DETERMINING THE BROODSTOCK ORIGIN AND PATHOGENS OCCURRENCE IN A GIANT FRESHWATER PRAWN (*Macrobrachium rosenbergii*) NUCLEUS BREEDING FARM

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FACULTY OF SCIENCE
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DETERMINING THE BROODSTOCK ORIGIN AND PATHOGENS OCCURRENCE IN A GIANT FRESHWATER PRAWN (*Macrobrachium rosenbergii*) NUCLEUS BREEDING FARM

ABSTRACT

The giant freshwater prawn, *Macrobrachium rosenbergii,* as known as udang galah in Malaysia, is the biggest freshwater prawn in the world. The Department of Fisheries in Malaysia is giving priority on *M. rosenbergii* as food products for consumption and export. However, the major limitation in shrimp production is diseases. Like other animals, prawns are affected by viruses, bacteria, fungi, and metazoan parasites. A giant freshwater prawn nucleus breeding farm located in in Kampung Chennah, Jelebu, Negeri Sembilan has no systemic approach to prevent prawn diseases caused by viruses and bacteria. This study was undertaken to implement a systemic approach of genetic, health surveillance and biosafety measures in the farm. 19 juveniles *M. rosenbergii* (cycle 1) and 30 juveniles *M. rosenbergii* (cycle 2) were sampled from the pond by seining for mitochondrion DNA analysis, viral screenings and bacterial screenings. The first part of this study is useful to create a genetic profile of *Macrobrachium rosenbergii* and to know the origin of broodstock by using mitochondrion DNA analysis. Cytochrome c oxidase I (COI) of mitochondrial gene was used for phylogenetic analysis by DNA amplification using the Polymerase Chain Reaction (PCR) method. MEGA X software was used to construct a phylogenetic tree from the aligned sequences, followed by Network 5 software was used to generate a median-joining haplotype network. The haplotype network diagram generated shows the broodstock are come from four states which are Kedah, Perlis, Johor, Negeri Sembilan. The second part of this project is virus screening of *M. rosenbergii* from the farm. DNA viruses include White Spot Syndrome Virus (WSSV), Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV),

Monodon Baculovirus (MBV), Baculovirus penaei Virus (BP) and RNA viruses include Yellow Head Virus (YHV), Taura Syndrome Virus (TSV), Infectious Myonecrosis Virus (IMNV), *Macrobrachium rosenbergii* nodavirus (*Mr*NV) were screened using multiplex PCR method. Dual priming oligonuclotide (DPO) primer was used to produce high PCR specificity by inhibiting mismatching and non-specific priming of the primers to the template under less optimal PCR conditions. The third part of this project is screening of *Vibrio parahaemolyticus* bacteria, which cause acute hepatopancreatic necrosis disease (AHPND) in giant freshwater prawn. Polymerase chain reaction was performed using primer AP3, which targets sequence 336 bp. All 19 juveniles *M. rosenbergii* collected from cycle 1 showed the absence of DNA viruses and RNA viruses. On the other hand, out of 30 juveniles *M. rosenbergii* collected from cycle 2, all showed the absence of DNA viruses, 5 samples show the presence of Yellow Head Virus (YHV) infection. The results showed the prevalence of YHV infection in prawn samples was 80%. The reasons for YHV infection in prawns could be contamination of broodstock, contamination of water supply and pond environment. For *Vibrio parahaemolyticus* screening, all samples show negative result. A systemic approach of biosafety measures is developed for the prawn farm to prevent diseases. These measures include prawn health surveillance, quarantine records, water quality monitoring, disinfection and hygiene, disease outbreaks investigation and standard operating procedures.

Keywords: *Macrobrachium rosenbergii*, broodstock, prawn viruses, *Vibrio parahaemolyticus*, biosafety measures.

MENGENAL PASTI ASAL STOK PEMBIAK BAKA DAN KEMUNCULAN PATOGEN DI LADANG PEMBIAKAN UDANG GALAH AIR TAWAR (*Macrobrachium rosenbergii*)

ABSTRAK

Udang galah air tawar, *Macrobrachium rosenbergii*, yang dikenali sebagai udang galah di Malaysia, merupakan udang air tawar terbesar di dunia. Jabatan Perikanan di Malaysia memberi keutamaan kepada *M. rosenbergii* untuk penggunaan sebagai produk makanan dan eksport. Walau bagaimanapun, halangan utama dalam pengeluaran udang adalah penyakit. Seperti haiwan lain, udang juga dipengaruhi oleh virus, bakteria, kulat, dan parasit metazoan. Ladang ternakan udang air tawar gergasi yang terletak di Kampung Chennah, Jelebu, Negeri Sembilan tidak mempunyai pendekatan sistemik untuk mencegah penyakit udang yang disebabkan oleh virus dan bakteria. Kajian ini dijalankan untuk melaksanakan pendekatan sistematik mengenai pengawasan genetik, pengawasan kesihatan dan keselamatan di ladang. 19 juvana *M. rosenbergii* (kitaran 1) dan 30 juvana *M. rosenbergii* (kitaran 2) telah dikumpulkan dari kolam untuk mitokondrion DNA analisis, pemeriksaan virus dan pemeriksaan bakteria. Bahagian pertama kajian ini berguna untuk membuat profil genetik *M. rosenbergii* dan mengetahui asal usul stok penetasan dengan menggunakan mitokondria DNA analisis. Cytochrome c oxidase I (COI) daripada gen mitokondria digunakan untuk filogenetik analisis dengan amplifikasi DNA menggunakan kaedah Reaksi Rantai Polimerase (RBP). Perisian MEGA X digunakan untuk membina pokok filogenetik dari urutan sejajar, diikuti dengan perisian Network 5 yang digunakan untuk menjana rangkaian haplotip median-sambungan. Gambar rajah haplotip yang dijana menunjukkan stok penetasan berasal dari empat negeri iaitu Kedah, Perlis, Johor, Negeri Sembilan. Bahagian kedua projek ini adalah pemeriksaan virus *M. rosenbergii* dari ladang

tersebut. Virus DNA termasuk virus bintik putih, *Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV)*, *Monodon Baculovirus (MBV), Baculovirus penaei Virus (BP)* dan RNA viruses termasuk virus kepala kuning, *Taura Syndrome Virus (TSV)*, Virus Myo, *Macrobrachium rosenbergii* nodavirus (*Mr*NV) diperiksa dengan menggunakan kaedah Reaksi Rantai Polimerase (RBP) berbagai. Dual priming oligonuclotide (DPO) primer digunakan untuk menghasilkan spesifikasi PCR yang tinggi dengan menghalang pemadatan tidak sepadan dan gabungan primer yang bukan spesifik kepada templat di bawah keadaan PCR yang kurang optimum. Bahagian ketiga projek ini ialah penyaringan bakteria *Vibrio parahaemolyticus*, yang menyebabkan penyakit nekrosis hepatopankreatic akut (AHPND) dalam udang air tawar gergasi. Reaksi Rantai Polimerase dilakukan menggunakan primer AP3, yang menyasarkan sekuen 336 bp. Kesemua 19 juvana *M. rosenbergii* yang dikumpulkan dari kitaran 1 menunjukkan ketiadaan virus DNA dan virus RNA. Sebaliknya, daripada 30 juvana *M. rosenbergii* yang dikumpulkan dari kitaran 2, semua menunjukkan ketiadaan virus DNA, manakala 5 sampel menunjukkan hasil positif jangkitan Virus Kepala Kuning. Keputusan menunjukkan kes jangkitan YHV dalam sampel udang adalah 80%. Sebab-sebab jangkitan YHV dalam udang boleh disebabkan oleh pencemeran stok penetasan, pencemaran bekalan air dan persekitaran kolam. Untuk pemeriksaan bacteria *Vibrio parahaemolyticus*, semua sampel menunjukkan hasil negatif. Pendekatan sistemik dalam pelaksanaan langkah-langkah keselamatan dibentukkan untuk ladang udang untuk mencegah penyakit. Langkah-langkah ini termasuk pengawasan kesihatan udang, rekod kuarantin, pemantauan kualiti air, pembasmian kuman dan kebersihan, penyiasatan wabak penyakit dan prosedur operasi standard.

Kata kunci: *Macrobrachium rosenbergii*, stok pembiak baka, virus udang, *Vibrio parahaemolyticus*, lagkah keselamatan.

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%	:	Percentage
^{o}C	:	degree celcius
Da	:	Dalton
kD	:	kiloDalton
mL	:	Milliliter
µl	:	Microliter
g	:	Gram
µg	:	Microgram
ng	:	Nanogram
mg	:	Microgram
µm	:	Micrometer
nm	:	Nanometer
cm	:	Centimeter
M	:	Molar
mM	:	Milimolar
µM	:	Micromolar
pmol	:	pico-mol
µmol	:	micro-mol
bp	:	base pair
kbp	:	kilobase pairs
ppm	:	Parts per million
rpm	:	rotation per minute
s	:	seconds
AHPND	:	Acute hepatopancreatic necrosis disease
BLAST	:	Basic Local Alignment Search Tool

BPV	:	Baculovirus penaei virus
COI	:	Cytochrome c oxidase subunit locus I
DNA	:	Deoxyribonucleic acid
dNTP	:	Deoxynucleotide triphosphate
DPO	:	Dual priming oligonuclotide
EB	:	Elution Buffer
ELF	:	Eukaryotic elongation factor
ELISA	:	Enzyme-linked immunosorbent assay
EMS	:	Early mortality syndrome
ETP	:	Economic Transformation Programme
FAO	:	Food & Agriculture Organization
IHHNV	:	Infectious hypodermal and haematopoietic necrosis virus
IMNV	:	Infectious myonecrosis virus
LB	:	Luria-Bertani
mA	:	Milliampere
MBV	:	Monodon baculovirus
MEGA	:	Molecular Evolutionary Genetics Analysis
$MgCl_2$	:	Magnesium chloride
*Mr*NV	:	*Macrobrachium rosenbergii* nodavirus
mtDNA	:	Mitochondrial DNA
NaCl	:	Sodium chloride
NKEA	:	National Key Economic Area
NS	:	Non-structural protein
OIE	;	World Organisation for Animal Health
ORF	:	Open reading frame
PCR	:	Polymerase chain reaction

PIB	:	Polyhedral inclusion body
Pir	:	*Photorhabdus* insect-related
PL	:	Postlarvae
RNA	:	Ribonucleic acid
RNase	:	Ribonuclease
rRNA	:	Ribosomal RNA
RT-LAMP	:	Reverse-transcriptase loop-mediated isothermal amplification reaction
RT-PCR	:	Reverse transcription polymerase chain reaction
ss	:	Single stranded
Sg.	:	Sungai
SPF	:	Specific pathogen free
SPR	:	Specific pathogen resistant
TBE	:	Tris/Borate/EDTA
TE	:	Tris-EDTA buffer
TEM	:	Transmission electron microscopy
TSV	:	Taura syndrome virus
UV	:	Ultraviolet
V	:	Voltage
WSSV	:	White spot syndrome virus
WTD	:	White tail disease
YHD	:	Yellow head disease
YHV	:	Yellow head virus

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CHAPTER 1: INTRODUCTION

Aquaculture is defined as the farming or cultivating of aquatic animals in an artificially controlled or half-controlled environment for commercial purposes (Stickney, 2009). The term 'aquatic' refers to various types of water environments, for example, freshwater, brackish water and marine. When we talk about the term 'aquatic animals', it can be further elaborated as living things that lives in water such as variety of plants, invertebrates and vertebrates (Stickney, 2009).

In tropical areas, the adult prawns are available throughout the year in freshwater prawn farms, the word 'broodstock' usually refers to the female prawns which are kept in hatcheries until their eggs hatch, followed by which they are discarded or sold. Freshwater prawn eggs are carried by the adult female prawns under the tail which are easily can be noticed (Figure 1.1). These prawns are called 'berried' females. Even though berried females can be easily obtained from farm ponds throughout the year, but the amount of berried females may differ depends on the time of year (FAO, 2002).

Figure 1.1: The eggs of *Macrobrachium rosenbergii* are carried by the berried female prawns until the eggs hatch. When they are ripen, the colour turns from orange to grey (New & Valenti, 2000).

In present years, the prawn industry in Malaysia is mainly dependent on wild broodstocks and pond-raised broodstocks for breeding purposes. Majority of the farmers in hatcheries are usually using berried wild female prawns grabbed from rivers as the starting batch of origin broodstocks. After the eggs of prawns are deposited, the postlarvae (PL) are raised in green-water, which is the water in shore on soundings, or clean water. Thus, the origin of broodstocks is a main concern, because it will affect the quality and grade of hatchery post-larvae. Nevertheless, there is not much documentation and research related to the disease status of the wild giant freshwater prawn in Malaysia (Hazreen Nita et al., 2011). Therefore, the first objective of this study is to examine the source and origin of *Macrobrachium rosenbergii* wild broodstock.

Metazoan mitochondrial DNA (mtDNA) marker is used to examine the source of broodstock in this study, because it is generally employed to study genetic diversity and population structure of aquatic organisms. Mitochondrial DNA marker is suitable for genetic studies because their mutation rate is higher compared to the nuclear genome (Brown et al., 1979; Thomas et al., 1997; Denver et al., 2004). Moreover, mitochondrial DNA marker does not have introns (Costanzo & Fox, 1990; Matsuzaki et al., 2004) and mode of inheritance mode is mono-parental (Shitara et al., 1998; Wan et al., 2004). Out of all the mitochondrial genes, the cytochrome c oxidase subunit locus I gene has been widely employed by many researchers in their studies (Hebert et al., 2004; Liu et al., 2011; Barman et al., 2014). COI gene is one of the most useful markers used for molecular systematic (Souza et al., 2015). COI gene used by these researchers for species barcoding and also to study the relationships between populations and previous demographic events such as population distribution, spatial changes in response to birth, migration, and death (De Jong et al., 2011; Li et al., 2014; Han et al., 2015). Moreover, mtDNA has a high degree of conservation, which means the gene is remained

essentially unchanged throughout evolution (Moritz & Cicero, 2004). mtDNA also has numerous rapidly evolving nucleotide sites, which enables easier to distinguish between evolved species (Nylander et al., 1999).

In recent decades, aquaculture industry sector is keep developing fast in the world to cover the production of freshwater, brackish and marine which includes fish farming, prawn farming and also aquatic plants. Aquaculture plays an important role in National Key Economic Area (NKEA), which is the central of the Malaysian Government's Economic Transformation Programme (ETP). This programme targets to expand economic activity to generate high income and sustainability. The global demand for aquaculture products is increasing from year to year; it is expected to increase to more than 635 million tonnes in 2020 from 251 million tonnes, which is at present. In 2003, the aquaculture production was at 194,139 tonnes (USD 308 million), which occupying roughly 20% of the total amount of the aquaculture production in Malaysia. Aquaculture is undeniable a vital and predominant sector to increase local production for food security and also at the same time, to increase export revenues in Malaysia (Banu & Christianus, 2016).

In Malaysia, the Department of Fisheries is giving priority on *M. rosenbergii* as food products for consumption and export (Ganjoor, 2015). Even though the giant freshwater prawn production is lower compared to other Asian countries such as Vietnam and Thailand, but it is developing quickly and it has big commercial market potential in the overseas market. Therefore, it can replace tilapia *Oreochromis sp.* and tiger shrimp, *Peneaus monodon* farming to meet the high demand as food products. Moreover, the growing rate of *M. rosenbergii* is fast, large in size and tolerant to certain diseases (Ranjeet et al., 2002).

In aquaculture industry, the commercialization production is always large-scale to meet the demand of consumption and economic. However, the major limitation in aquaculture production is diseases when aquaculture activities expand. Like other animals, prawns are affected by viruses, bacteria, fungi, and metazoan parasites. Among the pathogens, prawn viruses are the main reason causing huge economic losses, especially in the hatchery and nursery stages. Infectious disease agents can spread easily and rapidly. Reported prawn viruses include White Spot Syndrome Virus (WSSV), Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV), Monodon Baculovirus (MBV), Baculovirus penaei Virus (BP), Yellow Head Virus (YHV), Taura Syndrome Virus (TSV), Infectious Myonecrosis Virus (IMNV), *Macrobrachium rosenbergii* nodavirus (*Mr*NV). Some viruses are highly pathogenic and causing diseases in shrimp such as WSSV and TSV (Ganjoor, 2015). Therefore, the second objective in this study is to screen for these DNA and RNA viruses in *Macrobrachium rosenbergii* wild broodstock.

Other than viruses, *Vibrio parahaemolyticus*, which has a 70-kbp plasmid, is the etiologocal agent of acute hepatopancreatic necrosis disease (AHPND) (Lee et al., 2015; Tran et al., 2013), which is a common disease in aquaculture. The homologous of the *Photorhabdus* insect-related (Pir) toxins encoded by this plasmid are the causative agent for shrimp mortality caused by AHPND (Lee et al., 2015; Sirikharin et al., 2015). AHPND affects two shrimp species most frequently, which are the giant tiger prawn (*Penaeus monodon*) and the whiteleg shrimp (*Litopenaeus vannamei*) (NACA, 2012). AHPND usually appears within the first thirty days after the shrimp is stocked in ponds (NACA, 2012), and causes the mass mortality in shrimp with more than 70% (Kongrueng et al., 2014), which is a large amount. Hence, the third objective of this study is to screen for *Vibrio parahaemolyticus* in *Macrobrachium rosenbergii* wild broodstock.

Good biosafety measures in prawn farm are important to maintain healthy shrimps and mitigate the risk of diseases outbreaks in aquaculture farm to generate good quality product yield. These biosafety actions or plans for a shrimp farming facility involves disease monitoring, managing disease outbreaks, aquatic health tracking, water quality monitoring, and others general security precautions.

The outcome of the study will provide useful information and foundation to improve breeding programs in the hatchery to sustain the demand for industry in Malaysia to produce high quality locally potential broodstocks. Accordingly, a health surveillance program of the broodstock is essential and important as a biosafety measure to reduce the risk of disease outbreaks in the farm. Moreover, the information on prevalence of the particular viral and bacterial diseases provides the breeders information on the health status of broodstock. As *M. rosenbergii* becoming an important source for export and consumption, this practice can be applied to give rise specific pathogen free (SPF) broodstock generation in the future.

Objectives of Study

The aim of the study was to determine the broodstock origin and pathogens occurrence in the giant freshwater prawn (*Macrobrachium rosenbergii*) nucleus breeding farm. The specific objectives of the study include:

i. To examine the origin of *Macrobrachium rosenbergii* wild broodstock collected from a prawn farm located in Kampung Chennah, Jelebu, Negeri Sembilan using cytochrome c oxidase subunit locus I (COI) gene sequence;

ii. To screen for DNA and RNA viruses which includes White Spot Syndrome Virus (WSSV), Infectious hypodermal and haematopoietic necrosis virus (IHHNV), Monodon Baculovirus (MBV), Baculovirus Penaei Virus (BPV), Yellow Head Virus (YHV), Taura Syndrome Virus (TSV), Infectious Myonecrosis Virus (IMNV) and *Macrobrachium rosenbergii* Nodavirus (MrNV) in *Macrobrachium rosenbergii* wild broodstock;

iii. To screen for *Vibrio parahaemolyticus* in *Macrobrachium rosenbergii* wild broodstock.

