that the prevalence of IHHNV infection in wild female berried broodstocks was 20 % (6/30) (Table 3.3), but there was no clinical sign seen on the broodstocks. The findings were similar to that reported by Zhang in 2007, where all IHHNV PCR positive berried broodstocks showed no apparent symptoms indicating that the broodstocks were carriers of IHHNV (Zhang *et al.*, 2007).

The sequence of the 389-bp amplicon produced from Malaysian *M. rosenbergii* (broodstock) after amplification using IHHNV389F/R OIE primers was aligned and compared to the sequences of IHHNV available in the GenBank database. Based on the results of the alignment, the sequence could be suggested to have been derived from IHHNV infection (Figure 3.3). The identity of Malaysian IHHNV isolate from *M. rosenbergii* (GenBank Acc. No. HM536212) is suggested to be closely related to the Taiwanese IHHNV strains isolated from *M. rosenbergii* broodstock (GenBank Acc. No. DQ057982) and *M. rosenbergii* postlarvae (PL) (GenBank Acc. No. DQ057983) (Hsieh *et al.*, 2006) showing 98 % similarities. The Malaysian isolate also showed 98 % identities to six other IHHNV sequences available in the GenBank isolated from marine shrimp strains; GenBank Acc. No. AF218266 (Bonami *et al.*, 1990), Taiwan C strain (AY355308), Taiwan A strain (AY355306), Ecuador strain (AY362548) (Hsia *et al.*, 2003), AF273215 (Shike, 2000), and Fujian strain (EF633688).

TCS calculated a 90 % parsimony connection limit of 12 steps and resulted in a haplotype network of the sequences of partial non-structural protein 1 gene for IHHNV (Figure 3.5). There are 44 variable sites observed in all 12 haplotypes of partial non-structural protein 1 gene for IHHNV sequences (Figure 3.6). The sequence of 389-bp region, (Hap 1) showed only four nucleotide substitutions compared to Taiwanese strain GenBank Acc. No. DQ057982 (Hap 2) and six nucleotide substitutions compared to DQ057983 (Hap 3) with no deletion or insertion. The results suggest that there may potentially be more differences in other regions of the viral genome that could reflect the heterogeneity between the different isolates of *M. rosenbergii*. Since most of the published and commercialized IHHNV detection kits are based on the Hawaiian strains, this projected difference may be a reason as to why these kits were not able to detect IHHNV in Malaysian isolates (unpublished data).

A standardized nested PCR protocol that amplifies partial region of the non-structural protein 1 of IHHNV genome has been developed to overcome the problem of low virual copy number which occurred in the earlier study (unpublished data). The rationale of performing nested PCR instead of standard PCR is that these internal primers (nested primer; GF1_F/R) have the capability to reduce non-specific binding in amplification. In addition, nested primer will binds inside the first PCR product fragment which resulted in more specific amplification for IHHNV detection. The detection limit of the nested PCR assay was equivalent to 10 copies of IHHNV plasmid containing IHHNV389 region.

The specificity of the primer was validated using DNA and plasmid containing the available prawn viruses as templates. The concentration of each plasmid containing fragment of the viral DNA was 10 ng./ul. The two sets of primers produced the expected amplification products of 389 bp (external primer) and 247 bp (internal primer) in the presence of IHHNV DNA, whereas no such amplicons were obtained when the plasmid containing fragment of nodavirus (MrNV) DNA and hepatopancreatic parvo-like virus (HPV) were tested by nested PCR. The results suggested that the newly designed primer appears to be specific to detect the IHHNV nonstructural protein in *M. rosenbergii*. As the currently suggested primers for PCR detection of IHHNV are not capable of giving a specific and reliable result, it is hence showed the need of a specifically-designed primer set, coupled with a simple yet specific method for detecting IHHNV to overcome the detection problem. Nevertheless, the limitation of the PCR approach in detection of IHHNV in wild-caught freshwater prawn is that this method only amplifies short targets of interest (247 bp) which represents less than 1 % of the IHHNV whole genome (4 kb). Moreover, this approach can only detect the presence and absence of the virus DNA but cannot be used in detection of the infectious state of the disease.

The results in this chapter suggested that there are infections of IHHNV in freshwater prawn in Malaysia. This information will be used to enhance breeding programs in Malaysia hatcheries to cater for the growing needs of freshwater industry in producing high quality, domesticated potential broodstocks for the mass production of frys. Hence there is an important need to detect and control aquatic viral diseases by conducting comprehensive screening programs on broodstocks. This practice will also be practical for producing potential specific pathogen free (SPF) broodstock generations.

CHAPTER 4

SCREENING OF IHHNV IN GIANT FRESHWATER PRAWN, *Macrobrachium rosenbergii,* WILD BROODSTOCK

4.1 INTRODUCTION

Giant freshwater prawn which is native to Malaysia has the highest value in local and also international fisheries trade with the local price, RM50-RM60 per kilogram, depending on the size of the prawn. Currently the culture of this species, locally known as 'udang galah' are experiencing rapid expansion in many states in Malaysia. The aquaculture production for giant freshwater prawn in Malaysia has increased from 551.6 tonnes (2009) to 619.22 tonnes (2010) (www.dof.gov.my). However, little is known about the true disease status of this species which might cause economic losses and affect the growth of aquaculture industry.

In the previous study (Chapter 3), screening of IHHNV was conducted in a freshwater prawn hatchery. The sequence obtained from the screening result confirmed there are infections of IHHNV in freshwater prawn in Malaysia. By looking at the scenario of freshwater farming in Malaysia, most of the farmers use wild freshwater prawn as their broodstock without considering the optimal size or concerns on disease screening. This situation may increase chances for pathogens from the wild entering the culture which may result in mortality or slowing the growth of the prawn.

Hence, in Chapter 3, a pair of specific primers has been designed and will be use in this study as screening tools for IHHNV detection. Two rivers which are located in the state of Perak and the state of Negeri Sembilan in Peninsula Malaysia have been recognized as the common locations where the farmers usually catch the berried giant

freshwater prawn as their potential broodstock for their farm. With the assistance from Fisheries Research Institute (FRI) Penang, sampling for this study was conducted at these rivers throughout the year 2009/2010. The present study aimed to obtain prevalence of IHHNV infection as baseline data and to potentially assist farmer to recognize the best weight of freshwater prawn for broodstock collection which would minimize disease transfer to the hatchery.

4.2 MATERIALS AND METHODS

The experiment carried out to achieve this objective was to perform nested polymerase chain reaction (PCR), histopathological studies and *in situ* hybridization (ISH).

4.2.1 Study area and sample collection

The giant freshwater prawn broodstock were collected from two selected areas in Malaysia which are Sungai Rubana, Perak (latitude: 3^{o} 58' 60 N; longitude 100^{o} 55' 0 E) and Sungai Timun, Negeri Sembilan. (latitude 2^{o} 26' 44 N; longitude 102^{o} 3' 38 E). A total of 537 and 419 wild giant freshwater prawns were captured randomly from each location; Sungai Rubana (Perak), and Sungai Timun (Negeri Sembilan) respectively between January 2009 and December 2009. From the raw data obtained during the necropsy, all the specimens were grouped into six groups of weight classes and the prevalence of IHHNV infection will be discussed with respect of these groups (Table 4.1). During necropsy, pleopods of the prawns was collected and preserved in 95 % ethanol for DNA extraction.

Table 4.1 Groups of weight classes of *M. rosenbergii* collected from Sungai Rubana, Perak and Sungai Timun, Negeri Sembilan.

Weight (g)	Number of prawn captured in each location	
	Sungai Rubana, Perak	Sungai Timun, Negeri Sembilan
≤ 20	88	237
21 – 40	323	123
41 – 60	62	29
61 – 80	39	10
81 – 100	16	7
> 100	9	13
TOTAL	537	419

4.2.2 Molecular Study

The protocol of IHHNV detection using nested PCR approach was performed using the method obtained in the OIE manual (OIE, 2003) and the primer used in the screening of IHHNV infection was IHHNV389. This primer is chosen because it has been suggested to amplify the non-structural protein – coding region (ORF1) of the IHHNV genome. Another set of primers was used to amplify the specific region of non-structural protein 1 (NS1) which are the nested primer designed from the Malaysian IHHNV isolate (HM536212) (Chapter 3).

(a) DNA extraction

Genomic DNA was extracted using Qiagen DNAeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions. This kit is designed for rapid purification of total DNA including fresh and frozen animal tissues. Approximately, 25 mg tissue was cut and placed in 1.5 ml microcentrifuge tube. 180 µl of Buffer ATL was added into the tube.

Then, 20 µl of Proteinase K was added into the microcentrifuge tube. The mixture was mixed thoroughly by vortexing and was incubated at 56 ^{o}C until the tissue was completely lysed. During incubation the tube was vortex occasionally to disperse the sample. After vortexing for 15 seconds, 200 µl of Buffer AL was added to the sample and the solution was mix thoroughly. Then, 200 µl of ethanol was added and again the mixture was mix by vortexing the tube. The mixture solution was transferred into the DNeasy Mini spin column by pipetting and the column was placed in a 2 ml collection tube which was provided by manufacturer. The spin column was centrifuge at 8,000 rpm for a minute and the collection tube contained flow-through was discarded.