CHAPTER 2: LITERATURE REVIEW

2.1 Giant freshwater prawn, *Macrobrachium rosenbergii*

The giant freshwater prawn, *Macrobrachium rosenbergii*, also known as *udang galah* in Malaysia, is the largest freshwater prawn in the world. The male body size is approximately 32 cm with body weight more than 200 g. The adult female is smaller than male species with a size of about 29 cm (Ling, 1969). Formerly, the generic names are Cancer and Palaemon, while *M. rosenbergii* also called as *Palaemon carcinus*, *P. dacqueti*, and *P. rosenbergii* in the past (Ling, 1969).

Macrobrachium rosenbergii are found mostly in inshore freshwater areas such as lakes, rivers, marshes, river valleys, and ponds, and also in estuarine areas, where fresh water and salt water are mixed. Some of the species prefer live in clear water river, on the other hand, others prefer to live in extremely turbid or cloudy water. *Macrobrachium rosenbergii* is the one which living in turbid conditions (FAO, 2002).

Macrobrachium rosenbergii are scattered throughout the tropical area with sun-rays throughtout the years and subtropical areas, the regions adjacent to the tropics in the world. Holthuis (1980) studied on the distribution geographical region, local names, natural living environment and maximum scales for *Macrobrachium* commercial farming. The expert of the Food and Agriculture Organization (FAO), Shao-Wen Ling found that the giant freshwater prawn (*Macrobrachium rosenbergii*) larvae were able to survive in brackish water, which is an environment has more salinity than freshwater but not much as seawater, thus they can be found in estuaries where a river connects a sea.

Macrobrachium rosenbergii can be differentiated from other species in the genus because of some special appearance. It has a very long rostrum, with approximately 11-

14 dorsal teeth and 8-10 small ventral teeth. The tip of the last segment in the abdomen reaches distinctly beyond the back spines of the telson (Figure 2.1). The male species can be distinguished from female species as it has very long second claws, in addition, the segments are more lengthen and have edgeless spines. Moreover, adult male species has finger covered by a thick fur in the second chelipeds, and the fur is not found in fixed finger and other chelipeds.

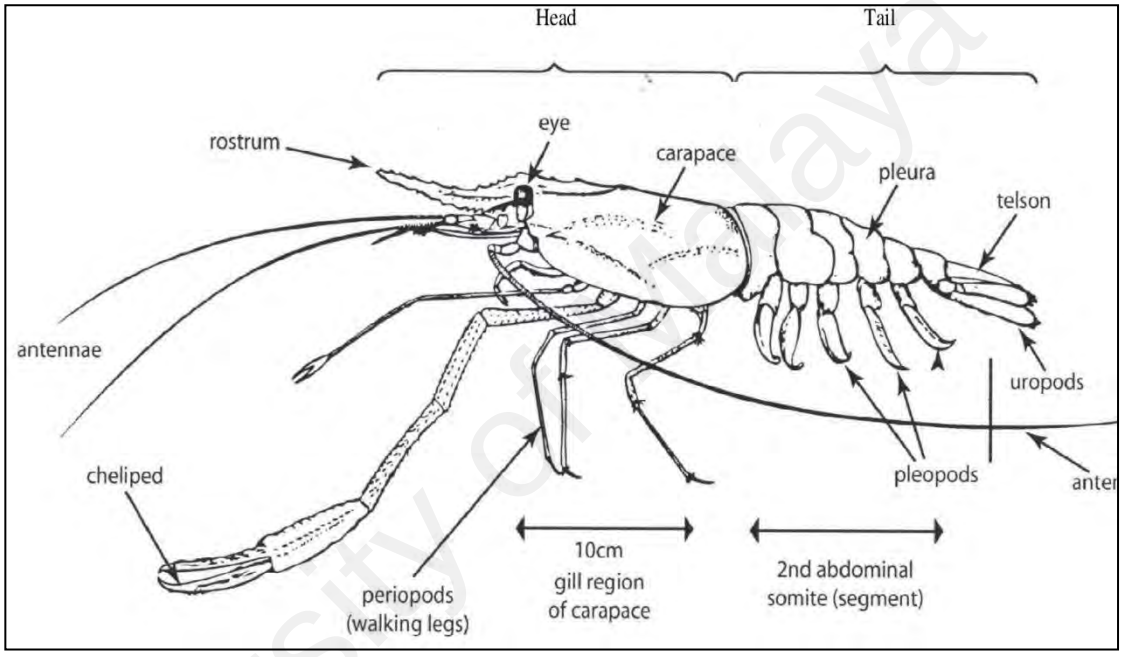

Figure 2.1: The external characteristics of *Macrobrachium rosenbergii* (New & Singholka, 1985).

The eggs from *Macrobrachium rosenbergii* freshwater prawn are compact, with the diameter between 0.6 to 0.7 mm, followed by the vivid orange colour that last until 2 to 3 days before they brood, the colour will develop to dark grey. The colour switches indicated the consumption of food reserve from the embryos. The larvae need to grow through the 11 stages (Uno & Kwon, 1969) before reconstruction followed by a few differentiation characteristics. Meanwhile, as the size growth from the 4th stage is different, this causes some employee, Ling (1969) to narrate the eight stages of growth. When the larvae are growing under first stage, the size is about 2 mm long, where counted from the rostrum tip to the telson tip. The upside down swim pattern is cause

by the light attraction of the thoracic appendages. Under the eleven stages, the larvae grow to around 7.7 mm in size. This included the newly metamorphosed postlarvae (PL) size. The reason is their motility pattern that performs just like the adult prawns. In this stage the light orange pink colour of the head and translucent will show.

The two main parts of the cephalothorax and abdomen which are the head and tail are located at the body of post larvae and matured prawn. The somites are the twenty segments of the bodies in the prawn. From the segments, 14 of them are in the head part, which are protected under the dorsal and lateral shield, which is called the carapace whether the structure is rigid and smooth, excluded the two spines at the side. The antennal spine is located beneath the body while the hepatic spine is located at the low and back side of the antennal spine. The front position of rostrum indicates the end point of carapace, where the structure is curved. Extension of rostrum is longer than the antennal scale (FAO, 2002).

Cephalon is the frontal part of the cephalothorax that contains six segments which also consists of the eyes and ten appendages. From the six segments, the last three parts are able to be observed as the prawn is flipped over. Cephalon segment plays important role for supporting the protruded eyes, the pair of antennae, where there are three parts of stalks, found in the three flagella tactile. It also support the second part of antennae, where there are five parts of penducles along with a flagellum, mandibles that is used to consume food, maxillae which is lamelliform located under the second maxillae, carries the function of food transferring into the oral part. The second maxillae have the same structure with the first, however it has additional use where the appendages is continuously moving and creating the water current along the gill. This draws water to the respiratory system (FAO, 2002).

In order for sensory function to perform, two pairs of antennae contain statocyst on the peduncles that act as the gravity receptor. The oral part and both maxillae locate the six sets of oral part. The thorax which is the back portion of cephalothorax, contain eight mixed segments. The appendages can be observed clearly. They contain three sets of maxillipeds, five sets of pereipods. Both parts have the function of oral. The third maxillipeds that look like legs are actually the oral part. The pincers (chelae) are found on the first and second pereiopods. They are also known as chelipeds. Although the first legs are lean but they are tougher than the other leg. In order to consume food, mating and attack, the second chelipeds play a role in these activities. The cheliped are stronger on the third, fourth and fifth legs which are known as walking legs. For females, the membrane covered eggs are located at the base of third pereiopods while for the male, this area is where the sperm released at the fifth base pereiopods. There are chemoreceptor cells which are carrying the function of food and salt extraction in the pereiopods. The pair of legs of *M. rosenbergii* is the same size. For adult males the legs are very long and able to achieve the tip of rostrum (FAO, 2002).

In the tail part, there are six parts with each a set of appendages use for swimming and walking. The primary five sets are soft for swimming purpose. For female prawn, their legs contain the auxiliary for keeping the egg sacs in the brood chamber. This part functions as copulation objective in the male prawn. The structure is known as appendix masculine. The hard and rigid uropods are also the sixth set of pleopods. The central appendage of telson consists of two wide vertebral columns that extend to the back of the tail. The tail part with fan-like shape builds up from the uropods and telson, which allows the prawn to reverse immediately.

Some of the *Macrobrachium* species are transferred to other parts of the world from their origin location for research study. *M. rosenbergii* is the most common used species

for large scale commercial farming and has been transferred to many countries. Overall, the production of *M. rosenbergii* in the world is reported more than 200,000 tonnes in the year of 2008 and increasing from year to year (FAO, 2010) (Figure 2.2). Some countries produce *M. rosenbergii* in a very large scale, for example, Bangladesh, Brazil, China, India, Malaysia, Taiwan, and Thailand (FAO, 2002). Other than these countries, there is more than 30 other countries are also reported with the production of *M. rosenbergii* in the year 2000. Vietnam is also one of the main producers of *M. rosenbergii* (New, 2000).

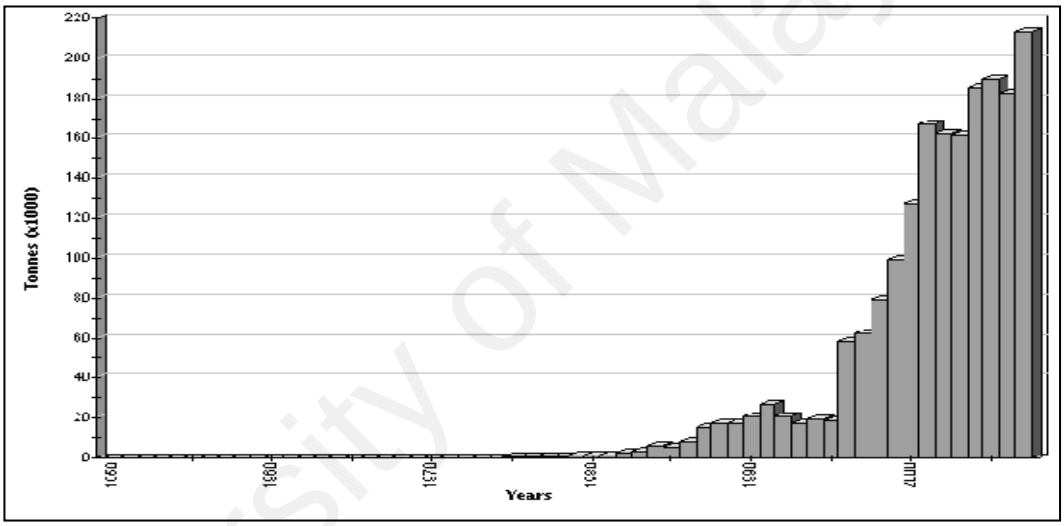

Figure 2.2: Aquaculture production of *Macrobrachium rosenbergii* in the world (FAO Fishery Statistic) (www.fao.org).

2.2 Biosecurity in shrimp farming

Biosecurity means "life protection", it can be described as preventing the entry, establishment and outbreaks of undesirable biological organisms or living things. Biosecurity, when it is being practically applied in shrimp aquaculture, is elaborated as the practice or actions to exclude some unwanted pathogens in farming aquatic organisms in broodstock facilities, hatcheries, and farms, or from the entire countries to prevent diseases (Lightner, 2003). Basically, farm biosecurity has the following three objectives: First, to minimize the chance of high-risk inputs introducing foreign

pathogens. Second, to surveillance and detect incursions when they occur. Third, responding to disease incursions by controlling the spread of unwanted disease agents (Perera et al., 2008).

In prawn farm, biosecurity involved practising sets of science-based approaches to prevent or mitigate the risk of a specific pathogen, which is infectious agent that resulting in disease – (a) getting into the farm, and (b) spreading in a pond or to adjacent ponds, and to other farms or to the surrounding environment.

The biosecurity program is farm-level and aimed to target highly infectious pathogens includes White Spot Syndrome Virus (WSSV), Yellow Head Virus (YHV), Taura Syndrome Virus (TSV) and Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV). These viruses are harzardous and harmful because they can cause mortality, highly transmissible to prawns, uncured and infect variety of hosts. In fact, these viruses and related diseases are studied and reported with a lot of information. The epidemiological information about the viruses, such as its carriers and its route of transmission, is generally known. Therefore, a specific, focused biosecurity programs can be implemented for each particular pathogen.

One of the ways to exclude pathogens from aquatic stock is by using quarantine and specific pathogen-free (SPF) or specific pathogen resistant (SPR) certified broodstocks, also, limiting the imports or entry of live and frozen shrimp which might be infected with pahogens (Lightner, 2003). To exclude pathogens from hatcheries and farms, potential vectors or organisms and possiblesources of contamination are excluded and at the same time, internal cross contamination is eliminated. On the other hand, in the domestic poultry industry, biosecurity can be explained as a crucial method to prevent, control, and eliminate economically infectious pathogens and diseases. Even biosecurity in this circumstance may have many different explanations, but the objective of this

application in shrimp farming is to control stock and exclude pathogens. This can be applied by stocking farms with diseases-free shrimps into farms with controlled water origin. A good layout design of farm and good water management are useful to control the water source, through the implementation of practices as “zero water exchange”, which means complete absence of water exchange during rearing and use of water treatment devices to eliminate possible vectors from water source (Browdy et al., 2001).

Horowitz and Horowitz (2003) suggested and discussed about some physical, chemical, and biological methods or measures and also second line defence against outbreaks of diseases. Physical measures include physical barriers, quarantine and water treatment to block the entry of vectors which carrying diseases to the farm, while chemical measures are the preparation or steps used to treat materials before emoving into the farm. For example, chlorination and ozonization are the most common method of water disinfection, while iodine and chlorine are used to treat possible other organisms that might transmit diseases. Biological measures can be applied by using SPF shrimps. A second line defence in shrimp industry can be applied by using specific pathogen-resistant shrimp.

The pathogens WSSV and IHHNV are the most common disease in shrimp industry. Both viruses have been found in wild penaeid shrimp in the Americas (Motte et al., 2003) and Asia (Fegan & Clifford, 2001). The occurance of these pathogens in wild shrimp stock in the Americas led to the modification of practice in shrimp farming. In the past, broodstock and postlarvae were obtained from the natural environment regardless their quality and health status. Moreover, in past days, shrimp farms in most of the isolated and aparted area, were operated and ran without a comprehensive, clear plan and protocol. After the appearance and epidemic of WSSV in Asia and followed by Americas, the farms have started to implement and apply a comprehensive biosecurity

action plans to prevent against diseases and harmful pathogens. Over and above that, the application of thebiosecurity measures and plans has reduced the burden of farmers caused by production losses due to disease infection and to improve the quality of shrimp products (Fegan & Clifford, 2001).

If pathogens or diseases are found in one of the pond, it is not necessary to discard all of the shrimps as some measures can be taken to avoid the complete loss of the shrimps and disease outbreaks to other ponds. Lightner (2003) suggested a method to get rid of pathogens at the beginning stock level and disinfection in the farm to remove bacteria. For instance, the disease infected ponds should be reduced density to avoid more shrimps are being infected. After that, disinfection process should be carried out to remove pathogens and lastly, restocked the pond with specific pathogen free stocks. On the other hand, Horowitz and Horowitz (2003) recommended preparing better biological environmetns to the infected regions to increase the capability to withstand diseases infection. They recommended three different types of measures: first, perform physical measures such as increase aeration into the pond, control temperature for shrimps' optimum growth, remove sludge and treat wastewater to remove contaminants and to provide better living environment. Second, perform chemical measures such as control pH and salinity of water, reduction of ammonia and nitrite level, and usage of antibiotics. Third, perform biological measures, consists the use of probiotics, which contain a mixture of bacterial species provide health benefits for the host. Probiotics was initially used to promote growth and health of animals, however, new discoveries have been found, such as their effect on stress tolerance, although this requires a more scientific development.

2.2.1 Managing risk of pathogen introduction

The most common pathogen introduction into aquaculture systems are (1) stock, such as post-larvae for stocking grow-out ponds, (2) feeds, (3) water, and (4) equipment such as nets, aerators, etc. that may be shared between farms. In addition, other inputs of pathogen may be caused by vaccines and instruments.

To minimize the likelihood of live pathogens are present in the farm inputs, some measures can be taken. Stock can be sourced from populations which determined to be specific pathogen free (SPF) or specific pathogen resistant (SPR). Moreover, populations can be monitored regularly and known to be of good health status or be quarantined or treated to reduce the risk (Perera et al., 2008).

The pathogens may be survive outside a host and to infect a variety of host species, means that aquatic animal pathogens can be introduced into aquaculture systems through intake water, either in fomites or in infected organisms. Intake water can be screened and filtered to exclude or reduce the presence of pathogens, fomites and infected organisms. Water treatment can be done prior to use, such as filtration, UV, ozone or chemical treatment can reduce the risks of pathogen present. Farm equipment such as aerators, harvest nets, footwear, containers and vehicles for transporting live prawns can be readily disinfected using chemical treatments, such as sodium hydroxide and iodophors (Perera et al., 2008).

Mohan et al. (2004) suggested a general summary on farm-level biosecurity measures that are commonly used. They also suggested better management practice at the farm level associated with white spot disease. These measures include pond preparation to exclude pathogens and carriers, stocking of WSSV negative post-larvae,

screening, disinfecting intake water and effecting physical barriers to prevent the entry of wild crustaceans into the ponds.

2.2.2 Surveillance to detect pathogen incursions

Early detection of pathogen incursion in a farm enables for more effective establishment of control system. Therefore, health surveillance is important to detect early incursions which can be either targeted to specific pathogens or more to general. Prawns can be periodically screened, the number of samples is ensured to be enough to produce an acceptable level of confidence in detecting the agent were it to be present. The health status of the prawns can also be monitored generally through microscopic examination. Both targeted and general surveillance requires specialised expertise, while general surveillance is cheaper (Perera et al., 2008).

2.2.3 Response to pathogen incursions

If there is disease incursion detected into a farm, actions and response plans are taken rapidly. Response plans are practical steps that should be carried out immediately in the event of outbreak. It is time-saving because the decisions and plans related to various circumstances are already be planned, thus enables for early action.

Response plans and control program should be made by based on some considerations such as location and design of the farm. For example, building shrimp culture ponds that can be fully drained and dried makes it cost-effective eradication of many aquatic pathogens agents from those ponds. Moreover, farm layouts can be designed in such a way when a disease outbreak occurs, so that the affected prawns are easily isolated, thereby prevent the disease from spreading to other parts of the farm or adjacent farms which provided with the same water source (Perera et al., 2008).

An effective disease response plan includes monitoring, containment and eradication. Monitoring includes surveillance; containment includes isolation of infected stock, tracing back, control clinical disease, emergency harvesting, control vectors; and eradication includes stock destruction, safe disposal, disinfection and treatment.

In addition, an important element for successful implementation of biosecurity strategies is ensuring the farm staff and managers have awareness on the potential risks and trained in biosecurity measures practices, and also understand their individual roles as respond to disease event. Training programmes are important for farm staff to create awareness and incorporate into policy or response plans. In this case, governments can assist in these programmes through the development of regional based resource material (Perera et al., 2008).

2.2.4 Cost and benefits

The cost of implementing biosecurity measures can be high, especially for marginal operations in which short term profit is important for business success. The cost for surveillance includes diagnostic testing to ensure the absence of farm inputs can be significant. The overall cost of biosecurity depends on the particular conditions and needs, at the same time, the range of available measures and that relatively low cost measures should be taken into account.