The DNeasy Mini spin column was placed in a new 2 ml collection tube which was provided in the kit, and approximately 500 µl of Buffer AW1 was added into the column. The spin column was centrifuge at 8,000 rpm for 1 minute and the collection tube contained flow-through was discarded. The DNeasy Mini spin column was placed again in a new 2 ml collection tube and 500 µl of Buffer AW2 was added into the column. The spin column was centrifuge at 14,000 rpm for three minutes to dry the DNeasy membrane .The collection tube contained flow-through was discarded and the spin column was placed in a clean 1.5 ml microcentrifuge.

Then 200 µl of Buffer AE was added directly onto the DNeasy membrane and the tube was incubated for a minute at room temperature. Lastly, the tube was centrifuged at 8,000 rpm for a minute in order to elute the DNA. The quality of the extracted DNA was analysed by gel electrophoresis and the quantity was measured using a NanoDrop spectrophotometer (NanoDrop Technologies, Inc.). The DNA extracted from both selected area were subjected to nested PCR for screening of IHHNV (See Section 3.2.7).

(b) Sequence analysis

The two sets of primers produced the expected amplification product of 389 bp (external primer)(OIE, 2003) and 247 bp (internal primer) in the presence of IHHNV DNA. The identity of 247 bp product was confirmed by DNA sequencing. The sequences were compared with related sequences available in GenBank in NCBI using NCBI-BLAST. Maximum parsimony phylogenetic tree (1,000 bootstraps) was constructed using MEGA software (Tamura *et al.*, 2007).

(c) Statistical analysis

The prevalence of IHHNV infection in *M. rosenbergii* captured from both sampling locations were determined (Bush *et al.*, 1997). The samples were grouped into six different weight classes. A one-way ANOVA test was performed to find significant differences in the IHHNV prevalence between weight classes. All statistical analysis was executed at the significant level of 0.05 using the statistical program SPSS Statistic 17.0.

4.2.3 Histopathology study

For the histopathological studies, normal histology was done according to Bell and Lightner (1988). The samples were cut and immersed in Davidson's fixative solution. Then, the samples were transferred to 70 % alcohol for subsequent histo-slide preparation.

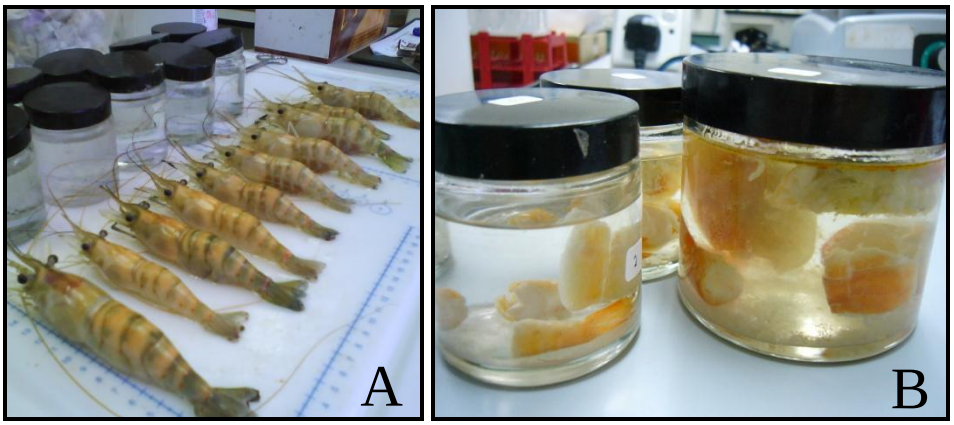


Figure 4.1 Histology samples. (A) *M .rosenbergii* sample that were injected with Davidson's solution (DS) fixative solution. (B) Samples were immersed in DS for 24 h to ensure tissue fixation.

(a) Tissue processor

The desired tissue which was enclosed within the embedding cassette was processed in an automatic tissue processor. The prawn tissue was processed using the following routine; 70 % ethyl alcohol (EtOH) (two separate one hour baths), 80 % EtOH (two separate one hour baths), 95 % EtOH (two separate one hour baths), 100 % EtOH (two separate one hour baths), clearing agent (xylene) (two separate one hour baths), and paraffin (two separate one hour baths).The soft, tissues were embedded in a hard paraffin block, which was then placed in a mold containing more molten wax and then it was allowed to cool and harden.

(b) Embedding

The embedding cassettes containing the dehydrated and infiltrated tissue samples were transferred to the cassette bath which was filled with molten wax to prevent the solidification of the wax. After the embedding was finished, the tissue was then sectioned into very thin (4 - 5 µm) sections using a microtome. Finally, the sections were stained with haematoxylin and eosin (Sheehan *et al.*, 1980).

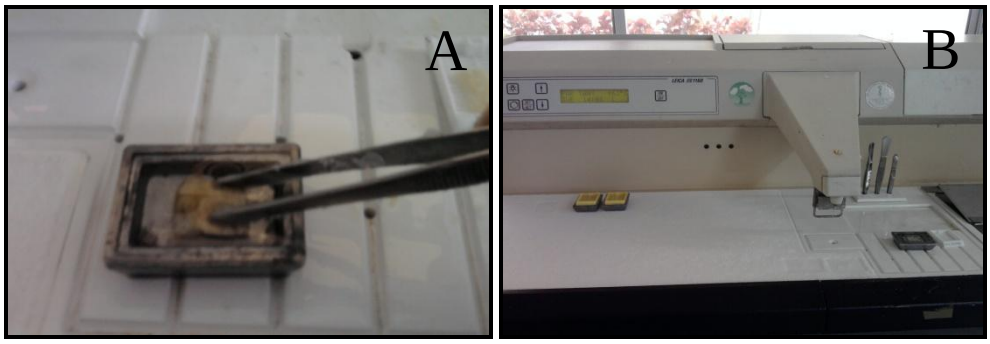


Figure 4.2 (A) Fixed sample is embedded in the paraffin. (B) Embedded machine that was used for this purpose.

(c) Sectioning

Trimming process begins by adjusting the aligned level of paraffin block with the edge of the knife on the microtome holder. The surface was trimmed to expose as large an area of the tissue as possible. The trimming can begin with the thickness gauge at 10 – 15 micrometer. When the section appears to be cutting evenly in ribbons, the microtome thickness gauge is set to 4 µm. The thickness section of four micrometers was gently teased off with the aid of the hair brush and the fine forceps before placed into water bath. The sample identity (ID) was written at the end of the glass slide with pencil. The slide should be dried on a hot plate for two hours or overnight in an incubator oven set at 40 ^{o}C.

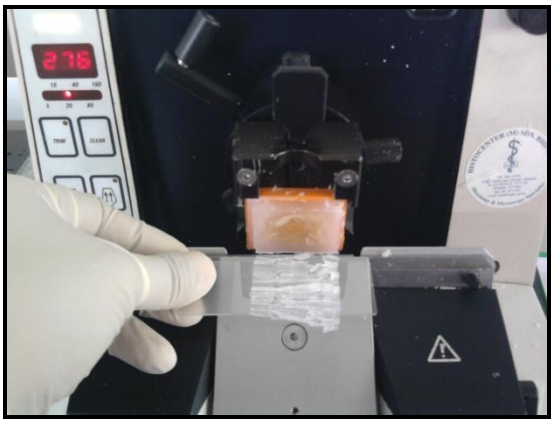


Figure 4.3 The sectioning of embedded paraffin using microtome.

(d) Staining with Hematoxylin and Eosin (H & E)

The histology slides were stained using Hematoxylin and Eosin (H&E) procedure (Sheehan *et al.*, 1980). After sectioning has done and the slides were incubated in the oven at 40 ^{o}C . Staining method begins with tissues dehydration using the following routine in Appendix A (Figure 4.4). The slide was placed in the last xylene until ready for cover slips. The slide was mounted with DPX before the section is covered with cover-slips. Finally, the slides were ready for examination under bright-field microscope (Leica DM5000B) which connected to a digital camera (Leica DFC 320) associated with computer software (Leica QWin).

Figure 4.4 Staining booth and the collection of histo-slide which were ready for microscopy observation.

4.2.4 *In situ* hybridization (ISH)

The *in situ* hybridization method used a DIG-labeled DNA probe which specific for IHHNV and generally it followed the method outlined by Mari *et al.* (1993) and Lightner D. V. (1996).

The embedded tissue in the paraffin was sectioned at 4 – 6 µm thickness and was put on the positively charged microscope slide. The slides were arranged in a slide rack and heated in an oven at 60 ^{o}C for 45 minutes. The tissues were dehydrated in the staining centre using the following routine; xylene (three separates five minutes baths),

absolute alcohol (two separate one minute baths), 95 % alcohol (two separate one minute baths), 80 % alcohol (two separate one minute baths), 50 % alcohol (one minute bath), distilled water (six rinses) and 1X TNE (five minutes bath).

The slide was flattened in a humid chamber and 1 ml of Proteinase K solution was pipetted on the tissue section. The slides were incubated for 15 minutes at 37 ^{o}C in a humid chamber. The slides were put back in the slide rack and the sections were fixed in 0.4 % formaldehyde for five minutes at room temperature. Then, the slides were incubated in 2X SSC for five minutes at room temperature. Later, 0.5 ml of prehybridisation buffer was added on the flat slide and incubated for 30 minutes at 37 ^{o}C in a humid chamber. The DIG-labeled probe was boiled for 10 minutes and was quenched on ice just prior to use. The probe was diluted to 25 ng ml^{-1} in prehybridisation solution and the tissue was covered with 250 µl of that solution. The slides were incubated overnight at 37 ^{o}C in a humid chamber.

During the incubation, the wash buffer was pre-warmed at 37 ^{o}C. After the hybridisation process, the slides were placed back in the slide rack and washing step followed with these routine; 2X SSC (two separate five minutes baths), 1X SSC (two separate five minutes baths), 0.5X SSC (two separate five minutes baths) and 0.1X SSC (two separate five minutes baths). Then, the slides were washed in Buffer I for five minutes at room temperature. The slides were placed flattened in a humid chamber and Buffer II was used for blocking the slide. The slides were incubated for 15 minutes at 37 ^{o}C. The anti-DIG AP conjugate was diluted to 1/1000 in Buffer II (1 µl anti-DIG AP per 1 ml buffer). The tissues were covered with 500 µl of diluted conjugate and incubated in a humid chamber for 45 minutes at 37 ^{o}C. Then, the slides were washed twice in Buffer

I for five minutes each time at room temperature. The slide was washed once again with Buffer III for five minutes.

The development solution was prepared by adding 4.5 µl NBT per 1 ml Buffer III and mixed them well. Then, 3.5 µl X-phosphate per 1 ml of the solution was added and mixed them well. 500 µl of the mixture was added on the slide and the slides were incubated in a dark humid chamber for 2 – 3 hours at room temperature. The reaction was stopped by returning the slides to the slide rack and the slides were washed in Buffer IV for 15 minutes at room temperature. The slides were counterstained by dipping in 0.5 % aqueous Bismarck Brown Y for five minutes. Then the slides were dehydrated in the staining centre with the following routine; 95 % alcohol (three separate one minute baths), absolute alcohol (three separate one minute baths), xylene (four separate one minute baths).

The slide was placed in the last xylene until ready for mounting and cover slips. The slide was mounted with DPX before the section is covered with cover-slips. Finally, the slides were ready for examination under bright-field microscope (Leica DM5000B) which connected to a digital camera (Leica DFC 320) associated with computer software (Leica QWin).

4.3 RESULTS

The present study was carried out to determine the prevalence of IHHNV infections in wild-caught giant freshwater prawn, *Macrobrachium rosenbergii* broodstock captured in two selective areas in Malaysia throughout one year period.

4.3.1 Gross signs of IHHNV

In this study, the screening results by nested PCR showed that IHHNV infection were common in the adult prawn, *M. rosenbergii* with prevalence of 48.7 % (Negeri Sembilan) and 64.4 % (Perak) (Table4.2). The economic impact caused by IHHNV in wild-caught *M. rosenbergii* was unknown as all the infected prawns did not show any mortality. However during necropsy, runt deformity syndromes (RDS) gross signs such as symptoms of black patches on telson and pleopods were seen on the infected prawns (Figure4.5) and deformity of rostrum (Figure 4.6 and Figure 4.7).

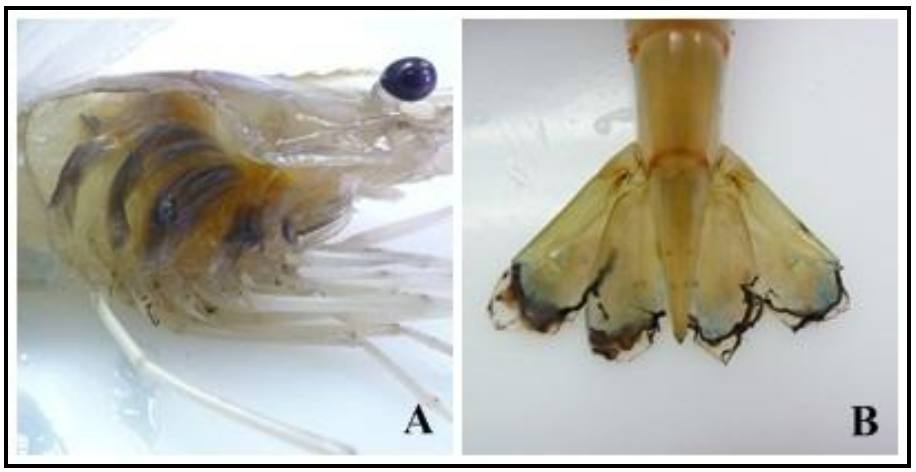


Figure 4.5 Symptom of (A) black gill and (B) black patches on telson of examined samples.

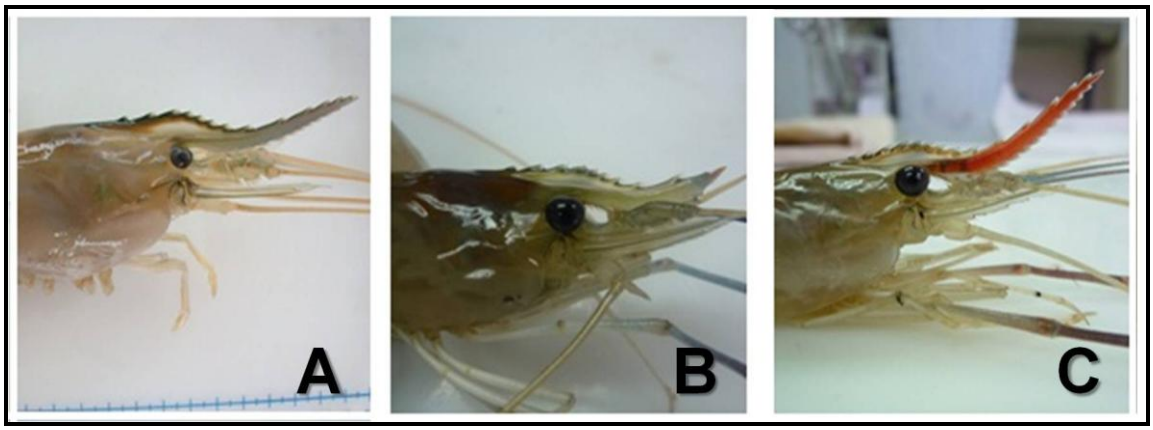


Figure 4.6 Deformity of rostrum were seen on the infected samples during study observation. Lateral view of (A) normal rostrum of *M. rosenbergii*; (B) short rostrum; (C) reddish rostrum.

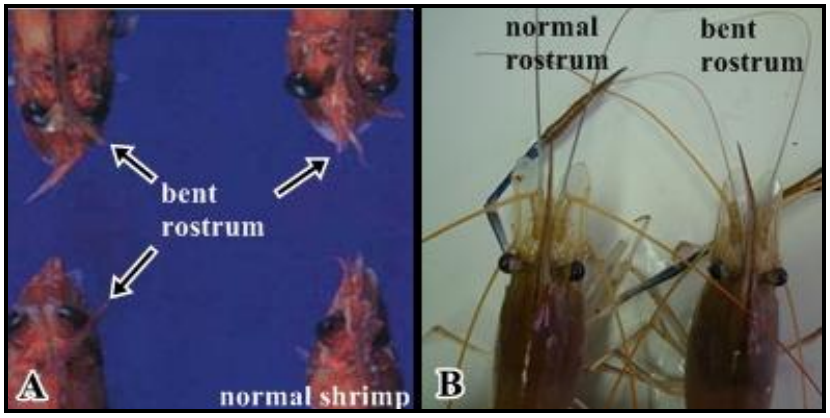


Figure 4.7 Deformity of rostrum. Dorsal view of (A) reference picture of juvenile *L. vannamei* showing bent rostrum (Bondad-Reantaso *et al.*, 2001); (B) studies observation: adult *M. rosenbergii* showing bent rostrum.

4.3.2 Prevalence of IHHNV in *M. rosenbergii* captured in Sungai Rubana, Perak and Sungai Timun, Negeri Sembilan

M. rosenbergii broodstocks with average weight of 32.0±17.8 g and 24.2±15.2 g were captured from Sungai Rubana, Perak and Sungai Timun, Negeri Sembilan respectively with sampling period of one year (January – December 2009). Throughout the screening process by nested PCR, prevalence of IHHNV in Sungai Rubana and Sungai Timun is 64.4 % and 48.7 % respectively (Table 4.2). In the present study, prawns captured in Sungai Rubana showed higher prevalence of IHHNV infection as compared to the prawns of Sungai Timun.

Table 4.2 Prevalence of IHHNV in *M. rosenbergii* sampled from two areas in Malaysia by using nested PCR.

Location	No. of prawn examined (n)	No. of IHHNV infected prawn	Prevalence of IHHNV infected prawn through the year (%)	IHHNV infected prawn's weight (Mean Weight ±Std.Dev)
Sungai Rubana, Perak	537	346	64.4	31.99 ± 17.79
Sungai Timun, Negeri Sembilan	419	204	48.7	24.2 ± 15.15

4.3.3 The prevalence of IHHNV infection with respect to the six weight classes of *M. rosenbergii*

IHHNV prevalence was determined from the nested PCR screening results and was classified into six classes based on prawn's weight (Table 4.3). The results showed the prawn with size less than 40 g has higher prevalence of IHHNV infection as compared to other weight classes. The highest prevalence of IHHNV was found in Sungai Rubana and Sungai Timun in the group weight of less than 20 g and 21-40 g with the prevalence of 75.0 % and 60.2 % respectively. The differences between the prevalence of IHHNV that were recorded in six different weight classes of *M. rosenbergii* from Sungai Rubana were statically significant ($p < 0.05$). A one-way ANOVA was also conducted to find significant differences in the IHHNV prevalence between weight classes in Sungai Timun prawns. There was also a significant differences between the group ($p<0.05$). Tukey post-hoc comparisons of the six weight classes indicate that the 21 – 40 g weight class (M= 1.40, 95 % Cl [1.31, 1.49]) gave significantly higher preference ratings than the >100g weight class (M= 1.92, 95 % Cl [1.76, 2.09]), p=0.04. Comparisons between other weight classes within the groups were not statistically significant at $p< 0.05$.