However, many farmers are not aware and lack of attention to biosecurity, shows that there is uncertainty of biosecurity measures value. Normally, the decisions made are based on short-term commercial grounds and at an individual enterprise stage, also regulatory controls are not well established.

Even though this relatively short-term actions are taken at an enterprise level, the short and longer term impacts to aquaculture significantly due to several key disease

problems (white spot disease, Taura syndrome disease) over the last decade. These resulted in slow but increasing attention to biosecurity by industry groups and governments through practising, regulation and education, and also by international organisations specifically Food and Agriculture Organization of the United Nations (FAO) and World Organisation for Animal Health (OIE), by establishing guidelines (Perera et al., 2008).

2.2.5 Risk assessment based resource allocation

The main objective of commercial aquaculture at the farm level or major commercial enterprises, which are small-scale, is to make profit. Therefore, all farmers would consider the need for implementing biosecurity measures in the context of resources availability. All recognize the potential business risks related with disease and allocate some resources to handle the risk. However, the resource allocation is rarely based on proper risk assessment. Under finite resources circumstance, it is important that resource allocation related with disease management is corresponding with the biosecurity risk. In this case it is important not to always equate the pathogen to clinical disease. This error causes unnecessary focus on the pathogen, when dealing with clinical disease may present a more cost-effective option (Hugh & Stoskopf, 1999).

Biosecurity risk can be defined as a measure of the likelihood of a disease outbreak in a farm and the short term and long-term negative effects of the outbreak. Biosecurity risk assessment at the farm level includes diseases of concern, an evaluation of the likelihood that the agents would enter the farm system under existing operating environment, and an evaluation of the overall financial losses to the farm caused by a disease outbreak (Perera et al., 2008).

Based on biosecurity risk assessment, farmers can determine the most suitable and cost-effective risk management measures to deal with the risk. The risk assessment approach is suitable for individual farmers, farming cooperatives and government regulators.

Farmers and industry groups need to consider wider and longer term negative impacts. For example, white spot disease causes huge losses in shrimp farms during the first few years following introduction of WSSV into systems. White spot disease caused approximately more than 50% in the main shrimp production state in India (Yap, 2001), between 70-75% in Ecuador (Hill, 2002; Yap 2001), arbout 45% in Bangladesh at 1996 (Mazid & Banu, 2002), and about 80% in Peru (Yap, 2001). Moreover, Thailand and China which are the major shrimp producing countries also have experienced huge production losses. Individual farmers are usually experienced these significant losses, however, industry groups and government regulators would need to look into these impacts. At the same time, industry groups and governments need to consider the negative impacts of aquaculture such as ecosystems damage or human health issues on chemical residues, as these issues are usually not considered at an individual farm stage.

2.3 Broodstock

2.3.1 Obtaining berried females

Berried female prawns can be obtained by using cast netting, followed by selection or total harvest. They can be found in rivers, canals and lakes at which they are indigenous. Some hatcheries choose to use berried females from natural waters because they believe that wild prawns produce better quality larvae than farm-reared prawns. However, there are some problems facing when collecting berried females from rivers or lakes. It might cause egg loss during transportation from collecting sites to hatcheries

farm. Hence, some hatcheries choose to use adjacent rearing ponds for their broodstock supplies (FAO, 2002).

During the beginning of the rainy season, berried females can be found in large amount. When *Macrobrachium rosenbergii* is raised in sub-tropical areas, broodstock are usually obtained from ponds during the harvest. During winter season in some countries, the broodstock are maintained in indoor environment which is environmentally-controlled conditions (FAO, 2002).

2.3.2 Genetic improvement

The issues related to broodstock selection and the advantages of maintaining specific broodstock facilities have been discussed (Daniels et al., 2000). Moreover, the topic of genetic selection of *Macrobrachium rosenbergii* is also been reviewed by Karplus et al., 2000. However, there is only little progress on genetic improvement of *Macrobrachium rosenbergii* even though this topic could be expected to bring improvements.

Macrobrachium rosenbergii which are hatched from eggs earlier appear to have advantage in grow-out because they are usually being called as the first ones as dominant blue claw males. However, there is no evidence shows that these 'early hatchers' have better quality and genetic than the late ones. Hence, it would be no use to choose prawn eggs from one part of the spawning period as larvae stocking. In addition, choosing larvae from one part of the spawning period could bring another consequence, which is reduction in genetic variation and increase in inbreeding (Tave, 1996).

Most of the farmers select larger prawns which carry more eggs, but this may not be a good choice. Instead of choosing large females six months after stocking, choosing berried females from ponds three months after stocking is a beter choice. These prawns give a better genetic effect and produce larger prawns at harvest (FAO,2002).

When the berried females *Macrobrachium rosenbergii* used in the hatcheries have been removed from grow-out ponds, the growth rate and survival of the prawns during grow-out was declined. This situation is due to occurrence of inbreeding or known as genetic degradation. It has been noticed in some countries such as Taiwan, Thailand, Martinique, and Province of China. In some countries where *Macrobrachium rosenbergii* is native, there is the problem as the broodstock for hatcheries are obtained from grow-out ponds and this is being repeated for many times for generations. On the other hand, for the countries where *Macrobrachium rosenbergii* is not native, the problem may be worse. This is because the farmed stock has normally arised from a very small amount of females or post-larvae, which were introduced into the country many years before (FAO, 2002).

When the yields of prawns decrease, it affects the farmers' incomes. Therefore, this brings to solutions which are using more wild broodstock prawn and improve the genetic ofbroodstock. In 1998, genetic improvement work has started in Thailand. A company has introduced a new strain of *Macrobrachium rosenbergii* and it has significantly improved the performance of the prawns (Anonymous, 2001).

2.4 Major diseases in shrimp farming

2.4.1 White spot syndrome virus (WSSV)

White spot syndrome virus (WSSV) was a viral disease which bring a serious impact to Thai shrimp aquaculture. All farmers, especially small-scale farmers whose main income was generated from shrimp farming are thought to have become indebted to banks because of a serious production lost due to shrimp disease.

It has been proven that spawning stress induces WSSV replication in *Penaeus monodon* (Flegal, 2007). White spot syndrome virus (WSSV) consists of a rod shaped

double-stranded DNA virus together with a large circular genome of 293 kbp. It is first appeared in China in 1992, has become epidemic in farming area around the world, and has been a main reason of poor productivity in shrimp aquaculture. This disease affects a wide range of host such as crayfish, lobsters, crabs, shrimps, and freshwater prawns (Escobedo-Bonilla et al., 2008). WSSV affects crustacean greatly and become a big problem in aquaculture farming (Walker et al., 2011). Some of the infected hosts will not kill by the virus, but act as carriers by transmitting infection to cultured host (Flegel, 2007).

WSSV causes white spot disease in shrimps with white spots appearance on shell (Figure 2.3). White spot disease can be transmitted horizontally and vertically. Many crustaceans such as crabs and copepods are either hosts or carriers of WSSV. Non-arthropod crustaceans such as *Balanus sp.* and annelid can also be a carrier of WSSV. This virus can survive in water without host for few days and longer period in soil. When larvae are stocked in ponds, they can be infected by WSSV through untreated water or carriers in the water. WSSV can bring about up to 100% accumulative mortality within two to ten days to cultured shrimp. For that reason, thorough investigations and comprehensive research sudies have done on WSSV in the past few decades to understand the infection mechanism. Researchers studied on the virus replication and viron biological process development and show that DNA replication and the formation of envelope occur in the nucleus starting from the beginning (Durand et al., 1997; Wang et al., 2000).

Figure 2.3: White spot syndrome virus (WSSV) infected shrimps with the appearance of white spots on shell (Aquaculture Pathology Laboratory, University of Arizona).

Histopathology examination by using light and electron microscopes can diagnose WSSV infection. In addition, in situ DNA hybridization also has been used to test with various species of cultivated shrimp in several Asian countries and the results showed gross signs of white spot syndrome. Other than that, real-time PCR and isothermal DNA amplification have also been used because these methods are rapid and specific. After the DNA hybridization probes for WSSV were developed in Thailand, primers of WSSV were designed and used widely for PCR detection. Other than PCR tests, immunological tests also been applied by emploting an antigen to detect presence of antibodies to a pathogen, or an antibody to detect the presence of an antigen, of the pathogen in the samples. Lateral flow chromatographic detection strips are available in Japan and Thailand now (Flegel, 2006).

2.4.2 Infectious hypodermal and haematopoietic necrosis virus (IHHNV)

Infectious hypodermal and haematopoietic necrosis virus (IHHNV) is a small and single-strand DNA-containing parvovirus with average diameter of 22 nm. IHHNV is enzootic mainly in Taiwan, Singapore, Malaysia, Indonesia, Thailand, Philippines and Australia. This virus is highly transmissible and contagious to many penaeids shrimps such as *Penaeus stylirostris, Penaeus vannamei, Penaeus monodon* and *Penaeus chinensis.* Infected hosts suffer from cannibalism, reduced food intake and increased the

risk of mortality. The infected shrimps move upwards to water surface repeatedly, spin over and drop to bottom. In some cases, the infected hosts survive from IHHNV infection and act as carriers which pass the virus onto their offspring and other populations of same species through vertical and horizontal transmission (Bell & Lightner, 1984; Lightner et al., 1983). In horizontal transmission, viruses are transmitted among individuals of the same generation, whereas vertical transmission occurs from mothers to their offspring.

2.4.3 Monodon baculovirus (MBV)

Monodon baculovirus (MBV) is a double stranded DNA. Its DNA structure is similar to white spot syndrome virus (WSSV) and hepatopancreatic parvovirus (HPV). MBV does not cause death, but its effect is also serious as it causes growth retardation of the shrimp or stunted growth and thus economic losses (Flegel, 2006). The size of infected shrimps is much smaller than healthy shrimps.

The viral inclusions, which are the formation of aggregates in the nucleus or cytoplasm of the infected cells, can be seen in the cuticle of early post-larvae specimens under the light microscope. Polymerase chain reaction (PCR) is a common and general method used for MBV detection because it produces rapid results. Currently, many diagnosis methods have been developed such as fluorescence microscopy, monoclonal antibody, immunohistochemistry, PCR, nested PCR, multiplex PCR, dot-blot hybridization, in-situ hybridization, and transmission electron microscopy (Flegel, 2006).

2.4.4 Baculovirus penaei virus (BPV)

Baculovirus penaei virus is commonly known as BP virus disease, nuclear polyhedrosis disease, baculovirus disease and also PIB ("polyhedral inclusion body") virus disease. BP virus is epidemic in cultured and also penaeids in the Americas, and enzootic in some areas such as Peru and Mexico.

This virus affects a wide range of host such as *Penaeus aztecus, Penaeus vannamei, Penaeus duorarum* and *Trachypeanaeus similis*, as known as roughback shrimp. BPV are transmitted by horizontal transmission, which means transmitted among individuals of the same generation, ususually to all life stages. Obvious infection signs may not be seen in shrimp larger than the ninth moult postlarvae (PL-9), except if the shrimps were suffered from stressful environments such as insufficient food and inappropriate temperature. The growth rate of infected shrimps are reduced, moreover, gill and surface fouling are also increased.

One of the diagnostic techniques of BP virus is squash preparations, on other words, the smearing of a tiny tissue specimen between two slides before microscopic analysis. In this case, single or multiple polyhedral occlusion bodies (PIBs) in epithelial cell nuclei of hepatopancreas, anterior midgut or fresh faeces are examined using bright field microscopy. Sample illumination is transmitted white light after staining with 0.001% aqueous phloxine, and contrast in the sample is caused by attenuation of the transmitted light in dense areas of the sample (Thurman et al., 1990). It is a rapid and specific diagnosis method. BP virus also can be diagnosed by using genomic probes through dot blot assays and in situ hybridization (Lightner et al., 1992).

2.4.5 Yellow head virus (YHV)

Yellow head virus (YHV) at first was wrongly interpreted as a baculovirus. However, it was found that its morphology different from baculovirus soon through purification and characterization. It is classified as *Ronivirdae* (Boonyaratpalin et al., 1993). Yellow head virus (YHV) belongs to the family Roniviridae, causes yellow head disease (YHD), which is highly lethal and kills the shrimps quickly. YHV-infected tissues show enveloped bacilliform virions under transmission electron microscopy (TEM). The length is approximately 150-200 nm and the diameter range from 40-50 nm. They are located in vesicles of cytoplasm in infected cells and intercellular spaces (Chantanachookin et al., 1993).

Yellow head disease was first discovered as an epizootic from Thai shrimp farms, and then YHD has spread widely and reported in many cultivated shrimp areas in Asia (OIE, 2003). In United States, YHV has been found in frozen imported shrimp (Durand et al., 2000), it may be caused by accidental introduction and spread of these pathogens into the shrimp culture industries may be significant. In addition, YHV has been reported incorrectly in farmed shrimp from the Americas due to the severe necrosis of the lymphoid tissue and organ. Based on Pantoja & Lightner (2003), the tentative histological diagnoses were due to infections with white spot virus which causes necrosis lymphoid organ was similar to the hispathology that appearing in severe acute YHD.

RT-PCR method is currently the best rather than in situ hybridization for the diagnosis of yellow head disease. Thailand and Australia cooperative work has more recently revealed non-pathogenic YHV variants in Vietnam, Thailand and India. It may be possible to use the kit to differentiate some types of YHV based on the geographical location. However, the kit is not effective to detect the types of yellow head virus found

in India. These RT-PCR probes are also useful for inspecting suspected carriers of YHV and testing whether they can transmit the virus to cultivated shrimp. The results of these studies are useful to establish disease control programs for shrimp farmers. Other than nucleic acid-based tests for YHV group viruses, monoclonal antibody assays have also been developed for diagnosis of yellow head disease by immunohistochemistry (Wongteerasupaya et al., 1997).

2.4.6 Taura syndrome virus (TSV)

Taura syndrome was known as a new emergence disease in the Americas in 1992, however its viral etiology was not established until 1994 (Lightner et al., 1995). Taura syndrome virus (TSV) is a small single-stranded RNA virus, a member of the family Picornaviridae as it was an enveloped virus. The TSV genome consists of about ten thousands nucleotides and two large open reading frames (ORFs). ORF 1 contains the sequence motifs of non-structural proteins, which means proteins encoded by a virus but that is not part of the viral particle, such as helicase, protease and RNA-dependent RNA polymerase. On the other hand, ORF 2 contains TSV structural proteins sequences, which are the three main capsid proteins VP1, VP2 and VP3, with 55, 40, and 24 kDa respectively (Robles-Sikisaka et al., 2001).

TSV started to emerge in Ecuador in 1991 and the source of virus was unclear. The disease was categorized as a main new disease of farmed *L. vannamei*. It was known as Taura Syndrome (Lightner et al., 1995). In Asian countries, TSV outbreaks were first reported in Taiwan in imported *P. vannamei* as living fry for commercial aquaculture ponds use (Tu et al., 1999).

Current diagnosis methods of this disease includes pathological methods, monoclonal antibody based methods, in situ hybridization, dot-blot method,

immunohistochemistry methods, DNA amplification method (PCR). Primers for an RT-PCR method that produces a 231bp TSV specific fragment have been developed (Nunan et al., 1998).

2.4.7 Infectious myonecrosis virus (IMNV)

Infectious myonecrosis virus (IMNV) is the most recent recognized shrimp viruses to appear in Asia. It is believed that the arrival was duo to introduction with contaminated cultured *L. vannamei* from northeast Brazil. The outbreaks of the disease are related to environmental and physical stresses, such as unfavourable salinity and temperature, and might also be caused by insufficient nutrient feeds. Even *L. vannamei* is the main host for IMNV, other species such as *P. monodon* are also possible infected by the virus (Tang et al., 2005).

Infectious myonecrosis causes disease and death in juvenile and sub-adult pond-cultivated stocks of *L. vannamei*. IMNV is 40 nm diameter spherical virus, icosahedral, and a single double-stranded RNA with approximately 7000 bp. Infectious myonecrosis presents as a disease in *L. vannamei* with an acute starting of gross signs and increased the rate of mortalities, but after that, it develops with a more persistent condition with continual low level mortalities. The infected shrimp appear with large white necrotic areas in the striped muscle and lesions with coagulative muscle necrosis.

The characterization of IMNV has been partially studied and some parts of its nucleic acid (RNA) genome are known, have been cloned and sequenced. The diagnosis of the disease and the the detection of IMNV can be done by using molecular probes and RT-PCR methods which have been developed (Lightner et al., 2004).

2.4.8 *Macrobrachium rosenbergii* nodavirus (*Mr*NV)

White tail disease (WTD) is associated with both *Macrobrachium rosenbergii*-nodavirus (*Mr*NV) and extra small viruses (XSV). *Mr*NV is related to viruses in the family *Nodaviridae*, and satellite virus particles found in *Macrobrachium rosenbergii* (giant freshwater prawn) infected with *Macrobrachium rosenbergii* nodavirus. WTD causes serious mortality in postlarvae of *M.rosenbergii* in the hatcheries and nurseries stage. It has been reported that white tail disease affected freshwater prawn hatcheries and nursery ponds in some regions of India, killing a large amount of shrimps and leading large economic losses (Ravi et al., 2009).

*Mr*NV is a very tiny and non-enveloped virus with average diameter of 26 nm. The *Mr*NV genome consisted of two types of ssRNA, which are RNA1 and RNA2, with 2.9 kb and 1.26 kb for each. Another single polypeptide of 43 kDa is found in the capsid. *Mr*NV can be identified by using many diagnostic methods which include reverse transcriptase-polymerase chain reaction technique (RT-PCR), histopathology, immunological ways and in-situ dot blot hybridization method using nucleic acid probes (Sudhakaran et al., 2006).

2.4.9 Acute hepatopancreatic necrosis disease (AHPND)

Acute hepatopancreatic necrosis disease (AHPND), was initially called as early mortality syndrome (EMS), is a new appearing disease in shrimp aquaculture. Its first outbreak was in 2009, which brought to serious impact to shrimp farming industry with huge production loss (Lee et al., 2015). The AHPND first emerged in China, causing serious economic losses in the shrimp industry in Asia (NACA, 2012). In Mexico, AHPND has reduced the production of *Penaeus vannamei* significantly in the northwest regions such as Nayarit, Sinaloa, and Sonora since 2013 (Nunan et al., 2014; Soto-

Rodriguez et al., 2015). The symptoms of affected shrimps are obvious with the appearance of inactivity movement, growth retardation, empty stomach, and white atrophied hepatopancreas (Tran et al., 2013).

Vibrio parahaemolyticus, which has a 70-kbp plasmid, is the etiological agent of AHPND (Lee et al., 2015; Tran et al., 2013). In other words, the homologous of the *Photorhabdus* insect-related (Pir) toxins PirA and PirB encoded by this plasmid are the causative agent for shrimp mortality in AHPND (Lee et al., 2015; Sirikharin et al., 2015). The toxins are released from VP_{AHPND} isolates that colonized the shrimp stomach and enter the hepatopacncreas to produce pathognomonic AHPND lesions (Lai et al., 2015).

Two shrimp species are infected by AHPND most commonly, which are the giant tiger prawn (*Penaeus monodon*) and the whiteleg shrimp (*Litopenaeus vannamei*) (NACA, 2012). AHPND usually appears within the first month after the shrimp is stocked in ponds (NACA, 2012), and the mass mortality can be more than 70% (Kongrueng et al., 2014).