Table 4.3 Prevalence of IHHNV in *M. rosenbergii* captured from Sungai Rubana, Perak and Sungai Timun, Negeri Sembilan based on weight range.

Weight (g)	Number of IHHNV infected prawn/ Total of examined prawn (Prevalence, %)	
	Sungai Rubana, Perak	Sungai Timun, Negeri Sembilan
≤ 20	66/88 (75.0)	110/237 (46.4)
21 – 40	214/323 (66.3)	74/123 (60.2)[a]
41 – 60	37/62 (59.7)	12/29 (41.4)
61 – 80	17/39 (43.6)	4/10 (40.0)
81 – 100	8/16 (50.0)	3/7 (42.9)
> 100	4/9 (44.4)	1/13 (7.7)[b]
TOTAL	346/ 537 (64.4)	204/419 (48.7)

[a] and [b] represented the significant group of the prevalence of IHHNV among the weight classes

4.3.4 Sequence alignment using MEGA 4

For the confirmation of positive amplicons, 15 out of 204 PCR products (partial non-structural protein 1; 247 bp) of Negeri Sembilan isolates and 5 out of 346 PCR products of Perak isolates had been sent for sequencing. Only few PCR amplicons were sequenced because of the money constraint for sequencing service. Moreover, most of the PCR amplicons that were sequenced showed similar nucleotide identities. The partial non-structural protein 1 gene sequences (247 bp) of IHHNV obtained from this study were compared with related sequences from GenBank database using NCBI-BLAST program. Malaysian isolate showed 100 % identities to GenBank Acc. No. DQ057983sequence isolated from postlarvae *M. rosenbergii* (Hsieh *et al.*, 2006) and 99 % identities similar with seven other IHHNV strains isolated from marine shrimps. The maximum parsimony phylogenetic tree (1000 bootstraps) was constructed using MEGA software (Tamura *et al.*, 2007).

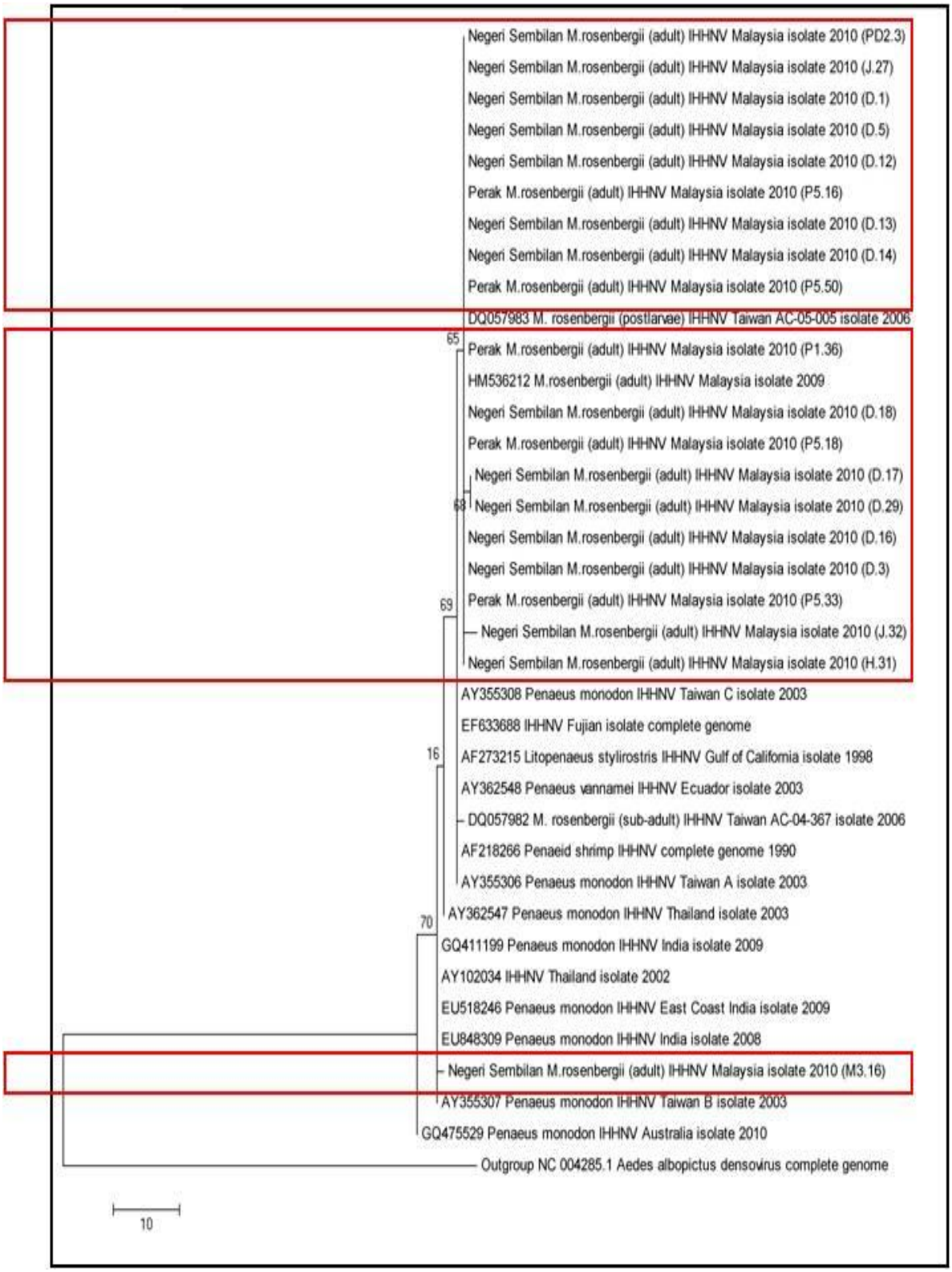


Figure 4.8 Phylogenetic tree generated from aligned *M. rosenbergii* and shrimp sequences available in GenBank. Aligned sequences consisted of partial non-structural protein 1 gene sequence (247 bp) were conducted using MEGA version 4 (Tamura *et al.*, 2007). Malaysian isolates of *M. rosenbergii* is clustered together with Taiwanese strain (DQ057983). Red boxes represent isolates from Malaysia.

4.3.5 Histopathology Examination

A total of 125 prawns from Sg. Timun, Negeri Sembilan and 100 prawns from Sg. Rubana, Perak are randomly collected for screening of IHHNV infection by using histology approach (Table 4.4). All the samples have been processed according to normal histology procedure by Bell and Lightner (1988) and had already been analyzed by microscopic observation. A one-year period for sample collection for histology was carried out together with sampling for the molecular study. However, both studies used different individual prawn from the same location due to limited samples. During the study period, all the prawn samples were purchased from fishermen nearby the river. The limited samples issue were pointed out due to the prawn catch was low during early rainy season and sometimes we could not get enough samples per month for both molecular and histology studies. Under histological examination, pathological changes were detected on the epithelial cells of the connective tissue and muscle tissues (Figure 4.9, Figure 4.10, and Figure 4.11). Few inclusion bodies were observed and compared with reference slides obtained from Prof. Lightner, University of Arizona, USA (Figure 4.12).

Table 4.4 Samples examined by histopathological method

Location	Sg. Timun, Negeri Sembilan		Sg. Rubana, Perak	
	No. of sample analysed	Prevalence of IHHNV infected (%)	No. of sample analysed	Prevalence of IHHNV infected (%)
TOTAL	**125**	**17/125 (13.5)**	**100**	**21/100 (21)**

(a) Pathological effects of IHHNV infection on hepatopancreas

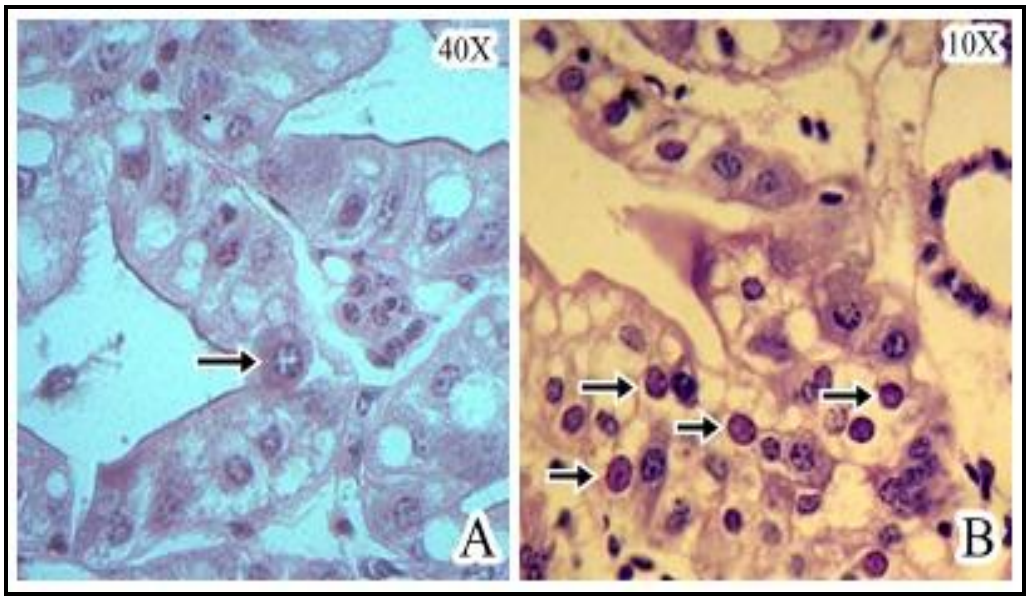


Figure 4.9 Hematoxylin and Eosin (H&E) histology of hepatopancreas of *M. rosenbergii* from Perak. (A) Normal epithelial cell at the connective tissue (arrow); (B) the nuclei of epithelial cells were hyperthrophied with margination of chromatin (arrows) and lacked clear halo information which known as Cowdry type B INIs indicated the IHHNV infection.