2.5 Current diagnosis methods of giant freshwater prawn diseases

Till now, there is no effective and successful treatment for viral infections in prawns, therefore prevention steps and actions are necessary to prevent the spreading of pathogens, and early disease diagnosis is an effective method to reduce the loss. Normally, clinical signs, abnormal symptoms, immune parameters and hispathological analysis are the first method used to survey animal health (Lightner et al., 2006). However, these methods are not sufficient to provide an accurate diagnosing, because many other factors can cause these identical signs. Therefore, more specific and

sensitive diagnostic methods are developed, especially those engaging molecular and immunological tools.

The first molecular techniques employed for shrimp viruses diagnosis is in situ hybridization in tissue parts. The main drawback of in situ hybridization is the viral genome might be degraded during fixation, especially when we deal with RNA viruses, it might give a false-negative results. Due to these reasons, methods which identify viral genome by amplification process produce more reliable and accurate results, such as reverse transcription polymerase chain reaction (RT-PCR), nested RT-PCR method. These are effective screening method for broodstock and post-larvae samples. However, the quantity of viral genome in samples amplified by one- or two-steps reaction may be lower than the detection limit of PCR, thereby give a false-negative results. Hence, a quantitative measure is widely used nowadays and desirable method by performing real time RT-PCR/ PCR. Moreover, a molecular method which is suitable for shrimp viruses screening is the reverse-transcriptase loop-mediated isothermal amplification reaction (RT-LAMP), followed by nucleic acid detection with a chromatographic lateral flow dipstick. The overall combined method produces specific, sensitive and rapid results, but it is expensive and not practical generally because the use of expensive reagents in the reaction (Lightner et al., 2006; Flegal, 2006).

Other than molecular techniques, monoclonal antibodies are also been developed for shrimp viruses and used in the development of enzyme-linked immunosorbent assay (ELISA), immunoblotting and immunohistochemistry (Flegal, 2006). All of the methods and procedures must be done by trained and experienced personnel by using certain equipment and machines in a lab, thus not suitable for pond-side detection. If the diagnosis is really need to be pond-side, assays developed as kits for farmers are the most suitable method for viral detection, as sometimes the rapid spreading of viruses

causes time constraint for sending samples to laboratory testing. Out of all the pond-side immunological tests, immunochromatographic strip tests are the most general and used because they are easy to use and provides rapid result (Seibert & Pinto, 2012).

For white spot syndrome virus, DNA hybridization probes have been developed by some laboratories in the past (Chang et al., 1996; Durand et al., 1996; Wongteerasupaya et al., 1996). In addition, the primers specific for WSSV detection by using PCR have also been developed by Lo et al. (1996). There are many choices and methods are available for the detection of the white spot disease virus, such as PCR (Nunan et al., 1998), in situ hybridization (Chang et al., 1996; Durand et al., 1996; Wongteerasupaya et al., 1996), dot blot hybridization (Wongteerasupaya et al., 1996; Sahul Hameed et al., 1998) and ELISA (Sahul Hameed et al., 1998). Primers for PCR based on the sequence of a cloned fragment of the white spot syndrome virus genome have been developed, at the same time, primers are used to detect WSSV from naturally infected shrimp and experimentally challenged shrimp (Tapay et al., 1999). In their study, one-step and two-step PCR protocols have been developed with high sensitivities of 10-100 pg and 100 femtograms. Moreover, *Macrobrachium rosenbergii* nodavirus (*Mr*NV) disease in giant freshwater prawn was diagnosed by standard protocols Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) by using specific primers and histopathology analysis (Koesharyani & Gardenia, 2014).

In Malaysia, Aznan et al. (2017) have conducted the health surveillance of freshwater prawn, *Macrobrachium lanchesteri* in Setiu Wetland, Terengganu, Malaysia in 2017. The study was aimed to survey the health status of *M. lanchesteri* in Nyatoh and Chalok Rivers in Setiu Wetland. The prawn samples were collected from each river by seining for bacterial, mycological screenings, viral screenings, and histopathogical examination. In this study, bacterial screening was done by culturing bacteria on

different types of agar, followed by BLAST analysis of 16S rRNA gene sequence. On the other hand, they also carried out viral screening for white spot syndrome virus (WSSV), Monodon baculovirus (MBV) and infectious hematopoietic and hypodermal necrosis virus (IHHNV) by using polymerase chain reaction. They have carried out histopathology and the results revealed degeneration of hepatopancreas tubule cells and loss of the star-shaped lumen profile in the samples from Chalok River. A total of 14 isolates were obtained from the prawn hepatopancreas and eggs, including *P. aeruginosa* isolate which could be potentially pathogenic and of public health concern. Even though there is no viruses were detected in the prawns in their study, the possible presence of the viruses in Malaysia could not be ruled out.

Dual priming oligonuclotide (DPO) primer was used in this study to screen for DNA and RNA viruses. In conventional polymerase chain reaction (PCR), it is usually associated with non-specific binding between the primer and template. The use of dual priming oligonuclotide (DPO) primer produces high PCR specificity by inhibiting mismatching and non-specific priming of the primers to the template under unfavourable PCR conditions.

The dual priming oligonuclotide (DPO) system has two different primer segments, the first one is a 5' conserved (18 to 25 nucleotides) region and the other one is a 3' specific (6 to 12 nucleotides) region, joined by a polydeoxyinosine [poly(I)] linker (Figure 2.8). The different location and number of nucleotides brings to different annealing preference for both segment. The longer 5′-segment binds to the DNA template and begins stable annealing, on the other hand, the 3′-segment selectively binds to its target site. Thus, the priming of 5′-segment and the 3′-segment of the DPO only produce target-specific extension. The application of dual priming oligonuclotide has been used to detect single nucleotide polymorphisms (SNPs) (Chun et al., 2007).

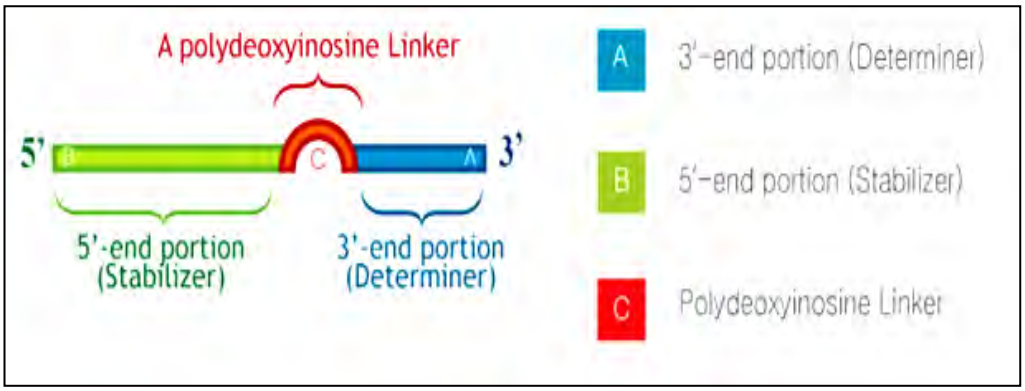

Figure 2.4: Dual priming oligonuclotide (DPO) has two different primer segments, a 5' conserved region and a 3' specific region (Chun et al., 2007).

The principle of DPO is that it composes two functional priming regions with one of the region is larger than the other region, joined by the poly (I) linker. Due to two uneven distribution of priming regions generate dual priming reactions based on the following scheme, therefore only target-specific product is produced.

Furthermore, a major advantage is, DPO makes primer design very easy and uncomplicated because two separate priming reactions give a primer with a high tolerance in annealing. The first stable priming reaction is started by Stabilizer of 5' end, while the second priming reaction done by Determiner, thus there is a chance to correct the specificity during second reaction. Therefore, DPO does not need a fixed optimization of PCR conditions, primer search for favourable parameters such as primer length, GC content, annealing temperature, and secondary structure (Chun et al., 2007).

When conducting multiplex PCR, it is also preferable because to its different functionalities formed by the poly (I) linker, causing differential annealing preferences (Chun et al., 2007). Hence, DPOs are widely applied in many researches to screen and diagnose viral infections (Chun et al., 2007; Chung et al., 2014; Kim et al., 2008; Kwon et al., 2014; Moon et al., 2010; Woo et al., 2008). Scientists have been using DPO system in specific multiplex RT-PCR assay for effective detection of influenza viral segments as a fast, cheap, and easy method (Lee et al., 2016).

CHAPTER 3: METHODOLOGY

3.1 Sample collection from prawn farm in Kampung Chennah, Jelebu, Negeri Sembilan

3.1.1 Sample collection

The *Macrobrachium rosenbergii* juveniles samples were collected from a prawn farm located in Kampung Chennah, Jelebu, Negeri Sembilan (Figure 3.1 & Figure 3.2). The total number of 19 *Macrobrachium rosenbergii* juveniles (cycle 1) was collected in year 2017. The total number of 30 *Macrobrachium rosenbergii* juveniles (cycle 2) samples was collected on 25th May 2018. The prawns were placed into a polystyrene box supplied with oxygen. The prawns were transported with aeration to the Genetics and Molecular Biology Laboratory at University Malaya for further works. The prawns were put separately into 1.5 mL microcentrifuge tubes and stored in -80 °C freezer.

Figure 3.1: Map of Peninsular Malaysia showing the location of studied *Macrobrachium rosenbergii* populations from a prawn farm located in Negeri Sembilan, Malaysia (Retrieved from malaysiavacationguide.com, 2019).

Figure 3.2: Map of Negeri Sembilan showing the location of studied *Macrobrachium rosenbergii* populations from a prawn farm located in Kampung Chennah, Jelebu, Negeri Sembilan, Malaysia (Retrieved from kissclipart.com, 2019).

3.1.2 Water quality test

During sample collection on 25th May 2018, the pond water (pond 10 and pond 15) quality was measured by using API® Freshwater Master Test Kit. After two months, the pond water (pond 10 and pond 15) quality was measured again. The physiochemical properties of pond water tested included (i) pH, (ii) ammonia level, (iii) nitrite level and (iv) nitrate level.

(i) pH test

A clean test tube was filled with 5 mL pond water to the line on the tube. pH Test Solution with 3 drops were added to the test tube. The dropper bottle was held upside down in a completely vertical position to assure uniformity of drops. The test tube was capped and inverted several times to mix solution. The test results were read by comparing the colour of the solution to the pH Color Chart. The tube was viewed in a well-lit area against the white area of the chart.

(ii) Ammonia test

A clean test tube was filled with 5 mL pond water to the line on the tube. Ammonia Test Solution #1 was added 8 drops to the test tube. The dropper bottle was held upside down in a completely vertical position to assure uniformity of drops. Ammonia Test Solution #2 was added 8 drops to the test tube. The dropper bottle was held upside down in a completely vertical position to assure uniformity of drops. The test tube was capped and shaken vigorously for 5 seconds. After that, the test tube was left for 5 minutes for the colour to develop. The test results were read by comparing the colour of the solution to the Ammonia Color Chart. The tube was viewed in a well-lit area against the white area of the chart.

(iii) Nitrite test

A clean test tube was filled with 5 mL pond water to the line on the tube. Nitrite Test Solution was added 5 drops to the test tube. The dropper bottle was held upside down in a completely vertical position to assure uniformity of drops. The test tube was capped and shaken for 5 seconds. After that, the test tube was left for 5 minutes for the colour to develop. The test results were read by comparing the colour of the solution to the Nitrite Color Chart. The tube was viewed in a well-lit area against the white area of the chart.

(iv) Nitrate test

A clean test tube was filled with 5 mL pond water to the line on the tube. Nitrate Test Solution #1 was added 10 drops to the test tube. The dropper bottle was held upside down in a completely vertical position to assure uniformity of drops. The test tube was capped and inverted several times to mix solution. Nitrate Test Solution #2 was added 10 drops to the test tube. The dropper bottle was held upside down in a completely vertical position to assure uniformity of drops. The test tube was capped and shaken vigorously for 1 minute. After that, the test tube was left for 5 minutes for the colour to develop. The test results were read by comparing the colour of the solution to the Nitrate Color Chart. The tube was viewed in a well-lit area against the white area of the chart.

3.2 Mitochondrion DNA analysis of giant freshwater prawn

3.2 .1 DNA extraction using EasyPure® marine animal genomic DNA kit method

Minced shrimp muscle tissues ≤ 30 mg were weighed and transferred into a 1.5 ml sterile microcentrifuge tube. Volume of 200 µl LB8 and 20 µl of RNase A were added to the tube. Samples were vortexed for 10 seconds and incubated at room temperature for 2 minutes. Samples were added with 20 µl of Proteinase K and mixed thoroughly by vortexing. Complete immersion of animal tissue in the solution was ensured. Samples were incubated at 55 °C until the completion of lysis. Samples were then vortexed in the middle of the incubation period for complete lysis. For the complete lysis, two hours were taken. Samples were added with 1.5 x volume (360 µl) of BB 8 and mixed thoroughly before transferring to spin columns. Samples were centrifuged at 12000 rpm for 30 seconds and the flow-through was discarded. Volume of 500 µl CB 8 was added

to the spin column and centrifuged at 12000 rpm for 30 seconds at room temperature, and the flow-through was discarded (this step was repeated). Volume of 500 µl of WB 8 was added to the spin column and centrifuged at 12000 rpm for 30 seconds at room temperature, and the flow-through was discarded (this step was repeated). Samples were centrifuged at 12000 rpm for 2 minutes at room temperature in order to completely remove the residual WB8. The spin columns were placed within sterile 1.5 ml microcentrifuge tubes. Volume of 50 µl preheated EB at 70 °C was added to the center of spin columns followed by incubation at room temperature for 2 minutes. The samples were then centrifuged at 12000 rpm for 1 minute at room temperature for genomic DNA elution. The spin columns were discarded and the microcentrifuge tubes containing eluted DNA were stored at -20 °C for further analysis.

3.2.2 Measurement of DNA purity

The quality and quantity of the extracted DNA was checked by NanoDrop spectrophotometer (NanoDrop Technologies, Inc.). The DNA sample was diluted 1:20 by dissolving 5 µl of DNA in 95 µl of autoclaved distilled water. The blank measurement was performed by dispensing 1 µl of buffer onto the lower optical surface. After the blank measurement is completed, 1 µl of each sample was loaded to be measured. The absorbency was measured at 260nm and 280nm.

3.2.3 Polymerase chain reaction (PCR) amplification

Polymerase chain reaction was performed using optimized primer which is universal DNA Folmer's primers for metazoan invertebrates (Folmer et al., 1994):

COI- forward primer : 5'- GGTCAACAAATCATAAAGATATTGG-3'

COI- reverse primer : 5'- TAAACTTCAGGGTGACCAAAAAATCA-3'

This pair of primer consistently amplified a 710bp DNA fragment of mitochondrial cytochrome c oxidase subunit I gene (COI) gene across the broadest array of invertebrates.

PCR amplification was performed using protocol described (Folmer et al., 1994) with minor modification. The reaction was performed using 25 µl reaction system containing 3 µl of the DNA extract as template, 0.5 µl of COI- forward primer and 0.5 µl of COI- reverse primer, 5 µl 10X PCR reaction buffer, 2 µl $MgCl_2$, 0.5 µl of dNTP, 0.25 µl of Taq polymerase (GoTaq®), 13.25 µl of nuclease free water. PCR reactions were amplified at following parameters: initial denaturation for 4 min at 94 °C, followed by 40 cycles of 30 s at 94 °C, annealing temperature of 48 °C for 30 s, and 1 min at 72 °C with the final extension at 72 °C for 10 min. Holding temperature was set at 4 °C.

3.2.4 Agarose gel preparation and gel electrophoresis

Dilution of 50 x TBE buffer into 1 x TBE buffer for gel use and tank use was carried out by adding 20 ml of 50 x TBE buffer and 980 ml of distilled water using measuring cylinder into 1 litre media bottle and mixed well. Agarose powder with 0.4 g (4.0 %) was weighed in an Erlenmeyer flask. Volume of 40 ml of 1 x TBE buffer was added into the flask and mixed well. The agarose solution was heated in a microwave oven until the agarose powder was completely dissolved into the solution. The molten agarose solution was left aside for brief cool down before the addition of ethidium bromide solution into it. The solution was mixed thoroughly through gentle swirling before poured into prior prepared gel casting tray attached with gel comb. The gel was ensured to be 3 mm thick with no air bubbles under or between teeth of comb. The gel was allowed for complete solidification for about 30 minutes at room temperature. The gel comb was removed gently from the solidified gel. The gel was mounted into the

electrophoresis chamber and 1 x TBE buffer was poured to cover the gel to a depth of approximately 1 mm above it. After that, volume of 5 µl 100 bp DNA ladder was loaded into the first well of submerged gel. The samples were slowly loaded into the wells of the submerged gel. The protective lid of electrophoresis chamber was closed and the electrical cables were attached to the correct ends of the chamber. The negatively charged DNA was migrated towards the positively charged anode with red cable connected. Volt of 80 V, current of 180 mA and time of 30 minutes were set on the power supply and the start button was pressed. The successful initiation of gel electrophoresis was confirmed by bubble confirmation at the cathode and anode of the chamber. The progress of gel electrophoresis was checked through the positions of the blue and yellow layers on the gel. After the completion of gel electrophoresis, the gel was removed from the electrophoresis chamber and placed in a transilluminator for band observation. Positive bands from PCR product were purified and sent for sequencing.

3.2.5 Data analysis

8 DNA sequences (cycle 1) and 26 DNA sequences (cycle 2) obtained were aligned and edited manually by using Mega X software (Tamura et al., 2013). Haplotypes were generated using Dna.SP v5 (Librado & Rozas, 2009) software. Genetic diversity within the respective populations was estimated by computing both haplotype (H) and nucleotide diversities (Tamura et al., 2013). Haplotype diversity represents the probability that two randomly sampled alleles are different, on the other hand, nucleotide diversity is defined as the average number of nucleotide differences per site in pairwise comparisons between DNA sequences (Tamura et al., 2013). A maximum likelihood phylogenetic tree of unique haplotypes was then constructed by Mega X to show haplotype pairwise differences, using the Maximum composite likelihood

calculated on the basis of a bootstrap of 1000 replicates. Median-joining haplotype network analyses were generated using the software package Network version 5 (Bandelt et al., 1999).

3.3 Viral screening of giant freshwater prawn

3.3 .1 DNA extraction using EasyPure® marine animal genomic DNA kit method

Minced shrimp muscle tissues of ≤ 30 mg were weighed and transferred into a 1.5 ml sterile microcentrifuge tube. Volume of 200 µl of LB8 and 20 µl of RNase A were added to the tube. Samples were vortexed for 10 seconds and incubated at room temperature for 2 minutes. Samples were added with 20 µl of Proteinase K and mixed thoroughly by vortexing. Complete immersion of animal tissue in the solution was ensured. Samples were incubated at 55 °C until the completion of lysis. Samples were then vortexed in the middle of the incubation period for complete lysis. For the complete lysis, two hours were taken. Samples were added with 1.5 x volume (360 µl) of BB8 and mixed thoroughly before transferring to spin columns. Samples were centrifuged at 12000 rpm for 30 seconds and the flow-through was discarded. Volume of 500 µl CB 8 was added to the spin column and centrifuged at 12000 rpm for 30 seconds at room temperature, and the flow-through was discarded (this step was repeated). After that, volume of 500 µl WB 8 was added to the spin column and centrifuged at 12000 rpm for 30 seconds at room temperature, and the flow-through was discarded (this step was repeated). Samples were centrifuged at 12000 rpm for 2 minutes at room temperature in order to completely remove the residual WB 8. The spin columns were placed within sterile 1.5 ml microcentrifuge tubes. Volume of 50 µl of preheated EB at 70 °C was added to the center of spin columns followed by incubation at room temperature for 2 minutes. The samples were then centrifuged at 12000 rpm for

1 minute at room temperature for genomic DNA elution. The spin columns were discarded and the microcentrifuge tubes containing eluted DNA were stored at -20 °C for further analysis.

3.3.2 RNA extraction using *TransZol* Up plus RNA kit method

The mortar, pestle, spatula, scissor and surrounding were sterilized with 70 % ethanol and RNase Zap prior to the transfer of sample to mortar. Frozen samples (juvenile *Macrobrachium rosenbergii* muscle tissue) in the range of < 30 mg were weighed and quickly transferred to a mortar. The samples were grinded thoroughly into powder form using liquid nitrogen. Complete grinding was ensured to increase RNA yield and quality. The grinded tissue powder was transferred to a new RNase-free microcentrifuge tube using spatula and 1 ml of *TransZol* Up was added into the tube. The tissue samples were then homogenized through vigorous shaking for 30 seconds or repeated pipetting up and down. Samples were incubated at room temperature for 5 minutes. Samples were added with 0.2 ml chloroform per ml of *TransZol* Up added previously and shaken vigorously for 30 seconds. Samples were treated with incubation at room temperature for 3 minutes followed by centrifugation at 10000 rpm for 15 minutes at 4 °C. After the centrifugation, the mixture was separated into three layers which were an upper colorless aqueous phase, an interphase, and a lower pink organic phase. The upper colorless aqueous phase with 50% volume of *TransZol* Up reagent contained the RNA. The upper colorless aqueous phase was then transferred to a new RNase-free microcentrifuge tube. A portion of upper colorless aqueous phase was left out to avoid DNA contamination from interphase. Equal volume of 100% ethanol was added to the tube and mixed gently by inverting the tube. The resulting solution and precipitate was transferred to spin column and centrifuged at 12000 rpm for 30 seconds at room temperature. The flow-through was discarded. This step was repeated when the

lysate volume was greater than the spin column hold volume. Volume of 500 µl CB9 was added to the spin column and centrifuged at 12000 rpm for 30 seconds at room temperature, and the flow-through was discarded. This step was repeated once. Volume of 500 µl WB9 was added to the spin column and centrifuged at 12000 rpm for 30 seconds at room temperature, and the flow-through was discarded. This step was repeated once. The samples were centrifuged at 12000 rpm for 2 minutes at room temperature for the complete removal of the remaining ethanol. The column matrixes were air dried for 5 minutes. The spin column matrix was placed into a new 1.5 ml RNase free tube and added with 100 µl of RNase-free water. The water was added by divisions, 2 times of elution with 50 µl water added each time to enable maximal elution of RNA. The tubes were incubated at room temperature for 1 minute. The tubes were centrifuged at 12000 rpm for 1 minute to elute the RNA. The spin column matrixes were discarded and the eluted RNA tubes were stored at -80 °C for further analysis.

3.3.3 cDNA conversion using Promega reverse transcription system method

PCR tubes were prepared and labeled. Volume of 4 µL RNA samples and 1 µL of Oligo (dT) 15 Primer were added into PCR tubes. The tubes were placed into preheated 70 °C heat block for 5 minutes. The tubes were chilled for 5 minutes. The tubes were centrifuged for 10 seconds and incubated in ice for 2 minutes. The reaction was performed using 20.0 µl reaction system containing 2.4 µl of $MgCl_2$, 4.0 µl of reverse transcription 10X Buffer, 1.0 µl dNTP mixture, 0.5 µl recombinant RNasin® ribonuclease inhibitor, 1.0 µl of reverse transcriptase, 6.1 µl of nuclease free water, 5.0 µl of mixed RNA Oligo (dT) 15 primer. PCR was ran at 25 °C for 5 minutes, followed by 42 °C for 1 hour and 70 °C for 15 minutes, the holding temperature was 4 °C. The samples were stored in -20 °C freezer until needed.

3.3.4 Measurement of DNA and RNA purity

The quality and quantity of the extracted DNA and RNA was checked by NanoDrop spectrophotometer (NanoDrop Technologies, Inc.). The DNA and RNA sample was diluted 1:20 by dissolving 5 µl of DNA in 95 µl of autoclaved distilled water. The blank measurement was performed by dispensing 1 µl of buffer onto the lower optical surface. After the blank measurement is completed, 1 µl of each sample was loaded to be measured. The absorbency was measured at 260nm and 280nm.

3.3.5 Polymerase chain reaction (PCR) amplification

The polymerase chain reaction (PCR) amplification was conducted by using PCR thermal cycler (Eppendorf Mastercycler ep Gradient S PCR). The reaction was performed using 20.0 µl reaction system containing 2.0 µl of the DNA extract as template, 4.0 µl of DPO primer (Table 3.1), 4.0 µl 10X PCR reaction buffer, 1.6 µl $MgCl_2$, 1.5 µl of dNTPs, 0.5 µl of Taq polymerase (GoTaq®), 6.9 µl of nuclease free water. PCR reactions were amplified at following parameters: initial denaturation for 10 min at 94 °C, followed by 40 cycles of 30 s at 94 °C, annealing temperature of 55 °C for 30 s, and 45 s at 72 °C with the final extension at 72 °C for 3 min. Holding temperature was set at 10 °C.

Table 3.1: DPO primer sequences for DNA and RNA viruses used in the present study.

Primer	Primer sequence (5' to 3')
WSSV	F: CAACAACATCTTCATCTGCAIIIIIAGCCACCTCT
	R: TCTCTGCAGTGTCGTCGTCCIIIIIGTCTTCATAT
IHHNV	F: TGGATGAACTTATATCTCTATGGIIIIIAAAGCAGTGACAG
	R: GGTCCGGTTGTTGGTATTTCIIIIICATACCAGTTT
MBV	F: TGGACGACCTTAGTGGAGATIIIIIGATGGTGCTC
	R: ACCCTTAGAATGTTCAACATTAGIIIIIGTGTTGTCACC
BPV	F: GTAATTTGTGCGAGTAAACCIIIIIAGCAGCAGAGA
	R: TACCCATATAGGATTCTACTGGIIIIICAAAGCAAGAAAC
YHV	F: ATCGAGATTCCTCAATCCTTIIIIITCGAACGCGG
	R: GTGTTTCCATGGGTTCGTCGIIIIIAAGGCTCATT
TSV	F: GTACGCTTGCTAAAGCAGCCIIIIICTTCAATTAT
	R: TAGCCTCAGTGACCACGGTAIIIIIACCTGGAGTA
IMNV	F: AGAACACAGCCTACTTTACAGGATIIIIITTGGGGTATA
	R: GTCGCCAAGTGTGAAATCGGIIIIICAGGATACAA
*Mr*NV	F: TAGTAACGCAAACGGCAAGCIIIIIAAGCGTAATC
	R: AATCCACAGACCCAATCTAAIIIIIGTGAACCTGAAA

F: Forward primer; R: Reverse primer

3.3.6 Agarose gel preparation and gel electrophoresis

Dilution of 50 x TBE buffer into 1 x TBE buffer for gel use and tank use was carried out by adding 20 ml of 50 x TBE buffer and 980 ml of distilled water using measuring cylinder into 1 litre media bottle and mixed well. Agarose powder with 0.4 g (4.0 %) was weighed in an Erlenmeyer flask. Volume of 40 ml 1 x TBE buffer was added into

the flask and mixed well. The agarose solution was heated in a microwave oven until the agarose powder was completely dissolved into the solution. The molten agarose solution was left aside for brief cool down before the addition of red safe solution into it. The solution was mixed thoroughly through gentle swirling before poured into prior prepared gel casting tray attached with gel comb. The gel was ensured to be 3mm thick with no air bubbles under or between teeth of comb. The gel was allowed for complete solidification for about 30 minutes at room temperature. The gel comb was removed gently from the solidified gel. The gel was mounted into the electrophoresis chamber and 1 x TBE buffer was poured to cover the gel to a depth of approximately 1 mm above it. The samples were then slowly loaded into the wells of the submerged gel. Volume of 5 µl 100 bp DNA ladder was loaded the last into one of the wells of submerged gel. The protective lid of electrophoresis chamber was closed and the electrical cables were attached to the correct ends of the chamber. The negatively charged DNA was migrated towards the positively charged anode with red cable connected. Volt of 50 V, current of 180 mA and time of 30 minutes for big gel were set on the power supply and the start button was pressed. The voltage used was determined based on the chamber distance between cathode and anode with 1-5 V applied for every cm. The successful initiation of gel electrophoresis was confirmed by bubble confirmation at the cathode and anode of the chamber. The progress of gel electrophoresis was checked through the positions of the blue and yellow layers on the gel. After the completion of gel electrophoresis, the gel was removed from the electrophoresis chamber and placed in a transilluminator for band observation.

3.4 *Vibrio parahaemolyticus* screening of giant freshwater prawn

3.4.1 DNA extraction using phenol-chloroform method

Samples with 50 mg were grinded in liquid nitrogen and put into 1.5 mL microcentrifuge tube. Volume of 200 µL lysis buffer and 18 µL proteinase K was added to the tube. The tubes were inverted for mixing. The tubes were incubated at 60 °C for 2 hours. After incubation, volume of 200 µL phenol-chloroform isoamyl alcohol (25:24:1) was added and the tubes were vortexed for 1 minute. The tubes were centrifuged at 27 °C, 16000x g for 5 minutes. Three layers were formed and 180 µL of upper aqueous layer was transferred to a new tube. Volume of 200 µL of chloroform isoamyl alcohol (24:1) was added and the tube was vortexed for 1 minute. The tubes were centrifuged at 27 °C, 16000x g for 5 minutes. Volume of 180 µL upper aqueous layer was transferred to a new tube. 0.5 volume of ammonium acetate was added to the tubes. Ethanol with 100%, which was 2.5 volume was then added to the tubes. The tubes were incubated at -80 °C for 1 hour. After incubation, the tubes were centrifuged at 4 °C, 16000x g for 30 minutes. The supernatant was discarded. 150 µL of 80% ethanol was added and centrifuged at 4 °C, 16000x g for 15 minutes. The supernatant was discarded. This step was repeated. The pallets formed were air dried and mixed with elution buffer. The tubes were stored at -20 °C (Sambrook & Russell, 2001). The quality of the extracted DNA was checked by gel electrophoresis while the quantity of DNA was obtained by NanoDrop spectrophotometer (NanoDrop Technologies, Inc.).

3.4.2 Measurement of DNA purity

The quality and quantity of the extracted DNA was checked by NanoDrop spectrophotometer (NanoDrop Technologies, Inc.). The DNA sample was diluted 1:20

by dissolving 5 µl of DNA in 95 µl of autoclaved distilled water. The blank measurement was performed by dispensing 1 µl of buffer onto the lower optical surface. After the blank measurement is completed, 1 µl of each sample was loaded to be measured. The absorbency was measured at 260nm and 280nm.

3.4.3 Polymerase chain reaction (PCR) amplification

The polymerase chain reaction (PCR) amplification was conducted by using PCR thermal cycler (Eppendorf Mastercycler ep Gradient S PCR). Polymerase chain reaction was performed using primer AP3 (Sirikharin et al., 2014):

AP3- forward primer : 5'- ATGAGTAACAATATAAAACATGAAAC -3'

AP3- reverse primer : 5'- GTGGTAATAGATTGTACAGAA -3'

This pair of primer consistently amplified a 336 bp DNA fragment. The reaction was performed using 25.0 µl reaction system containing 1.2 µl of the DNA extract as template, 0.5 µl of AP3 forward primer and 0.5 µl of AP3 reverse primer, 5.0 µl 5X PCR reaction buffer, 1.4 µl $MgCl_2$, 0.4 µl of dNTPs, 0.2 µl of Taq polymerase (GoTaq®), 15.8 µl of nuclease free water. PCR reactions were amplified at following parameters: initial denaturation for 5 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, annealing temperature of 53 °C for 30 s, and 40 s at 72 °C with the final extension at 72 °C for 5 min. Holding temperature was set at 4 °C.

3.4.4 Agarose gel preparation and gel electrophoresis

Dilution of 50 x TBE buffer into 1 x TBE buffer for gel use and tank use was carried out by adding 20 ml of 50 x TBE buffer and 980 ml of distilled water using measuring cylinder into 1 litre media bottle and mixed well. Agarose powder with 04 g (4.0 %) was weighed in an Erlenmeyer flask. Volume of 40 ml 1 x TBE buffer was added into

the flask and mixed well. The agarose solution was heated in a microwave oven until the agarose powder was completely dissolved into the solution. The molten agarose solution was left aside for brief cool down before the addition of red safe solution into it. The solution was mixed thoroughly through gentle swirling before poured into prior prepared gel casting tray attached with gel comb. The gel was ensured to be 3 mm thick with no air bubbles under or between teeth of comb. The gel was allowed for complete solidification for about 30 minutes at room temperature. The gel comb was removed gently from the solidified gel. The gel was mounted into the electrophoresis chamber and 1 x TBE buffer was poured to cover the gel to a depth of approximately 1 mm above it. The samples were then slowly loaded into the wells of the submerged gel. Volume of 5 µl 100 bp DNA ladder was loaded the last into one of the wells of submerged gel. The protective lid of electrophoresis chamber was closed and the electrical cables were attached to the correct ends of the chamber. The negatively charged DNA was migrated towards the positively charged anode with red cable connected. Volt of 50 V, current of 180 mA and time of 30 minutes for big gel and were set on the power supply and the start button was pressed. The voltage used was determined based on the chamber distance between cathode and anode with 1-5 V applied for every cm. The successful initiation of gel electrophoresis was confirmed by bubble confirmation at the cathode and anode of the chamber. The progress of gel electrophoresis was checked through the positions of the blue and yellow layers on the gel. After the completion of gel electrophoresis, the gel was removed from the electrophoresis chamber and placed in a transilluminator for band observation.

CHAPTER 4: RESULTS

4.1 Mitochondrion DNA analysis of giant freshwater prawn

The Cytochrome c oxidase I (COI) gene can be amplified clearly in the 34 samples out of 49 samples. The sizes of the COI gene were about 710 bp. The COI sequences were adjusted and aligned, and 599 bp consensus sequences were obtained from 120 individuals resulted in 24 haplotypes (Elsheikh et al., 2015).

Based on the haplotype network diagram (Figure 4.1), only two haplotypes were found to be shared between all the populations, which are haplotypes 1 and 9, while haplotype 8 shared between three of the populations. The distribution of haplotypes between populations was not regular or uniform, with unique haplotypes 11, 12 and 13 for Kedah, haplotypes 22, 23 and 24 for Perlis, haplotypes 14, 15, 17, 18, 19 and 20 for Johor, haplotype 21 for Negeri Sembilan, and lastly haplotypes 3, 5, 6 is the populations collected from the farm.

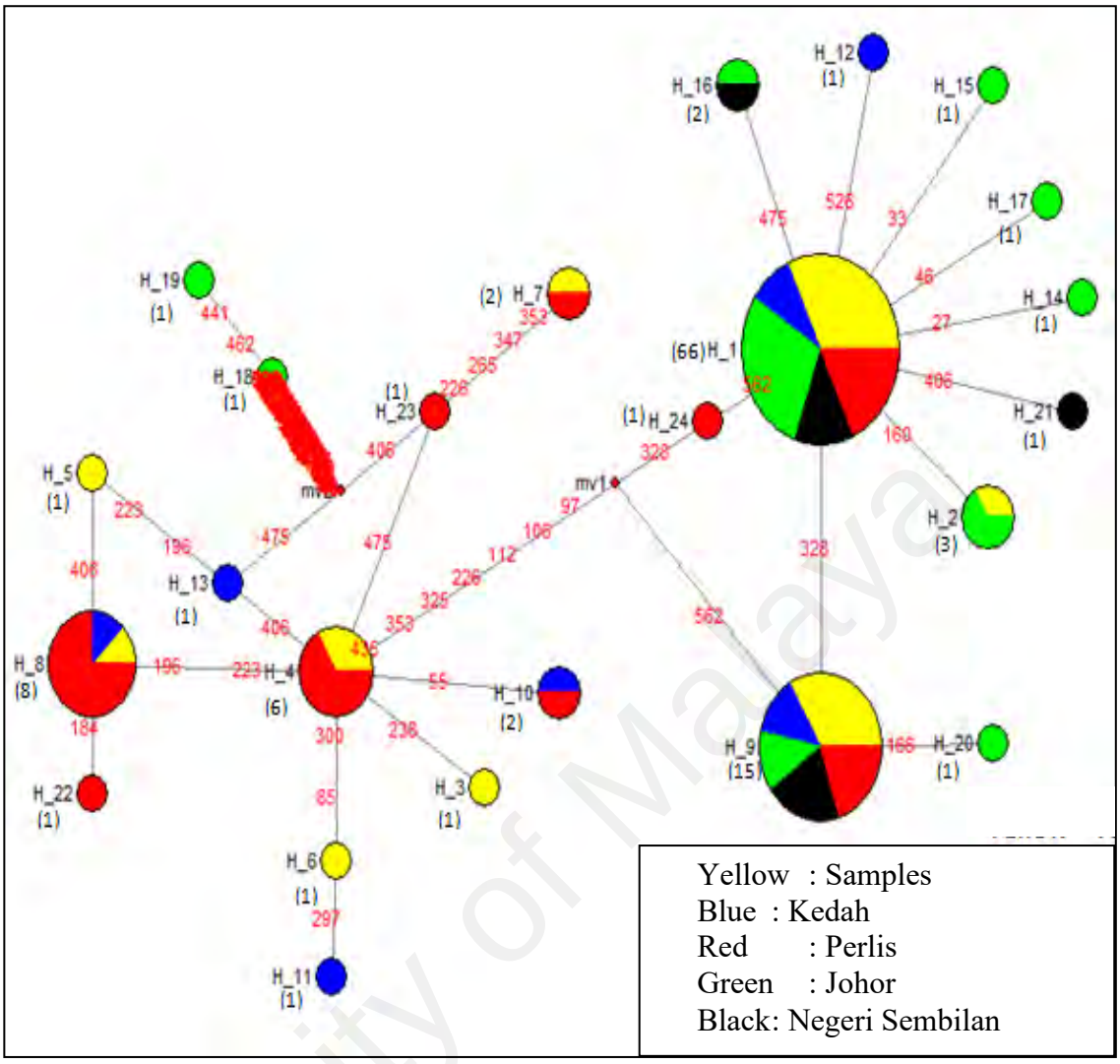

Figure 4.1: Haplotype networks of the *Macrobrachium rosenbergii* haplotypes from a farm located in Kampung Chennah, Jelebu, Negeri Sembilan. Each *Macrobrachium rosenbergii* population is represented by one color. Each node (pie diagram) represents a unique haplotype.