(b) Pathological effects of IHHNV infection on connective tissue of muscle

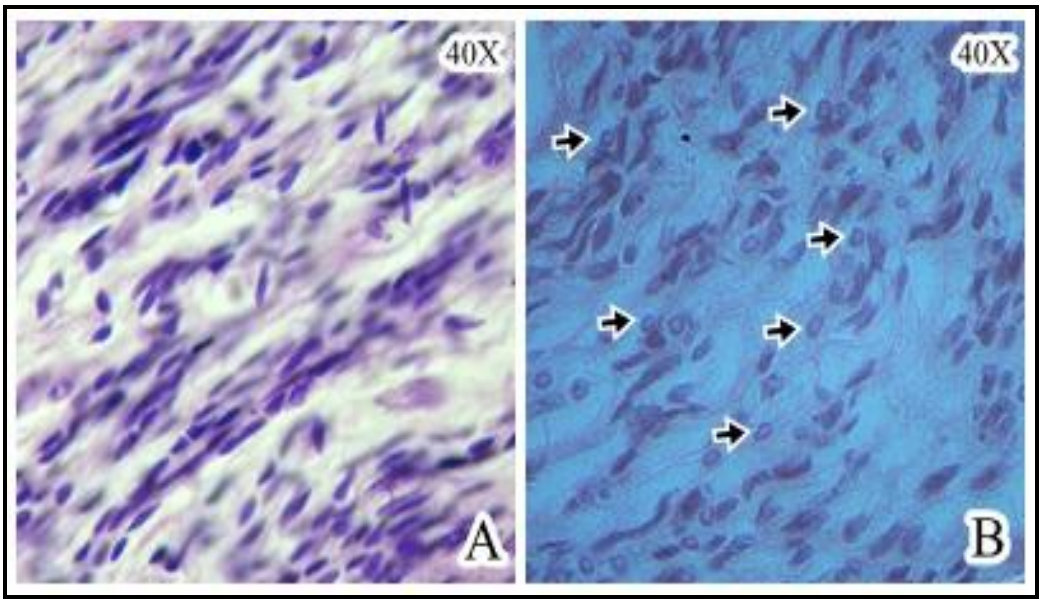


Figure 4.10 Hematoxylin and Eosin (H&E) histology of muscle's connective tissue of *M. rosenbergii* from Negeri Sembilan. (A) Normal histology structure of connective tissue. (B) A lot of inclusion bodies (Cowdry Type B-like inclusion) are found on the connective tissue of *M. rosenbergii*.

(c) Some other inclusion bodies found on the examined samples

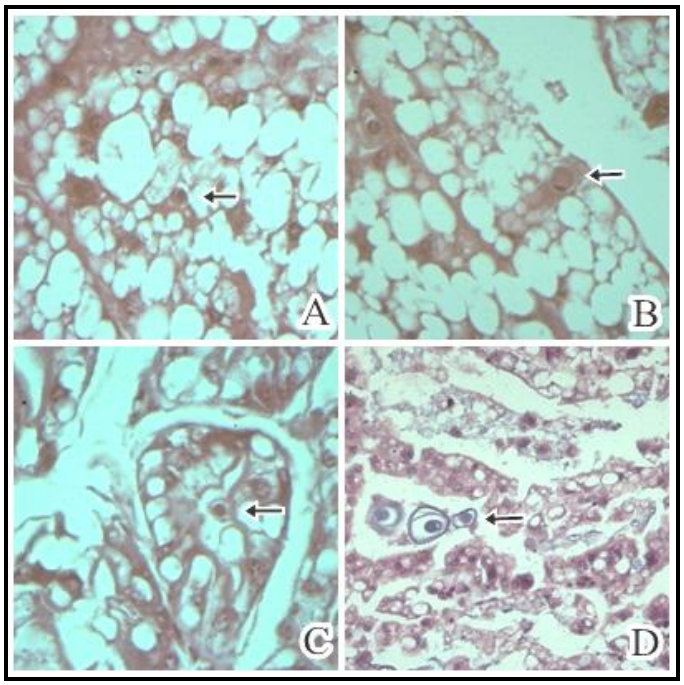


Figure 4.11 Hematoxylin and Eosin (H&E) histology of some other inclusion bodies found on the samples collected from both sampling sites. (A) Cowdry Type A-like inclusion body; (B) Cowdry Type B-like inclusion bodies; (C) Unknown inclusion bodies found in the epithelial cell of hepatopancreatic tissue; (D) The presence of hepatopancreatic lesions of basophilic inclusion indicates HPV infection.

(d) Comparison of IHHNV pathological effects found on *M. rosenbergii* and penaeid species.

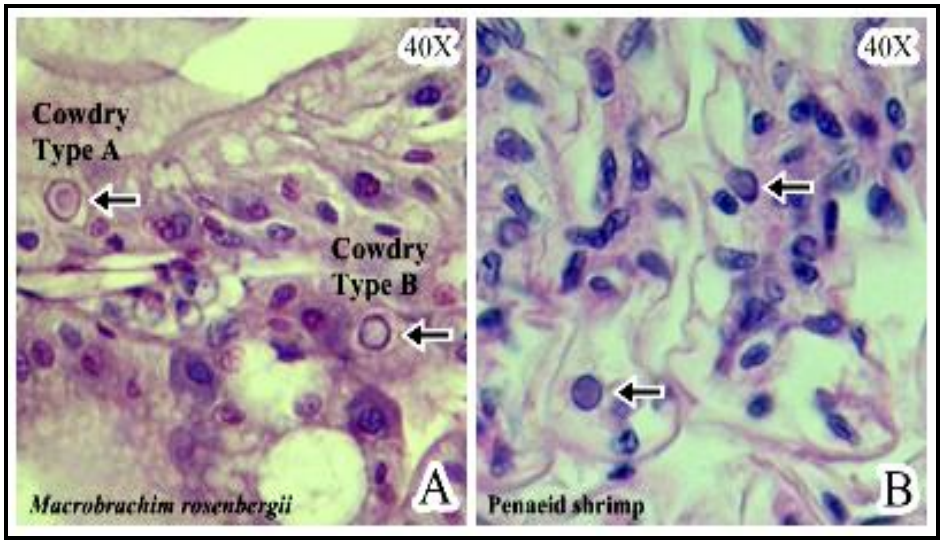


Figure 4.12 Hematoxylin and Eosin (H&E) histology of both infected prawn and shrimp. (A) Inclusion bodies Cowdry Type A & B were found on the epithelial cell of hepatopancreatic tubular. (B) The Cowdry Type B (arrow) found on the IHHNV infected shrimp which was used as reference slide. (Source: Prof. Dr. Lightner, University of Arizona, USA).

4.3.5 *In situ* hybridization detection

In situ hybridization (ISH) of IHHNV using DNA probe is one of the detection methods that may provide greater diagnostic sensitivity than conventional histology approaches. For the IHHNV infection confirmation by ISH, dark-blue or black precipitation can be observed on the slides which mark sites where IHHNV DNA is present. In present studies, histology slides with suspected inclusion bodies were used for ISH as confirmation of infection.

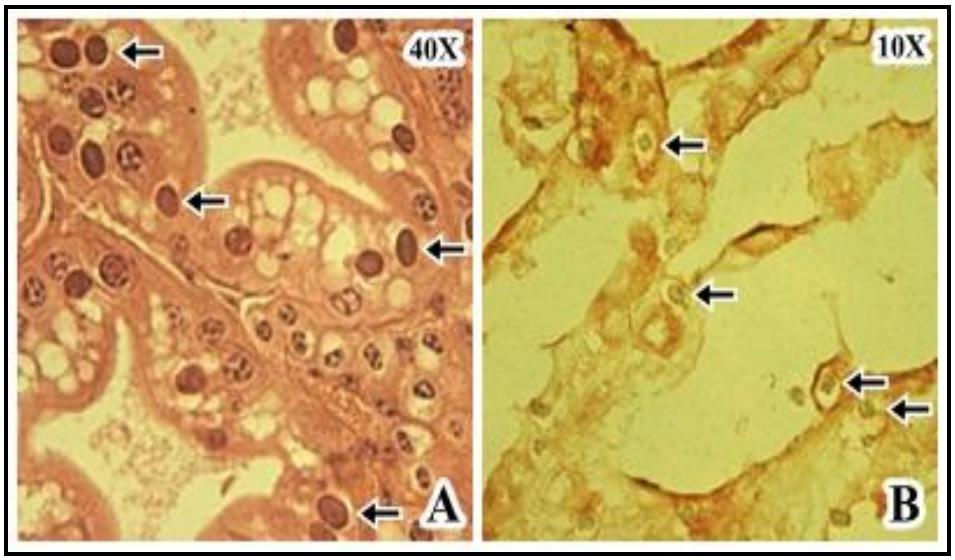


Figure 4.13 *In situ* hybridization detection using DNA probe of IHHNV (B) showed faint black precipitation as compared with histology slide stained with H&E stain (A).

4.4 DISCUSSION

M. rosenbergii were found to be infected with high prevalence at both sampling sites with gross sign of bent rostrum in some of the examined samples (Figure 4.7). Black gill symptom and black patches or spots were seen on the pleopods and telson of the organisms (Figure 4.5). The black spot lesions found on the prawns' bodies are due to melanisation which is a consequence of inflammation. Some RDS symptoms such as short rostrum were also seen on some prawns. Additionally from the data collected, it is showed that throughout the year 2009/2010, most of the prawns captured in both rivers were small in size with the weight of 40 g or below. The finding indicated that the prawns's sizes are greatly decreasing over the years. If this continues, it might lower prawns' market price value and cause economic disorder in the freshwater industry. Although RDS is suspected to be associated with IHHNV, the cause of RDS is still largely unknown.

In the present study that applies nested PCR detection, the prevalence of IHHNV infection in wild *M. rosenbergii* captured in Sg. Rubana and Sg. Timun may be considered as high with the percentages of 64.4 % and 48.7 % respectively (Table 4.2). By looking at the percentages, it is surprising that almost 50 % of the wild giant freshwater prawn broodstocks were infected with IHHNV yet there is no mortality case has reported or documented thus far. It has been reported that back in the 1990s, IHHNV was found to have been introduced to America by way of live *P. monodon* stocks from Asia (Lightner, 1996).

In a previous study and application of PCR primers, 3 kb fragments of IHHNV genome were sequenced from geographical specimens of infected samples and the results revealed that IHHNV of Hawaii was almost identical to the Philippines (Tang *et*

al., 2003). We could speculate that the inflow of IHHNV had already happened in Malaysian streams and the virus had somehow remained latent in *M. rosenbergii*. This could also account for the detection of the virus only using nested PCR instead of the conventional PCR using the OIE primers (OIE, 2003) as the viral load would be low in latent infections.

The prevalence of IHHNV was grouped into six classes based on the prawn's weights. Statistically, the result showed a significant difference between the weight classes ($p < 0.05$). According to the infection group, prawns with size less than 40 g have higher prevalence as compared to other weight classes (Table 4.3). However, this result could not be compared with other shrimp species as there is no documentations or reports on the distribution of size of infected organisms' size. Generally, female prawns become reproductively mature at the weights of between 15 and 20 g (New, 2005). However, based on our statistical data, we could suggest that the suitable weight for giant freshwater prawn broodstock candidate is 40 g and above since the selection of broodstocks from wild environment is typically based on ripeness of eggs without consideration to body size. Nevertheless, disease screening should be performed before the spawning and on the larvae after the egg hatched. With early detection of this disease, emergency harvesting could be possible to prevent the spread of the disease.

In the screening of IHHNV, 247 bp amplicon produced from positive samples by nested PCR were sent for sequencing to obtain the partial non-structural protein 1 gene sequence. There were 15 isolates from Sg. Timun and 5 isolates from Sg.Perak that were sequenced and were compared with the sequences available in GenBank using NCBI-BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The Malaysian isolates that we

obtained showed 100 % identities with Taiwanese IHHNV isolate from postlarvae *M. rosenbergii* (GenBank Acc. No. DQ057983) and 99 % similar nucleotide identities with seven other strains isolated from marine shrimp. All Malaysian isolates were clustered together with the Taiwanese strains. The sequence results were similar to the finding in previous chapter (Chapter 3) (Hazreen Nita *et al.*, 2011) and we believe that the virus could come from other aquatic animal in nearby habitats.

In this study, another screening method that was used to identify infected prawn is histopathology. Histopathology used Hematoxylin and Eosin (H&E) stains for recognization of the tissues type and morphological changes in disease diagnosis. Hematoxylin has a deep blue-purple colour which stains nucleic acids while eosin is pink which stains cytoplasm and protein nonspecifically (Fischer *et al.*, 2006). Under histopathology examination, pathological effects such as eosinophilic intranuclear inclusion bodies were found in the epithelial cell of the infected prawn. Based on the examination of all the samples, the prevalence of IHHNV infections for both sampling sites were 21.0 % (Perak) and 13.5 % (Negeri Sembilan) (Table 4.4). Histo-slides which were prepared for samples from Perak and Negeri Sembilan revealed the presence of hyperthrophied nuclei of epithelial cells with margination of chromatin and lacked clear halo information which is so-called Cowdry type B INIs (Figure 4.9). These characters of inclusion bodies were also found on the epithelial cells of connective tissues of hepatopancreas and muscle of *M. rosenbergii* (Figure 4.10). Besides, the presence of some other inclusion bodies was found in the connective tissue of the prawns (Figure 4.11). We could also see the presence of hepatopancreatic lesions of basophilic inclusion that indicated HPV infection.

The connective tissue is known to be one of the target organs of IHHNV. The shape of inclusion bodies of Cowdry Type B which were found in suspected infected prawns from Perak and Negeri Sembilan have identical and similar criteria as described in the previous study by Hsieh *et al*. (2006) (Figure 2.6). A few Taiwanese studies that observed inclusion bodies in epithelial cells of hepatopancreas of *M. rosenbergii* share similar findings to the current studies. However, the difference between our findings on *M. rosenbergii* and those finding on marine shrimp is that are IHHNV in shrimp species are almost always associated with tissues of ectodermal and mesodermal origin (Lightner, 1996; Bondad-Reantaso *et al.*, 2001; Flegel, 2006). There is no report of IHHNV infection associated with endodermal origin such as tubule epithelial cells of hepatopancreas.

The findings that have been observed were compared with positive IHHNV-infected shrimp tissue that which showed similar character of inclusion bodies (Figure 4.12). Although the inclusion bodies which were found in both *M. rosenbergii* and shrimp tissue (reference slide from Prof. Lightner, University of Arizona, USA) were similar in character, it may not confirm the infection of IHHNV. In addition, histology application had low sensitivity of detection due to the presence of the abnormal cells with many shapes, and characters. Thus, *in situ* hybridization (ISH) is be used in the current study to confirm the results of IHHNV infection in infected prawns obtained via histological observation. All histology slides with pathological effect of IHHNV were selected and later *in situ* hybridization assay was performed.

By using DNA probe which was designed from nested PCR primers, *in situ* hybridization may provide greater sensitivity in IHHNV detection. This method was performed in all the suspected tissues but the hybridization signal of IHHNV DNA was

too low (Figure 4.13). The low signal may be caused by low viral load in the tissue section. Optimization of the protocol was done, but it is still failed to get greater hybridization signal. The limitation of this method is due to the lack of positive-IHHNV tissue sections of *M. rosenbergii* as positive control. Furthermore, the deficiency some other virus positive controls such as MHPV, MBV and WSSV, restricted further optimization and validation of the method to be performed at this stage.

As a conclusion of this chapter, the screening of IHHNV for wild giant freshwater prawns was done throughout 2009 and it was found that these prawns could be a reservoir of IHHNV disease. Even though this species were infected with IHHNV with high prevalence and some signs of abnormalities, there is no documentation of serious mortality reported. Frequent monitoring of viral disease is likely to constitute an effective method for choosing healthy broodstocks from the wild environment for hatchery production and controlling the spread of the disease in Malaysia.

GENERAL DISCUSSION AND CONCLUSION

IHHNV caused acute disease in juvenile *P. stylirostris* with high mortalities more than 90 %. In this species, the juvenile and sub-adult stages shrimp are the most severely affected (Bell *et al.,* 1984). In *L. vannamei,* runt deformed syndrome (RDS) is often occur as a result of IHHNV infection. This chronic form of IHHNV disease has also been observed in cultured stock of *L. vannamei*, *P. stylirostris* and *P.monodon* (Lightner, 1996; Lightner *et al.*, 1997; Kim *et al.*, 2011a). The infected shrimp with RDS may display a bent or deformed rostrum, deformed sixth abdominal segment, wrinkled antennal flagella, cuticular roughness and other cuticular deformities (Bell *et al.*, 1984; Bondad-Reantaso *et al.*, 2001; Flegel, 2006). To date there is no documentation that had been reported on the clinical signs observed in IHHNV-infected giant freshwater prawn. However, present study found that infected *M. rosenbergii* did not show any sign of the mortalities but deformity of rostrum was observed in some of the samples.

Based on the screening of IHHNV results, we observed an unexpected high prevalence of IHHNV infection. Almost 50 % of the prawns were found infected with IHHNV but there is no mortality occurs in the population from both sampling sites. Some could hypothesize that this scenario might be related to the presence of virus-related sequence integrated into the genome of the prawn. Previous studies were reported that IHHNV –related sequence has been found to be inserted in genome of *P. monodon* from Australia and Africa (Tang *et al.*, 2006; Tang *et al.*, 2007). This integrated sequence is not active and was identified as ‘Type 3A’ and ‘Type 3B’ forms of IHHNV (Tang *et al.*, 2006). There are several methods of PCR assay which developed to discriminate between the infectious type of IHHNV and virus-related

sequence (Tang *et al.*, 2007; Rai *et al.*, 2009). However there is no report of IHHNV in giant freshwater prawn, *M. rosenbergii* related to this non-infectious type. There is no laboratory bioassay has been conducted to prove this fact. Furthermore, in a monitoring study of IHHNV-survivor cultured giant freshwater prawn showed an increase in IHHNV viral load by using real-time PCR on 6^{th}-week cultured juvenile and 15^{th}-week cultured juvenile prawn from one farm suggesting that this virus is active and not integrated in the genome of prawn (unpublished data). This indicates that these prawns have low copy of virus which does not cause disease. This virus may establish persistence infection in *M. rosenbergii* where the viral loads can be extremely low.