There are a few commonalities between all haplotype networks: First, each node represents a unique haplotype; second, the diameter of the circle is usually proportional to the number of individuals of that haplotype sampled; and third, haplotypes are connected to one another based on their similarity.

The *Macrobrachium rosenbergii* samples collected from a farm located in Kampung Chennah, Jelebu, Negeri Sembilan are haplotype 1 which means the broodstock are come from four states which are Kedah, Perlis, Johor, Negeri Sembilan. Corrected (K2P) pairwise group genetic distances among the *M. rosenbergii* are shown in Table

4.1, with the Pacific white shrimp, *Litopenaeus vannamei*, also included for comparison as a distant relative. The table revealed relatively high intra-specific genetic distances (0.005-0.018%) between *Macrobrachium rosenbergii* from the farm with *Macrobrachium rosenbergii* from Negeri Sembilan, Kedah, Perlis, Johor in Malaysia, but much greater distances when compared to *Litopenaeus vannamei* (1.049%). The lowest inter-specific divergence (0.005%) was observed between *Macrobrachium rosenbergii* from the farm and *Macrobrachium rosenbergii* from Negeri Sembilan, identifying a close genetic distance between these groups.

Table 4.1: Percent pairwise corrected (K2P) group genetic distance among the *Macrobrachium rosenbergii* from the farm, Negeri Sembilan, Kedah, Perlis, Johor, *Litopenaeus vannamei* Cytochrome c oxidase I sequences.

	Sample	1	2	3	4	5
1	*M. rosenbergii* farm	-				
2	*M. rosenbergii* Negeri Sembilan	0.005	-			
3	*M. rosenbergii* Kedah	0.008	0.007	-		
4	*M. rosenbergii* Perlis	0.010	0.009	0.011	-	
5	*M. rosenbergii* Johor	0.018	0.015	0.020	0.022	-
6	*Litopenaeus vannamei*	1.049	1.045	1.052	1.057	1.056

The phylogenetic tree was constructed using neighbour-joining method (Appendix A). The results showed small genetic distance between the *Macrobrachium rosenbergii* from the farm and the *Macrobrachium rosenbergii* from Negeri Sembilan, Kedah, Perlis and Johor. Most samples showed genetic distances of less than 0.1 between them; the highest genetic distance was between *Litopenaeus vannamei* (outgroups) and *Macrobrachium rosenbergii* and was well supported, with 1 100% bootstrap support in

the neighbor-joining analysis. The phylogenetic tree indicated that *Macrobrachium rosenbergii* from the farm is paraphyletic, with the *Macrobrachium rosenbergii* from Negeri Sembilan, Kedah, Perlis and Johor, reflecting the low divergence observed between the populations in pair-wise estimates.

4.2 Viral screening of giant freshwater prawn

4.2.1 Viral screening of giant freshwater prawn (cycle 1)

19 juveniles *Macrobrachium rosenbergii* (cycle 1) samples were collected from prawn farm in Kampung Chennah, Jelebu, Negeri Sembilan.

Table 4.2 and Table 4.3 showed the quantitative analysis of the DNA and RNA samples of the *Macrobrachium rosenbergii* (cycle 1). Overall concentration of DNA samples are in the range 100 ng/µl to 5000 ng/µl while RNA samples are in the range of 50 ng/µl to 520 ng/µl. The purity (A 260/280) of DNA and RNA are good in between ratio of 1.8 to 2.0. For (A 260/230), a pure DNA/ RNA sample will have a ratio of 2 to 2.2. Most of the DNA samples are approximately at the ratio of 2. For RNA samples, some of samples are in the range. For those samples that did not achieve the range, it showed there are some contaminations found in the sample.

Table 4.2: Quantitative analysis of DNA samples for *Macrobrachium rosenbergii* (cycle 1) using Nanodrop system.

Sample name	Sample type	Nucleic acid concentration (ng/µl)	A 260/280	A 260/230
M4G	DNA	546.7	1.77	1.40
M5G	DNA	755.7	1.78	1.41
M6G	DNA	564.2	1.83	1.42
M7G	DNA	556.1	1.93	1.82

Table 4.2, continued.

Sample name	Sample type	Nucleic acid concentration (ng/µl)	A 260/280	A 260/230
M8G	DNA	524.5	1.86	1.09
M9G	DNA	4896.1	1.97	2.19
M10G	DNA	109.7	1.96	2.07
M11G	DNA	292.4	2.03	2.30
M12G	DNA	355.2	1.95	2.01
M13G	DNA	107.9	1.13	0.70
M14G	DNA	120.4	2.00	2.31
M15G	DNA	349.4	1.93	1.92
M16G	DNA	331.0	1.95	2.00
M17G	DNA	156.3	0.93	0.84
M18G	DNA	143.1	2.02	2.22
M19G	DNA	406.0	1.97	2.21
M20G	DNA	586.0	1.97	2.14
M21G	DNA	278.3	1.98	1.95
M22G	DNA	566.3	1.94	2.11

Table 4.3: Quantitative analysis of RNA samples for *Macrobrachium rosenbergii* (cycle 1) using Nanodrop system.

Sample name	Sample type	Nucleic acid concentration (ng/µl)	A 260/280	A 260/230
M4m	RNA	245.1	2.13	2.15
M5m	RNA	517.7	2.10	2.22
M6m	RNA	284.8	2.13	2.42

Table 4.3, continued.

Sample name	Sample type	Nucleic acid concentration (ng/µl)	A 260/280	A 260/230
M15m	RNA	159.3	2.16	1.60
M16m	RNA	73.0	2.22	1.18
M17m	RNA	89.8	2.18	1.58

The screening of DNA virus was done by using gel electrophoresis. The gel consisted of 100bp DNA marker, positive control which include four types of DNA viruses band, and negative control. Figure 4.2 and 4.3 showed the agarose gel picture of *Macrobrachium rosenbergii* (cycle 1) for disease screening using DNA samples amplified by DPO system. In lane 1 was the 100bp ladder. Lane 2 showed 4 bands of DNA viruses as an indicator of the screening of WSSV (300bp), IHHNV (458bp), MB (515bp), BP (721bp), while lane 9 (Figure 4.2) and lane 16 (Figure 4.3) was the negative control. All the samples showed the absence of DNA viruses.

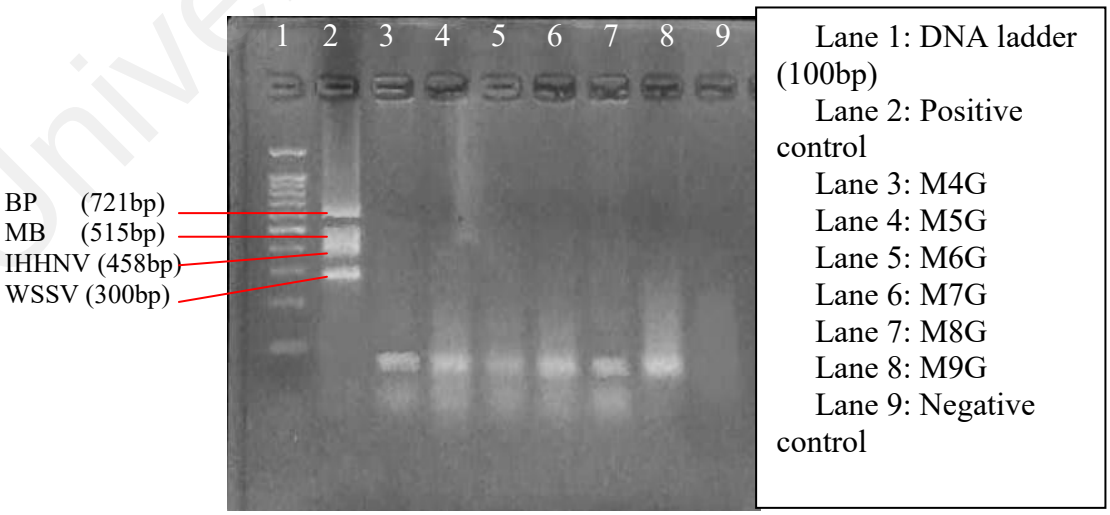

Figure 4.2: Gel imaging of PCR products of *Macrobrachium rosenbergii* (M4G-M9G) for disease screening using DNA samples amplified by DPO system.

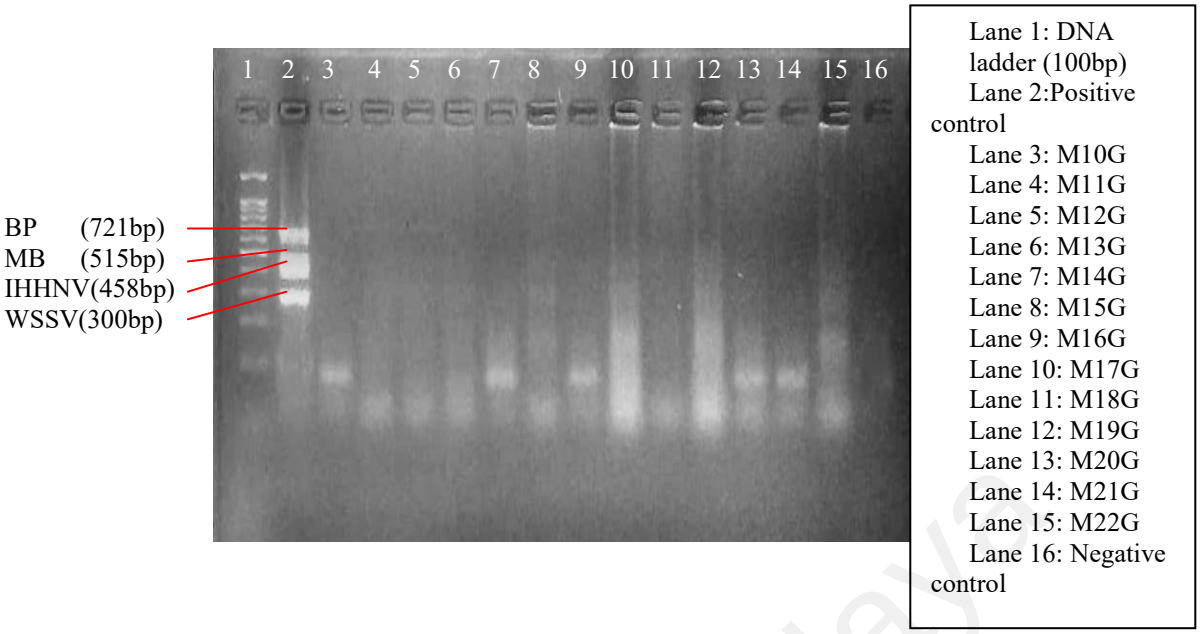

Figure 4.3: Gel imaging of PCR products of *Macrobrachium rosenbergii* (M10G-M22G) for disease screening using DNA samples amplified by DPO system.

Figure 4.4 showed the agarose gel picture of the *Macrobrachium rosenbergii* (cycle 1) for disease screening using cDNA samples converted from RNA amplified by DPO system. In lane 1 was the 100bp ladder. Lane 2 showed 4 bands of RNA viruses as an indicator of the screening of YHV (349bp), TSV (545bp), IMNV (591bp), MrNv (681bp), while lane 9 was the negative control. All the samples showed a band at 150bp which is the eukaryotic elongation factor (ELF) gene, which acts as an internal control. All the samples showed the absence of RNA viruses.

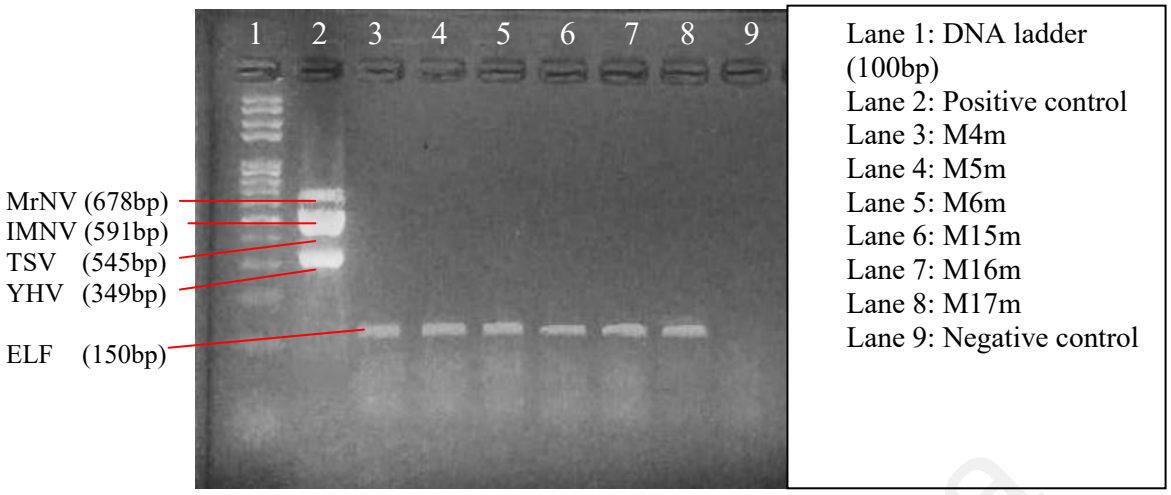

Figure 4.4: Gel imaging of PCR products of *Macrobrachium rosenbergii* (M4m-M17m) for disease screening using cDNA samples converted from RNA amplified by DPO system.

4.2.2 Viral screening of giant freshwater prawn (cycle 2)

30 juveniles *Macrobrachium rosenbergii* (cycle 2) samples were collected from prawn farm in Kampung Chennah, Jelebu, Negeri Sembilan.

Table 4.4 and Table 4.5 showed the quantitative analysis of the DNA and RNA samples of the *Macrobrachium rosenbergii* (cycle 2). Overall concentration of DNA samples are in the range 20 ng/µl to 450 ng/µl while RNA samples are in the range of 400 ng/µl to 1600 ng/µl. The purity (A 260/280) of DNA and RNA are good in between ratio of 1.8 to 2.0. For (A 260/230), a pure DNA/ RNA sample will have a ratio of 2 to 2.2. Most of the DNA samples are approximately at the ratio of 2. For RNA samples, some of samples are in the range. For those samples that did not achieve the range, it showed there are some contaminations found in the sample.

Table 4.4: Quantitative analysis of DNA samples for *Macrobrachium rosenbergii* (cycle 2) using Nanodrop system.

Sample name	Sample type	Nucleic acid concentration (ng/µl)	A 260/280	A 260/230
M1	DNA	74.7	2.20	1.28
M2	DNA	78.1	1.82	1.06
M3	DNA	31.6	1.80	0.63
M4	DNA	33.7	2.14	0.97
M5	DNA	68.4	1.87	0.69
M6	DNA	23.3	1.82	0.94
M7	DNA	76.6	1.90	1.49
M8	DNA	127.8	1.88	1.47
M9	DNA	136.8	1.96	1.79
M10	DNA	49.2	1.88	0.85
M11	DNA	95.3	1.86	1.10
M12	DNA	93.4	1.77	0.92
M13	DNA	227.8	1.87	1.59
M14	DNA	68.2	2.15	1.59
M15	DNA	20.5	1.65	0.25
M16	DNA	61.3	2.10	1.11
M17	DNA	105.8	1.91	1.74
M18	DNA	144.6	1.94	1.83
M19	DNA	235.0	1.86	1.77
M20	DNA	57.8	2.16	1.63
M21	DNA	60.2	1.74	1.52
M22	DNA	40.1	1.74	0.55

Table 4.4, continued.

Sample name	Sample type	Nucleic acid concentration (ng/µl)	A 260/280	A 260/230
M23	DNA	419.2	1.82	1.70
M24	DNA	76.4	1.83	1.20
M25	DNA	100.2	1.93	1.46
M26	DNA	56.0	1.87	1.33
M27	DNA	107.2	1.87	1.52
M28	DNA	153.3	1.85	1.65
M29	DNA	112.7	1.86	1.56
M30	DNA	132.5	1.88	1.26

Table 4.5: Quantitative analysis of RNA samples for *Macrobrachium rosenbergii* (cycle 2) using Nanodrop system.

Sample name	Sample type	Nucleic acid concentration (ng/µl)	A 260/280	A 260/230
M1	RNA	440.5	2.13	1.44
M2	RNA	559.4	1.95	1.10
M3	RNA	475.1	2.10	1.79
M4	RNA	455.0	2.10	2.13
M5	RNA	1514.5	2.14	2.06

The screening of DNA virus was done by using gel electrophoresis. The gel consisted of 100bp DNA marker, positive control which include four types of DNA viruses band, and negative control. Figures 4.5, 4.6 and 4.7 showed the agarose gel picture of *M. rosenbergii* (cycle 2) for disease screening using DNA samples amplified

by DPO system. In lane 1 was the 100bp ladder. Lane 2 showed 4 bands of DNA viruses as an indicator of the screening of WSSV (300bp), IHHNV (458bp), MB (515bp), BP (721bp), while lane 13 was the negative control. All the samples showed the absence of DNA viruses.

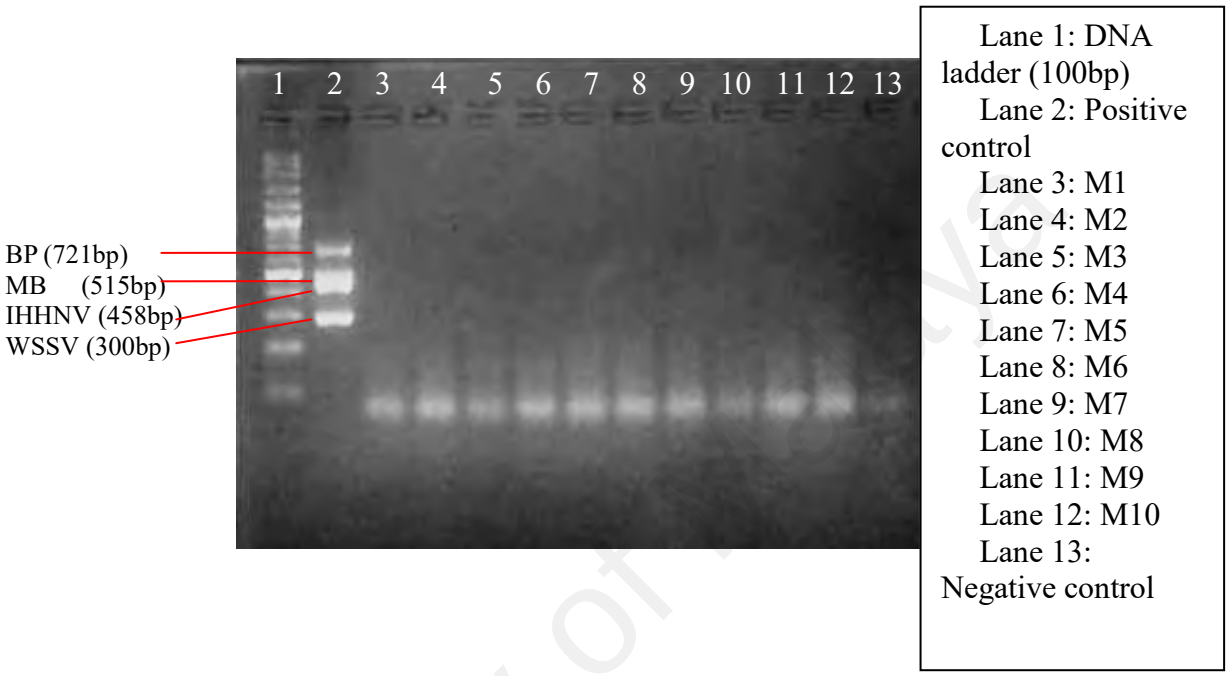

Figure 4.5: Gel imaging of PCR products of *Macrobrachium rosenbergii* (M1-M10) for disease screening using DNA samples amplified by DPO system.

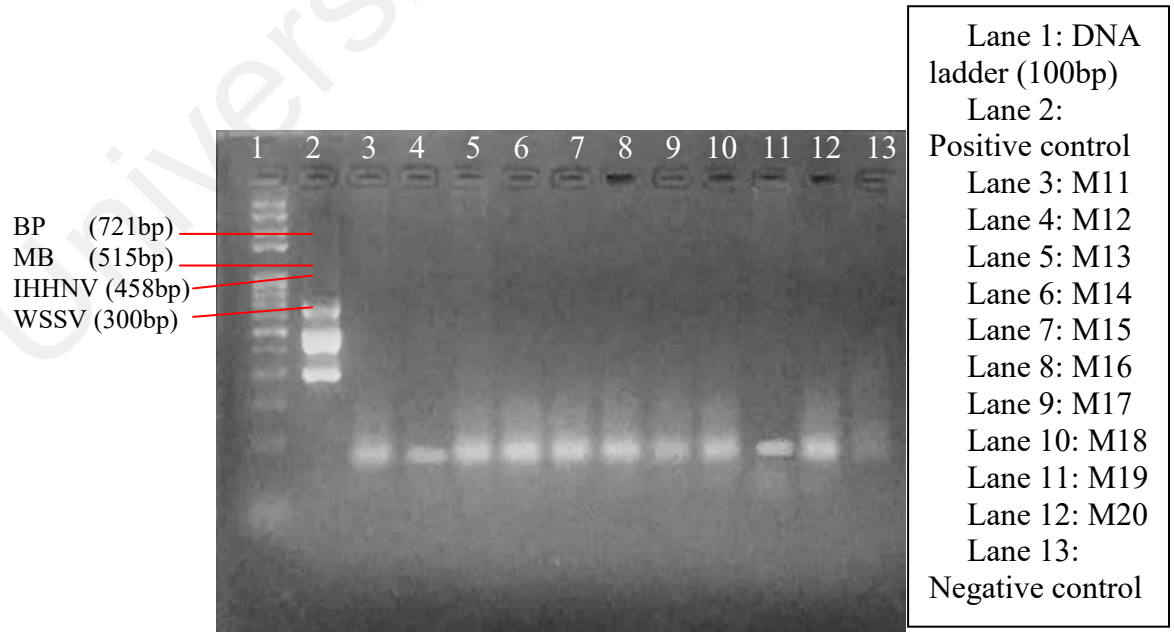

Figure 4.6: Gel imaging of PCR products of *Macrobrachium rosenbergii* (M11-M20) for disease screening using DNA samples amplified by DPO system.

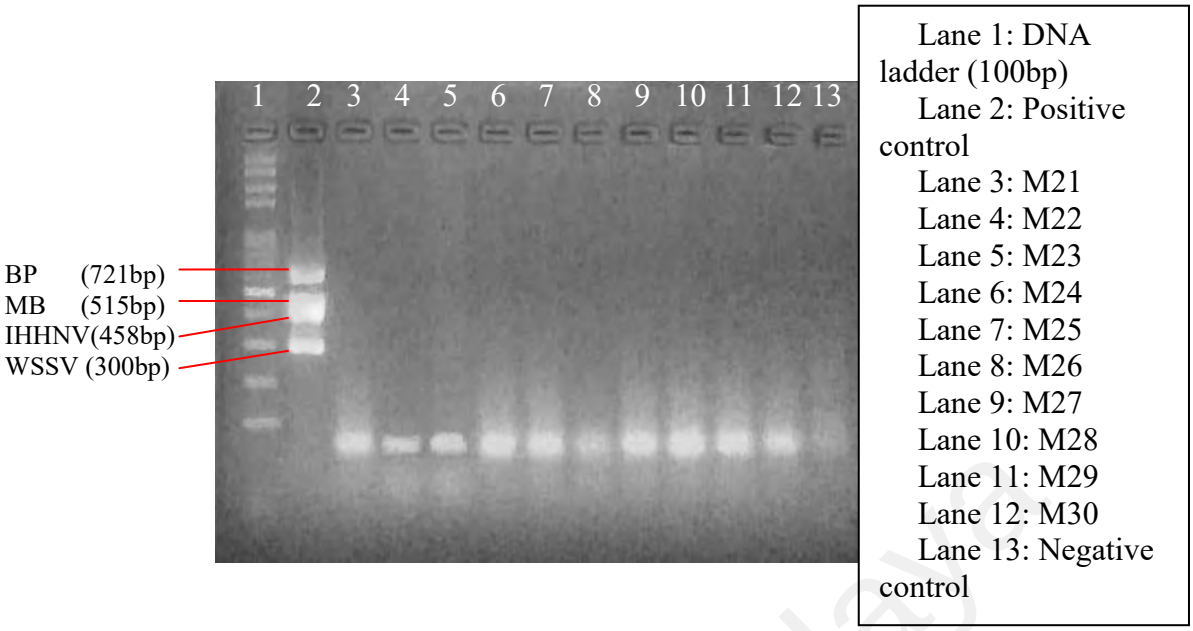

Figure 4.7: Gel imaging of PCR products of *Macrobrachium rosenbergii* (M21-M30) for disease screening using DNA samples amplified by DPO system.

Figure 4.8 showed the agarose gel picture of the *M. rosenbergii* (cycle 2) for disease screening using cDNA samples converted from RNA amplified by DPO system. In lane 1 was the 100bp ladder. Lane 2 showed 4 bands of RNA viruses as an indicator of the screening of YHV (349bp), TSV (545bp), IMNV (591bp), MrNv (681bp), while lane 8 was the negative control. All the samples showed a band at 200bp which is the eukaryotic elongation factor (ELF) gene, which acts as an internal control. The lane 3, 4, 5 and 6 showed a band at 349bp which indicating the presence of YHV infection in M1, M2, M3 and M4 samples.

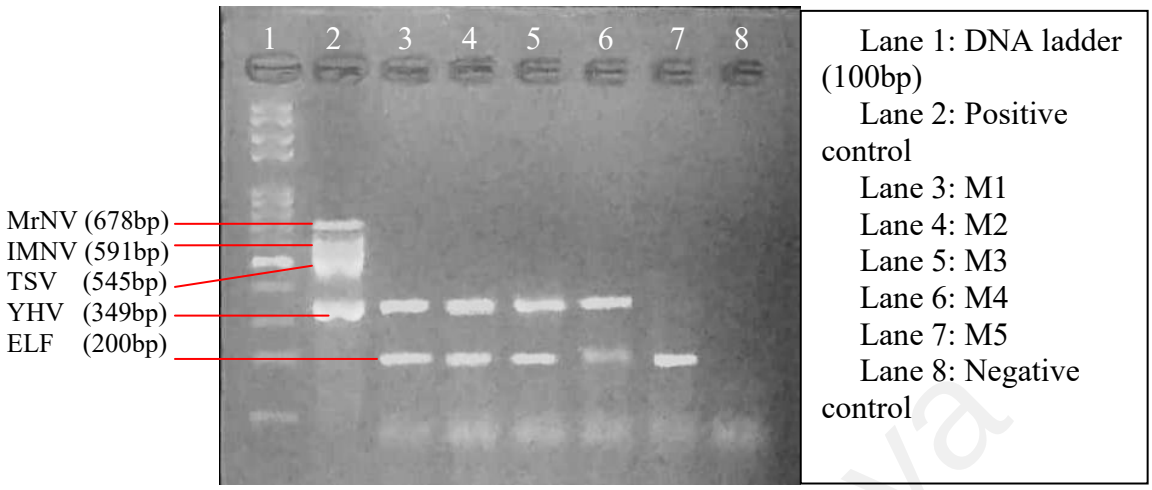

Figure 4.8: Gel imaging of PCR products of *Macrobrachium rosenbergii* (M1-M5) for disease screening using cDNA samples converted from RNA amplified by DPO system.

Table 4.6 showed the prevalence of samples positive with viruses based on DPO primer. For cycle 1, there was no DNA and RNA viruses detected in all the samples tested using multiplex PCR method. For cycle 2, there was no DNA viruses detected in all the samples tested using multiplex PCR method, but RNA virus was found in the samples. Yellow head virus (YHV) was detected in four samples out of five samples tested. The results showed the prevalence of YHV infection in prawn samples was 80%.

Table 4.6: PCR amplification results of viruses based on DPO primer.

Viruses	Cycle 1 (Positive with viruses)	Cycle 2 (Positive with viruses)
WSSV	0/19 (0%)	0/30 (0%)
IHHNV	0/19 (0%)	0/30 (0%)
MBV	0/19 (0%)	0/30 (0%)
BPV	0/19 (0%)	0/30 (0%)
YHV	0/6 (0%)	4/5 (80%)
TSV	0/6 (0%)	0/5 (0%)
IMNV	0/6 (0%)	0/5 (0%)
*Mr*NV	0/6 (0%)	0/5 (0%)

4.3 *Vibrio parahaemolyticus* screening of giant freshwater prawn

4.3.1 *Vibrio parahaemolyticus* screening of giant freshwater prawn (cycle 1)

Figures 4.9 and 4.10 showed the agarose gel picture of *Macrobrachium rosenbergii* (cycle 1) for *V. parahaemolyticus* bacteria screening using DNA samples amplified by amplified AP3 primer. In lane 1 was the 100bp ladder. Lane 2 showed a band of *V. parahaemolyticus* bacteria as positive control, while lane 9 (Figure 4.9) and lane 16 (Figure 4.10) was the negative control. All the samples showed a band at 150bp which is the eukaryotic elongation factor (ELF) gene, which acts as an internal control. All the samples showed the absence of *V. parahaemolyticus* bacteria.

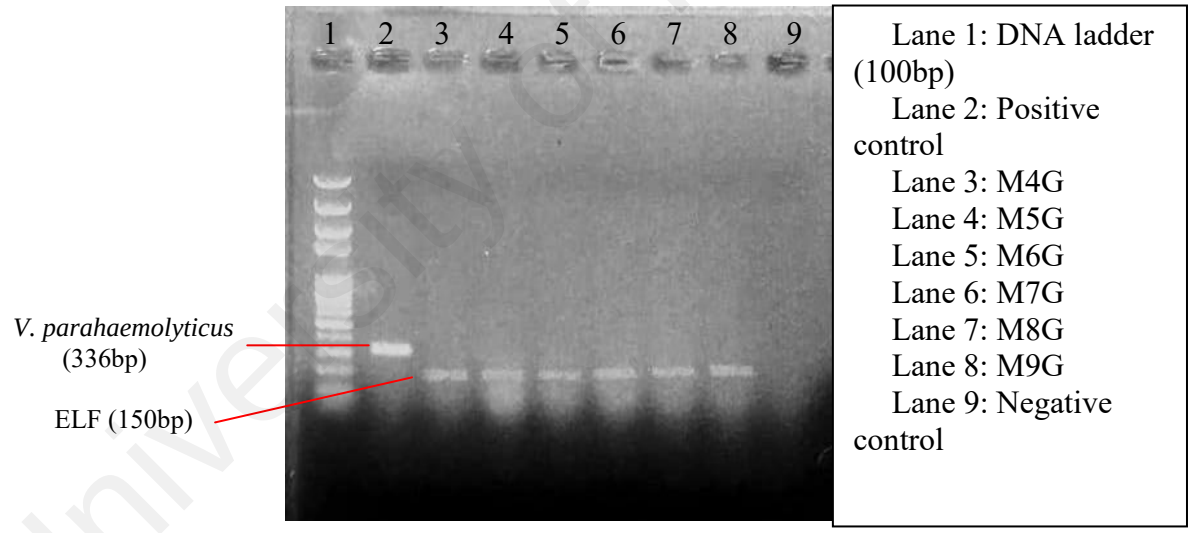

Figure 4.9: Gel imaging of PCR products of *Macrobrachium rosenbergii* (M4G-M9G) for *V. parahaemolyticus* bacteria screening using DNA samples amplified by AP3 primer.

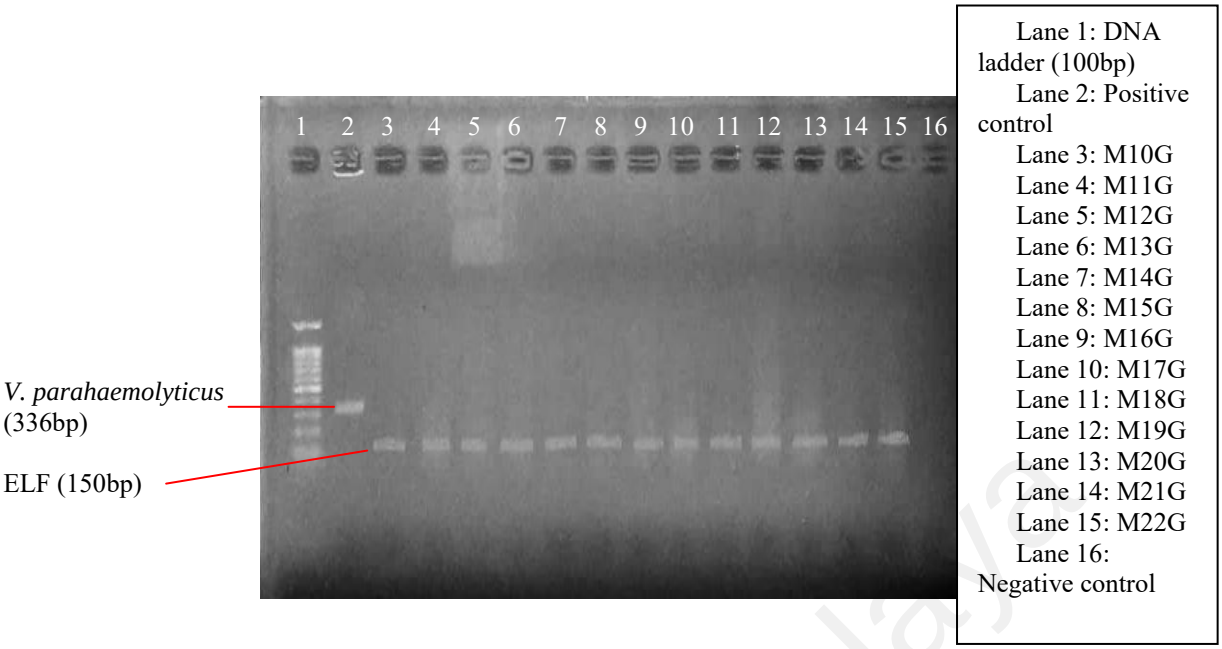

Figure 4.10: Gel imaging of PCR products of *Macrobrachium rosenbergii* (M10G-M22G) for *V. parahaemolyticus* bacteria screening using DNA samples amplified by AP3 primer.

4.3.2 *Vibrio parahaemolyticus* screening of giant freshwater prawn (cycle 2)

Figures 4.11, 4.12 and 4.13 showed the agarose gel picture of *Macrobrachium rosenbergii* (cycle 2) for *V. parahaemolyticus* bacteria screening using DNA samples amplified by amplified AP3 primer. In lane 1 was the 100bp ladder. Lane 2 showed a band of *V. parahaemolyticus* bacteria as positive control, while lane 13 was the negative control. All the samples showed a band at 150bp which is the eukaryotic elongation factor (ELF) gene, which acts as an internal control. All the samples showed the absence of *V. parahaemolyticus* bacteria.

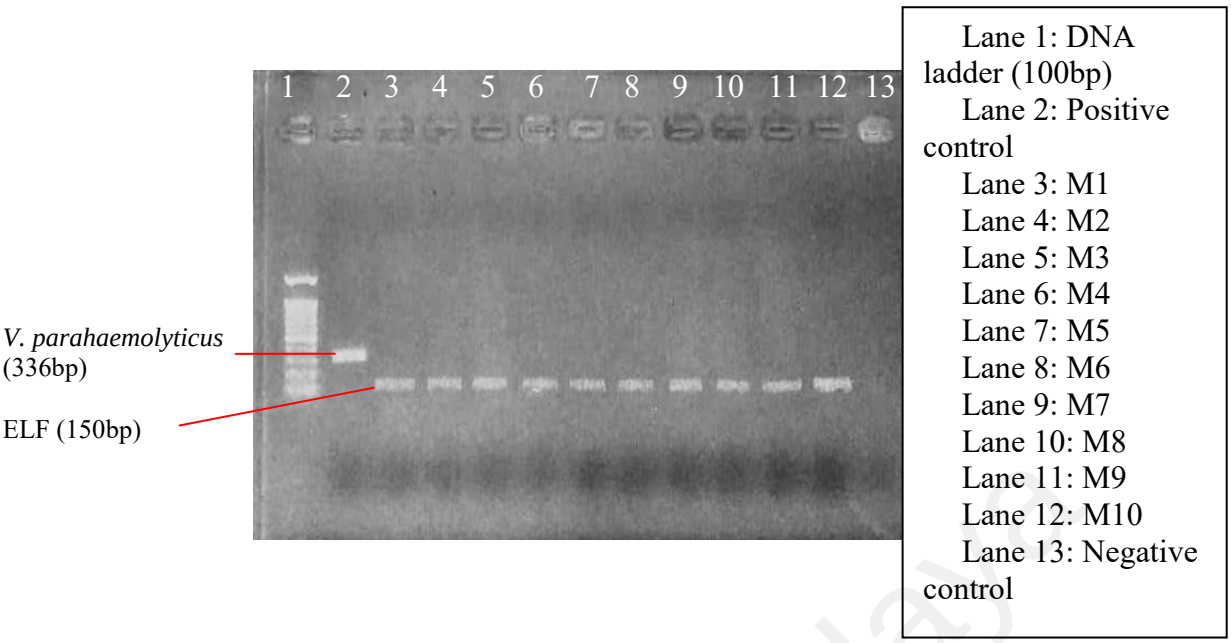

Figure 4.11: Gel imaging of PCR products of *Macrobrachium rosenbergii* (M1-M10) for *V. parahaemolyticus* bacteria screening using DNA samples amplified by AP3 primer.