It is well known that the best detection methods of this virus are through polymerase chain reaction (PCR) and sequence analysis of the target genes. To date many sets of primer were designed and published in order to develop a specific detection of IHHNV in shrimps which isolated from different regions (Nunan *et al.*, 2000; Tang *et al.*, 2007; Saksmerprome *et al.*, 2010; Teixeira *et al.*, 2010). In fact, some of the primers were designed specifically for discriminating the non-infectious and infectious form of IHHNV which occurs in *P. monodon* (Tang *et al.*, 2006; Tang *et al.*, 2007). Back in 2006, there is a preliminary study that revealed the occurrence of IHHNV infection in *M. rosenbergii* in Malaysia (Kua, unpublished). The source of the virus and viral transmission were unclear as there is no sign of disease observed in the population. Unfortunately, due to the limited number of sample collected, further investigation cannot be conducted.

In order to isolate the virus and determine the prevalence of infection, screening of IHHNV was conducted in freshwater prawn hatchery and wild environment. To achieve these objectives, a pair of nested primer was designed which will pair together with another set of primer suggested by OIE for nested PCR. This nested primer was designed from local IHHNV strain isolated from freshwater prawn (data unpublished). By understanding the prevalence of infection IHHNV in the selected sampling sites, we should have a better idea in selecting suitable broodstock for breeding program. We also should know which sampling area is the best for broodstock collection from wild environment. Thus, the present study was focused on obtaining the infection prevalence as a baseline data of IHHNV infection in freshwater broodstock.

IHHNV genome is comprised of three partially overlapping open reading frame (ORF); ORF1 (non-structural protein 1, NS-1), ORF2 (NS-2) and ORF3 (capsid rotein) (Dhar *et al.*, 2010; Kim *et al.*, 2011b). To achieve the objectives of this study, the prevalence of the infection is determined based on the presence of partial sequence of ORF1 (NS-1). ORF1 of IHHNV genome encoded a major non-structural protein of 2001bp (666 amino acids) in length (Rai *et al.*, 2001; Kim *et al.*, 2011b). In this study, the reason of selection ORF1 as detection region is because this ORF contains highly conserved replication initiator motifs, NTP-binding and helicase domain which common to all parvovirus. In addition, it is well established that these motifs involved in viral replication (Shike, 2000; Rai *et al.*, 2001; Dhar *et al.*, 2010; Kim *et al.*, 2011b).

In the present study, the prevalence of IHHNV infection from selected hatchery and wild environment were determined. Random sampling of giant freshwater prawn broodstocks (n=30) was done in a hatchery and were subjected to DNA extraction and detection of the virus by PCR. Six out of 30 samples (20%) were identified as positive

IHHNV. Sequence alignment of these positive isolates (389-bp) in GenBank revealed close identities (98%) to Taiwanese IHHNV strains isolated from *M. rosenbergii* in Taiwan. The local isolates sequences of 389-bp showed only four nucleotide substitution and six nucleotide substitution compared to Taiwanese strains, GenBank Acc. No. DQ057982 and DQ057983 respectively. Further analysis of the virus genome could not be performed due to limited sample obtained from the hatchery.

Sampling was done in two selective areas in Malaysia in order to determine the prevalence of IHHNV in wild environment. Fifty samples were collected every month in a year and were subjected to DNA extraction and nested PCR in order to obtain the infection prevalence. Few gross signs of the disease were observed but there is no mortality has been reported. We found high prevalence in both sampling sites (Sungai Rubana, Perak and Sungai Timun, Negeri Sembilan) and significant results between infection prevalence and weight of the prawn. From the statistic results, we would have better suggestion for farmers who use to take their broodstock from wild environment. We could suggest the best weight of broodstock captured from wild must be more than 40 g. But screening of disease should always be performs before spawning in order to control the spread of the disease. By knowing the status of disease, emergency harvesting is possible to avoid the disease being spread to another cultured farm.

IHHNV infection in giant freshwater prawn was also studied by histopathology and *in situ* hybridization (ISH). Basically, histopathology involves the embedding tissue in paraffin and micro-sectioning of the paraffin block followed by staining with H&E. Since IHHNV infection can be identified through pathological effects such as eosinophilic intranuclear inclusion bodies, histopathology diagnosis has been used as presumptive method and later the infection is confirmed with transmission electron

microscope (TEM) and molecular methods. In histopathological studies, 125 samples were examined and we observed low intensity of eosinophilic intranuclear inclusion bodies in connective tissue of epithelial cells in hepatopancreas and muscle tissues in some of the samples. However, contrast to our finding, as described in Chapter 2, in other shrimp species inclusion bodies is reported to be found in hematopoietic tissue, ovary and gill (Morales-Covarrubias *et al.*, 1999; Flegel *et al.*, 2010). Our findings on histopathology revealed low intensity of infection which we believed could not contribute to the mortality. The signs of an infected cells are; 1) margination of the chromatin, 2) cells become slightly hyperthrophic, and 3) the nucleolus becomes eosinophilic (Owens *et al.*, 1992).

In situ hybridization (ISH) involves the use of DIG-labelled DNA probe which developed through PCR method. This method was an additional method used in the present study which was applied to those histo-slides that gave positive signs of the presence of inclusion bodies that related to IHHNV. Basically, positive signals of ISH may give dark-blue or black precipitation which marks the presence of IHHNV DNA in the tissues. However in our study as describe in Chapter 4, we only observed faint black precipitation on the suspected infected tissues. Due to lack of positive control of other prawn viruses such as MBV, WSSV, MHPV and IHHNV, this method is unsuccessful to be optimize and validate as detection method in this study. We believed the low signal of ISH is due to low viral load in the suspected sample. This could also be related to the low intensity of inclusion bodies found in the tissues. Further investigation to improve this detection method is necessary as ISH could provide greater diagnostic sensitivity than other conventional histology methods.

In conclusion, the present study has successfully identified the partial sequence of ORF1 of IHHNV genome which leads to the first report on the occurrence of IHHNV infection in giant freshwater prawn, *M. rosenbergii* in Malaysia. This study had covered the screening of IHHNV infection in giant freshwater prawn in hatchery and wild environment. The infection prevalence was high is both sampling sites for the wild environment but no mortality cases has been reported. Future work should be done to understand the transmission and epidemiology of this disease in giant freshwater prawn in Malaysia.

FUTURE STUDY

Studies on screening of infectious hypodermal and hematopoietic necrosis virus (IHHNV) in giant freshwater prawn broodstock in Malaysia were conducted in selected hatchery and wild environment. These studies have described the prevalence of IHHNV infection in *Macrobrachium rosenbergii* which currently become an important sector in freshwater aquaculture industry in Malaysia. This is the first documentation reported that IHHNV is now had been shown to be present in wild *M. rosenbergii* in selective areas of Malaysia.

In Chapter 3, it was shown that 20 % of the broodstock from a selected hatchery were found infected with IHHNV by PCR. Since the broodstock used in the hatchery was collected from the wild environment, we believed that the virus had already been present in the river. The transmission of the virus might come from the cultured shrimps which were located near the river. As described in Chapter 2, this virus is likely to infect shrimps species and nowadays, many shrimp farms were established. When disease wave comes and attack their cultured shrimp, some farmers may take an easy approach by releasing the water from the infected ponds into the nearby river without being treated. These lead to the transmission of the infection to new hosts which one of the creature that live in the river is giant freshwater prawn.

It is not fully understand that how the virus affects the giant freshwater prawn as there is no mortality being observed. And the first question arises now is does the IHHNV infection in giant freshwater prawn related to the non-infectious type of IHHNV which found in *P. monodon* as reported in previous studies (Tang *et al.*, 2006;

Tang *et al.*, 2007; Saksmerprome *et al.*, 2010)? Answers to this question can give us clear view on the disease status of giant freshwater prawn in Malaysia.

In Chapter 4, high prevalence of the infection was found in Sungai Rubana, Perak and Sungai Timun, Negeri Sembilan with prevalence of 64.4 % and 48.7 % respectively. We observed RDS symptoms on few samples but there is no mortality that had been reported so far. The investigation on the prevalence of IHHNV infection with respect to weight of the prawn from two selective wild environments was also conducted. It is surprised to discover that most of prawns captured from both rivers were small in size with weight of 40 g below, already spawning and detected positive with nested PCR. The first question arises here is why the body sizes of this population are greatly decreasing? Secondly, does the environment or genetic factors influence the prevalence of the infection and growth rate of prawn? Answers to these questions may help us to understand the spread of the virus and could give us clues on how to control the disease spread.

The prevalence of IHHNV varies with different weight of the prawn obtained in the present study also suggested that the suitable weight of prawn for broodstock which captured direct from river must be more than 40 g. This suggestion is based on our monitoring and screening data throughout 2009/2010. Apart from PCR, screening of IHHNV was conducted via histopathology and *in situ* hybridization. Histopathological examination revealed the presence of eosinophilic intranuclear inclusion bodies in connective tissues of epithelial cells of hepatopancreas and muscle tissues. The Cowdry-type inclusion bodies that were found in this study have similar morphology cell as described in previous studies (Morales-Covarrubias *et al.*, 1999; Hsieh *et al.*, 2006; Flegel *et al.*, 2010). Moreover, there is also basophilic inclusion found in the

connective tissues of the prawns indicated HPV infection in some of the samples. In the present studies, *in situ* hybridization study was also carried out using DNA-labelled probe which may give greater sensitivity for IHHNV detection. However, due to lack of positive-IHHNV control, the hybridization signal of IHHNV DNA was too low to be optimized. We believed that this is due to the low viral load in the sample that was embedded in paraffin block.

Recently, Saksmerprome *et al.* (2011) revealed the first experimental support for the hypothesis-based prediction regarding the random insertion of viral sequences into the genome of shrimp. In their studies, they reported that random inserts of IHHNV sequences is common in the genome of *P. monodon*. This occurrence may lead to false positive results for some PCR detections. In order to cover the whole genome of IHHNV, they suggested a set of seven overlapping primer pairs which failure of some pairs may give a random pattern of putative viral inserts (Saksmerprome *et al.*, 2011). Specimens were considered as positive IHHNV, if they are positive with all seven overlapping sets of primer. This method could be used in the future study in order to determine whether inserts occurs in giant freshwater prawn.

The present studies have determined the prevalence of IHHNV infection which may acts as baseline information for IHHNV infection status on *Macrobrachium rosenbergii*. Although there is no documentation of serious mortality has been reported, this is the early documentation of IHHNV infection in giant freshwater prawn in Malaysia. Currently, we are trying to grow this virus on C6/36 cell line in order to increase the yield of the virus. More studies should also be carried out in order to understand the viral etiology and to study the epidemiology of IHHNV. Monitoring program should be carried out in other selective river in Malaysia in order to get clear

view on the disease status in giant freshwater prawn. To date, there is no definite treatment for this disease, thus frequent monitoring of the viral disease in prawns and shrimps should be conducted in order to control the spread of the disease.

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APPENDICES

APPENDIX A : HISTOPATHOLOGY

1. STAINING METHOD OF HEMATOXYLIN AND EOSIN (H&E)

Xylene I	3 minutes
Xylene II	3 minutes
100 % Alcohol	3 minutes
95 % Alcohol	3 minutes
80 % Alcohol	3 minutes
70 % Alcohol	3 minutes
Running water	3 minutes
Erlich Hematoxylin	10 minutes
Quick Dip in Acid -Alcohol	Quick dip
Running water	3 minutes
Water and LiCo3	2 or 3 times (1 -2 seconds)
Eosin	2 minutes
95 % Alcohol	3 minutes
100 % Alcohol	3 minutes
Xylene III	3 minutes
Xylene IV	3 minutes

2. PREPARATION OF DAVIDSON FIXATIVE (DF)

Formaldehyde	2 parts
Glycerol	1 parts
Glacial acetic acid	1 part
Absolute alcohol	3 parts
Sea water	3 parts

APPENDIX B : *IN SITU* HYBRIDIZATION

1. 0.4% Formaldehyde

5.4ml of 37% Formaldehyde

495ml ddH_2O

Store at 4^oC for up to 2 months

2. 20X Denhardt's Solution

0.4% BSA	0.4g Bovine Serum Albumin, Fraction V
0.4% Ficoll	0.4g Ficoll 400
0.4% PVP 360	0.4g Polyvinylpyrrolidone 360
ddH_2O	100ml

Filter through 0.45 µm filter; store at 4^oC

3. **25% Dextran Sulfate**

25g Dextran Sulfate

80ml ddH_2O (qs to 100ml)

Dissolve dextran sulfat in water, heat on LOW to facilitate mixing, dispense 10ml per tube; store frozen at -20 ^oC

4. Salmon Sperm DNA (10mg/ml)

500mg Salmon Sperm DNA (10mg/ml)

40ml ddH2O (qs to 50ml)

Add DNA slowly to water while stirring and heatin on LOW

Shear DNA by passing through an 18 gauge needle several times

Autoclave to sterilize and to further denature the DNA; dispense 2.5ml in small tubes; store at -20 ^oC

5. 10% Polyvinyl Alcohol

10g polyvinyl alcohol (30,000-70,000 MW)

90ml ddH_2O (qs to 100ml)

Stir to dissolve PVA (heat on LOW to facilitate mixing); dispense 10ml per tube; store at -20 ^oC

6. 0.5% Bismarck Brown Y

5g Bismarck Brown Y

1000ml ddH_2O

Stir until completely dissolved;filter through Whatman #1 filter paper; store at RT

7. 10X Tris NaCl EDTA (TNE)

500mM Tris-HCl	60.57g Tris Base
100mM NaCl	5.84g NaCl
10mM EDTA	3.72g EDTA.2H_2O (disodium salt)
ddH_20	990ml (qs to 1L)

pH to 7.4 with HCl; autoclaved;store at 4 ^oC

1X TNE, dilute 100ml of 10X TNE in 900ml ddH_2O;filter through 0.45µm filter

8. PK Solution (prepare just prior to use)

1X TNE	10ml
Proteinase K	1 mg

9. 20X SSC (sodium chloride/sodium citrate) Buffer

3M NaCl	175.32g NaCl
0.3M Na citrate	88.23 g Na.citrate.2H_2O
ddH_2O	900ml (qs to 1L)

pH to 7.0;autoclave;store at 4 ^oC

10. Hybridization Buffer (50mL) store 4^oC

4X SSC	10ml of 20X SSC
50% Formamide	25ml 100% Formamide
1X Denhardt's	2.5ml of 20X stock
5% Dectran Sulfate	10ml of 25X stock
0.5mg/ml DNA	2.5ml (10mg/ml of salmon sperm DNA)

11. <u>10X Buffer I</u>

1M Tris-HCl	121.1g Tris Base
1.5M NaCl	87.7 g NaCl
ddH_2O	900 ml (qs to 1L)

pH to 7.5 with HCl;autoclave;store 4^oC

1X Buffer; dilute 100ml of 10X in 900ml ddH_2O;filter thr 0.45µm

12. <u>Buffer II</u>

0.5% Blocking agent	0.5g Blocking Reagent #11 (Genius^{TM} 1 Kit)
1X Buffer I	100ml

Heat on LOW with stirring 30 min, store at 4^oC up to 1 wk

13. <u>Buffer III</u>

100mM Tris-HCl	12.11g TrisBase
100mM NaCl	5.84g NaCl
ddH_2O	990ml

pH to 9.5 with HCl;filter 0.45µm;store 4^oC

14. **<u>10X Buffer IV</u>**

100mM Tris-HCl	12.10g TrisBase
10mM EDTA	3.72g EDTA.2H_2O
ddH_2O	990ml

pH to 8.0 with HCl;store 4^oC

15. <u>Development Solution (add NBT and X-phosphate just prior to use)</u>

1X Buffer III	90ml Buffer III
1% PVA	10ml 10% Polyvinyl Alcohol
1mM Levamisole	24mg Levamisole

Mix and store at 4 ^oC

Just prior to use, add	**4.5µl NBT**
	3.5 µl X-phosphate
	1ml Development solution

APPENDIX C : RESEARCH ACHIEVEMENT, PUBLICATION OR CONFERENCE PRECEEDING

I. Honors and Awards

Silver Medal for BioInnovation Award at BioMalaysia Exhibition and Conference held in KLCC on 21st -23rd November 2011. **Title of invention**: Rapid Detection Kit of Infectious Hypodermal and Haematopoetic Necrosis Virus (IHHNV) for *Macrobrachium rosenbergii*.

II. Publications

1. **Hazreen Nita, M. K**., B. C. Kua, S. Bhassu and R. Y. Othman. 2011. Detection and genetic profiling of infectious hypodermal and haematopoietic necrosis virus (IHHNV) infections in wild berried freshwater prawn, *Macrobrachium rosenbergii* collected for hatchery production. *Molecular Biology Reports* .**39**:3785-3790.

2. Kua Beng Chu, Choong, F.C., **Hazreen Nita, M.K**., Muhd. Faizul, H.A.H., Bhassu, S., Imelda, R.R. and Mohammed, M. 2011. Parasitic and IHHNV infections in wild giant freshwater prawn *Macrobrachium rosenbergii* from Rejang River at Kuching, Sarawak *Tropical Biomedicine*. **28**(1): 85–89.

III. Publication in submitting process

Hazreen Nita M.K., Kua B.C., Bhassu, S. and R.Y. Othman. Occurrence of infectious hypodermal and haematopoietic necrosis virus (IHHNV) infections in wild-caught freshwater prawn, *M. rosenbergii* in Sungai Rubana, Perak and Sungai Timun, Negeri Sembilan, Malaysia. *Paper presented in 8th Symposium on Diseases in Asian Aquaculture* (2011). (Manuscript submitted to *Journal of Fish Disease*).

IV. Patent

1. A Method For Detecting Infectious Hypodermal And Haematopoietic Necrosis Virus

   Priority Date: 19 May 2011

   PI No.: PI 2011002235 (UM)

   - Won a silver medal during BioInnovation Award 2011, BioMalaysia in KLCC on 21st – 23rd Nov 2011