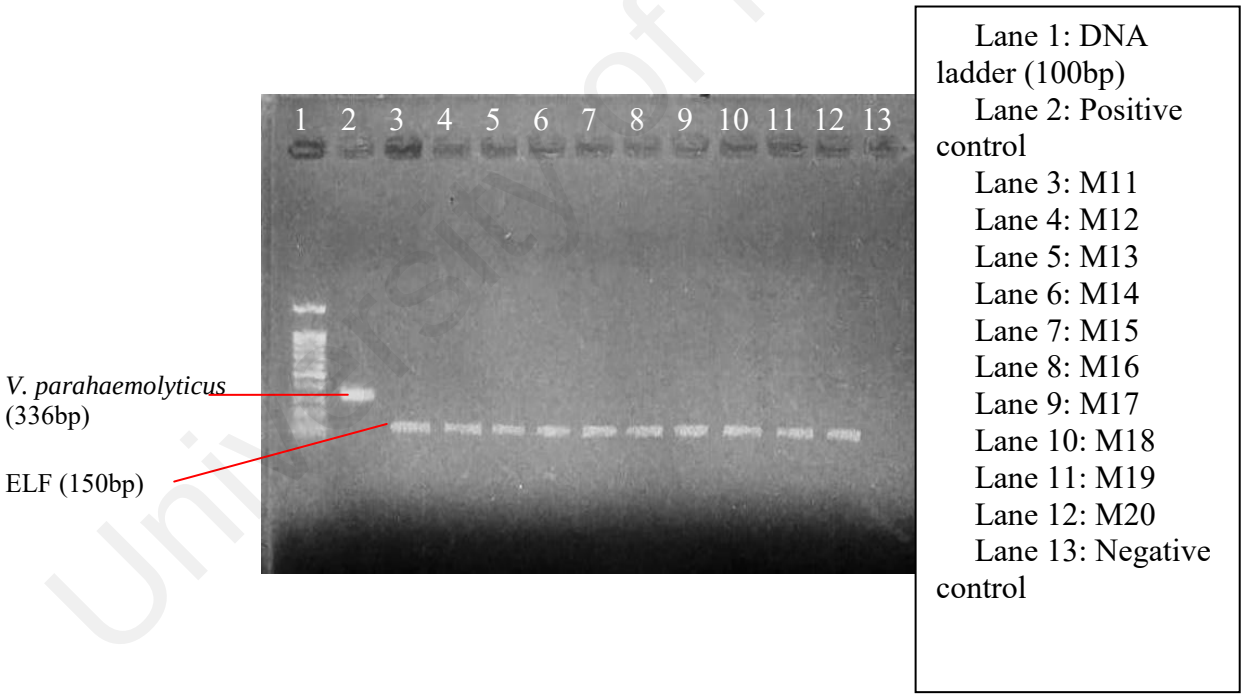

Figure 4.12: Gel imaging of PCR products of *Macrobrachium rosenbergii* (M11-M20) for *V. parahaemolyticus* bacteria screening using DNA samples amplified by AP3 primer.

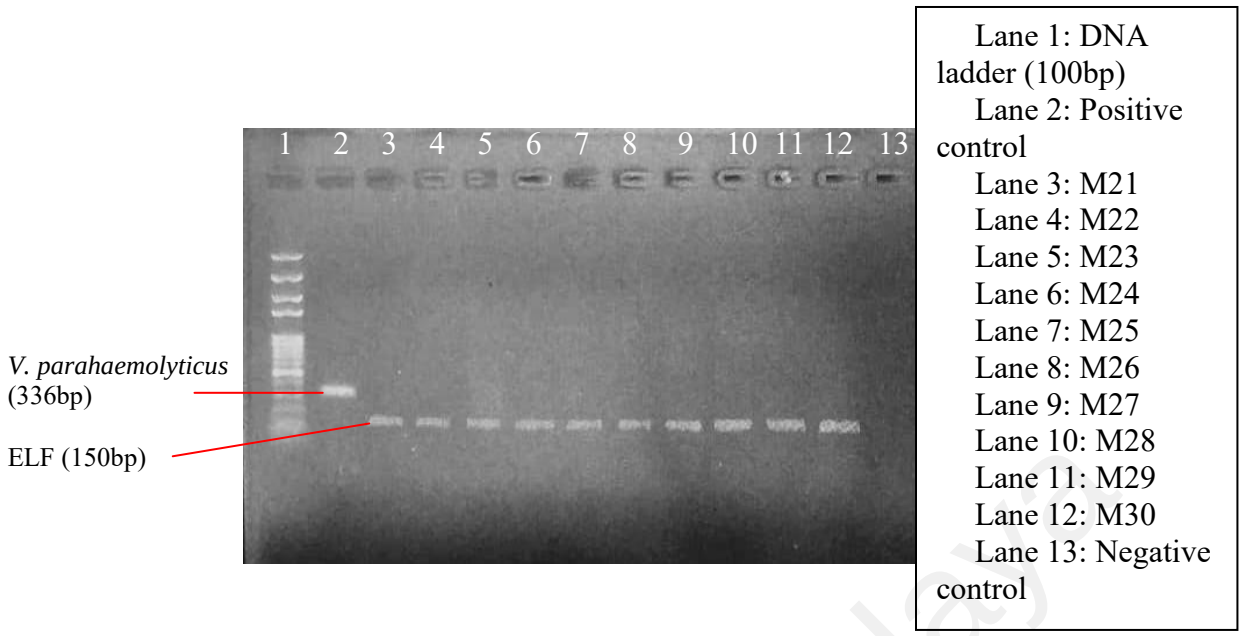

Figure 4.13: Gel imaging of PCR products of *Macrobrachium rosenbergii* (M21-M30) for *V. parahaemolyticus* bacteria screening using DNA samples amplified by AP3 primer.

Table 4.7 showed the prevalence of samples positive with viruses based on AP3 primer. There was no *V. parahaemolyticus* detected in all the samples tested using AP3 method. The results showed the prevalence of AHPND infection caused by *V. parahaemolyticus* in prawn samples was 0%.

Table 4.7: PCR amplification results of bacteria based on AP3 primer.

Bacteria	Cycle 1 (Positive with bacteria)	Cycle 2 (Positive with bacteria)
V. parahaemolyticus	0/19 (0%)	0/30 (0%)

4.4 Water quality test

On 25th May 2018, the pH of the pond water (pond 10 and pond 15) is 7.6 and ammonia level at 0.25 ppm. No nitrite content was detected in pond 10 while 0.25 ppm of nitrite was detected in pond 15. Moreover, both ponds showed no nitrate content in water.

After two months since sample collection, on 31st July 2018, the pH of pond 10 was 6.8 while pH of pond 15 remained unchanged, pH 7.6. In addition, there were no ammonia, nitrite and nitrate content were detected in both pond water. Table 4.8 and Table 4.9 show the pond water quality during the sample collection and after two months since sample collection.

Table 4.8: The pH, ammonia level, nitrite level and nitrate level of ponds in the farm tested on 25 May 2018.

Parameter	Pond 10	Pond 15
pH	7.6	7.6
Ammonia (ppm)	0.25	0.25
Nitrite (ppm)	0	0.25
Nitrate (ppm)	0	0

Table 4.9: The pH, ammonia level, nitrite level and nitrate level of ponds in the farm tested on 31 July 2018.

Parameter	Pond 10	Pond 15
pH	6.8	7.6
Ammonia (ppm)	0	0
Nitrite (ppm)	0	0
Nitrate (ppm)	0	0

CHAPTER 5: DISCUSSION

5.1 Mitochondrion DNA analysis of giant freshwater prawn

From the phylogenetic tree and median joining networking (Figure 4.1), the samples collected from the farm (population M) appearing in every clusters. On the other words, the *Macrobrachium rosenbergii* from the farm would have mixed with populations from other four states which are Kedah, Perlis, Johor and Negeri Sembilan.

One assumption could be that the *Macrobrachium rosenbergii* population in Malaysia was artificially transferred into other rivers between different states by human beings. Some wild prawns might have escaped and moved into other rivers and mixed with native populations. This explanation would be matched with the studies conducted by some researchers as they have reported on the effects of genetic diversity due to the introduction by humans on some freshwater aquatic organisms. Human introduction of freshwater organisms is a common phenomenon and it was found in *Macrobrachium asperulum* (Liu et al., 2011), freshwater crab (Shih et al., 2004), freshwater fishes (Wang et al., 2004), and the bamboo viper (Creer et al., 2001) in Taiwan.

The evolution of variations between populations from indigenous ranges to within-population variations by mixing genetic variations from different origin location of populations has brought out populations that have more genetic variation than indigenous native populations (Facon et al., 2003; Mamuris et al., 2005; Dlugosch & Hays, 2008). A high genetic diversity of shrimps in the farm may due to a rapid development or enlargement, various transmission, and also genetic variations caused by mutation.

Another possibility would be weather reason. In Malaysia, there are two types of monsoon winds seasons, the Southwest Monsoon occurs from April to September,

whereas the Northeast Monsoon occurs from October to March. The monsoon seasons in Malaysia is likely to bring some significant effect, which include elevating sea levels, increasing rainfall amount and increasing flooding risk in rivers. During this season, freshwater prawns may easily be transferred from mountains into estuarine as part of the floating pieces and then to offshore islands rivers. If the prawns were floated on rafts of floating plants during this period, this would have reduced their contact with the salt water and their chances of surviving is higher, by arriving on distant areas safely. Some researchers have reported a similar mode of dispersal of other animals. For instance, Censky et al. (1998) studied on the transfer of green iguanas to other islands by rafting during heavy rain. In addition, Toda et al. (1998) also studied on the Indian rice frog populations in Lanyu and Lyudao, and then they found that they were originated from eastern Taiwan.

Wild stocks are major resource for genetic variation and also important for genetic improvement of aquaculture stocks in the future. However, frequent harvesting of wild stocks to restock the aquacultures leads to population declines and thus loss of genetic diversity eventually. In Malaysia, the practice of using wild broodstock from natural water decreased in 2009, due to discovery of diseases in the stocks. Therefore, it had led to an establishment of management program involving the Department of Fisheries and universities for stock improvement in some locations of Peninsular Malaysia to ensure sufficient supply of stock. The presence of genetic diversity is useful in breeding programs regarding the maintenance of greater genetic variation in culture and the identification of genetically diverse broodstock (See et al., 2008).

The outcome of this study yields an analysis of the genetic structure, genetic diversity, distribution and phylogeography of five populations of the *M. rosenbergii*, including the population collected from the farm located in Kampung Chennah, Jelebu,

Negeri Sembilan, by studying on the cytochrome c subunit I (COI) locus, an mtDNA gene commonly applied by many researchers (Liu et al., 2011; Hsu et al., 2013; Xue et al., 2014).

Macrobrachium rosenbergii is cultured popularly in aquaculture, thus the data on its genetic structure, genetic diversity and phylogeography is useful and of high attention. Malaysia is one of the major top ten producers of freshwater prawns (New & Nair, 2012), is also paying high attention on the genetic and relationships between indigenous populations. Malaysia has implemented a genetic improvement planning and program on *Macrobrachium rosenbergii*.

5.2 Viral screening of giant freshwater prawn

5.2.1 DPO method to detect DNA and RNA viruses

Due to absence of drugs or vaccines for viral diseases in shrimp, therefore a sensitive and accurate diagnosis is important to implement appropriate control measures of technologies by using PCR or RT-PCR that have been used to screen for viruses at post-larvae stage before the sowing. However, there was no other viruses detected except YHV and this might be a false negative test as the amount of virus can be lower than the detection limit of multiplex PCR. A sensitive method with TaqMan in real-time PCR was developed which posses a wide dynamic range of detection that can detect up to 109 copies of WSSV and to less than two copies per reaction. This method has high sensitivity and wide dynamic range of detection, which is ideal for the detection of wide range of infections of WSSV in hatcheries and post-larvae stage (Jang et al., 2009).

Panichareon et al. (2011) states that, in a multiplex real-time PCR for YHV detection, it was less sensitive when comparing to conventional RT-PCR (Wongteerasupaya et al., 1997) and RT-PCR (Wijegoonawardane et al., 2010).

However, the sensitivity of multiplex real-time PCR can be used to diagnose and detect virus in ponds or carriers.

Multiplex PCR detection always analyzes with the positive control, the negative control, and the test samples. The proper identification is the presence of a positive band at the positive control lane and the absence of band at the negative control lane in the same gel comparing with DNA marker. The test samples can be observed by comparing to the DNA marker, the positive control, and the negative control. This method helps to distinguish the virus infected samples and healthy samples. The multiplex PCR analysis is sensitive, accurate and a suitable method to detect the infection though it is cost effective.

The PCR assay for detection of viruses was optimized using DNA extracted from purified DNA containing the viruses as the template. Optimal parameters were set to screen for the infection of viruses in the samples collected from the farm. Some of the bands shown in gel electrophoresis of the PCR products were faint and not clear. For example, ELF gene was not amplified in some of the shrimp samples. This might due to some reasons related to cycling times and temperature in PCR, for example, too few cycles were used and extension time was too short. In addition, PCR components may also cause the faint bands in gel such as primers contained impurities, primer concentration was too low or not enough template in the reaction. Primer-dimers were shown in some of the gel image. It might due to too many cycles were used in PCR reaction, therefore excessive cycling increases the chance for nonspecific amplification. Too much of primer was used might also contributed to primer-dimers. A high concentration of primers might cause the binding of primers to nonspecific sites of the template or to other primers.

For cycle 1, there was no DNA and RNA viruses detected in all the samples tested using multiplex PCR method. For cycle 2, there was no DNA viruses detected in all the samples tested using multiplex PCR method, but RNA virus was found in the samples. Yellow head virus (YHV) was detected in four samples out of five samples tested. A single 349 bp product was amplified and detected in four out of five samples collected from the farm. The results showed the prevalence of YHV infection in prawn samples was 80% (Table 4.6). This indicates a high prevalence of YHV infection of prawn samples in the farm.

One of the reasons for YHV infection in prawns could be contamination of broodstock. Prawns are easily infected by YHV starting from the late post-larval stage onwards. The clinical signs and symptoms are obvious on its appearance and the mortality rate is the highest during the early to late juvenile stages (Spann et al., 1992). During hatchery stage, shrimp are easily getting infected from other shrimp when the virus is transmitted through the food and water, as if they consumed tissues that are contaminated by dead shrimp (Wang et al., 2004). In some cases, crustaceans and other living organisms might become disease-carrying vectors.

Another factor of YHV infection in prawns (cycle 2) might be due to water supply contamination. The virus may be originated from river water from Sungai Chennah, especially when the river water was mixed with pond water during rainy season. In addition, the pond environments also could be the reason for YHV infection in prawns. For example, the high density of the prawns in the pond causes the easier outbreak of diseases. The water quality such as pH, temperature and salinity also affect the disease outbreak.

5.2.2 Field research on prawn viruses occurrence in Malaysia

Field research was carried out and the occurrences of White Spot Syndrome Virus (WSSV) in Malaysia aquaculture farm were discovered in 1998 and 1999. Dark-field microscopical observation and PCR was used to diagnose WSSV, and followed by PCR of PAV was used to confirm the virus (Momoyama et al., 1995).

In year 1998, field work was carried out in February, June and August, at the same time, researches were carried out at Penang Island, Nibong Tebal and Sungai Petani in Penang, Perak, and Kedah State, respectively. In year 1999, field researches were carried out at Taiping in Perak State, Bukit Tambun in Penang State, and Johor Bahru and Batu Pahat in Johor State. On the other hand, samples collected from Kuching in Sarawak State were studied and diagnosed on March and May 1999. Before the study, Sarawak State was free of White Spot Syndrome Virus (Momoyama et al., 1995).

By using PCR method, WSSV was detected in infected prawns in Penang (1998), Kedah (1999), and Sarawak State (1999), where the outbreaks of this disease were occurred. In the study, WSSV could not be detected in other states. It can be hypothesized that not all the prawn farms in Malaysia were contaminated by WSSV, or that all the surviving prawns became virus carriers. Since that, many farmers have given up to run a prawn farm due to heavy loss incurred by viruses until 1997. Thus, the farmers started to change their ponds into fishing ponds (Momoyama et al., 1995).

In 2006, IHHNV infection was reported on the giant freshwater prawn, *Macrobrachium rosenbergii* in southern Taiwan. It caused high mortalities in post-larvae *Macrobrachium rosenbergii* (Hsieh et al., 2006). On the other hand, Nita (2013) studied on IHHNV infection in wild *Macrobrachium rosenbergii* in Malaysia. The study has identified the partial sequence of ORF1 of IHHNV genome which causes the

first report on the occurrence of IHHNV infection in *Macrobrachium rosenbergii* in Malaysia by screening the giant freshwater prawn in hatchery and wild environment. The study revealed a high IHHNV infection prevalence in the sampling fields for wild environment. However, there was no mortality cases occurred.

This study revealed that there are infections of yellow head virus in freshwater prawn in Malaysia. The outcome of the study is useful to build a foundation of prawn breeding programs in Malaysia hatcheries to fulfill the growing need of freshwater prawn industry by generating high quality and domesticated potential broodstock. This method is also useful for producing potential specific pathogen free (SPF) broodstock generations.

5.3 *Vibrio parahaemolyticus* screening of giant freshwater prawn

5.3.1 PCR method to detect AHPND

There were many tentative PCR used to detect AHPND strains which are AP1, AP2 and AP3 methods published in 2014 (Flegal & Lo, 2014). AP3 method was chosen for this study because AP3 primer set showed the best sensitivity and specificity.

Two interim PCR detection methods based on primers AP1 and AP2 were introduced in December 2013. At first, AP2 performed as the better primer as it gave about 3 % false-positive results. Hence, the method was used to detect a high prevalence of VP_{AHPND} in live broodstock feeds, pond-reared and hatchery broodstock, and in post-larvae used to stock shrimp farms.

However, AP2 method was thought to give the false-positive PCR test results because of the mutation of plasmids that lack of the toxin gene. To overcome this problem, a more sensitive AP3 detection method based on AHPND strain detection and

use of the gene sequence of smaller 12.7 kDa toxin with 100 % sensitivity and specificity was released (Flegal, 2014). Moreover, PCR-based method for AHPND detection was also developed by using two AHPND-specific contig sequences located on an AHPND-related plasmid (Chen et al., 2014).

The AP3 method also been used in Thailand and other areas since June 2014 and the feedback has been positive. Based on the results accumulated from Thailand, VP_{AHPND} is revealed as prevalent in pond water and residues, in feces of broodstock, some batches of post larvae (PL) and some living animals which used to feed broodstock shrimp. This has created awareness of biosecurity risks for shrimp farmers. It suggests that the application of PCR screening of broodstock and PL can help to minimize the probability of AHPND outbreaks by reducing transmission to rearing ponds through PL. Furthermore, PCR monitoring during pond preparation and shrimp cultivation helps to reduce the transmission of VP_{AHPND} by identifying its environmental reservoirs.

5.3.2 Acute hepatopancreatic necrosis disease (AHPND) of farmed shrimp in Malaysia

Among bacterial shrimp diseases, diseases caused by *Vibrio alginolyticus*, *V. parahaemolyticus*, and *V. harveyi* are the the main concern in shrimp farming (Rajasekar et al., 2011; Wei & Wendy, 2012; Zhou et al., 2012; Tran et al., 2013; Nunan et al., 2014). Acute hepatopancreatic necrosis disease (AHPND) or early mortality syndrome (EMS) caused by *V. parahaemolyticus* first appeared in Asia in 2009 (Tran et al., 2013), brings to huge economic losses.

In 2011, the Fisheries Research Institute through the National Fish Health Research Division (NaFisH) received reports regarding cultured white-leg shrimps producing white feces and followed by death from a farm in Perak. After few months, the

Department of Fisheries (DOF) of Malaysia received reports regarding high mortalities rate in most of the shrimp farms in Peninsular Malaysia. Soon, the affected in Malaysia included Perak, Penang, Kedah and Pahang. The etiology of the disease was then confirmed as AHPND based on histopathological changes in the hepatopancreas of affected shrimps and diagnostic results. After that, samples collected from Sabah (2012), Sarawak (2012), Terengganu (2013), Melaka (2014) and Johor (2014) were also shown positive results for AHPND by hispathology (Chu et al., 2016).

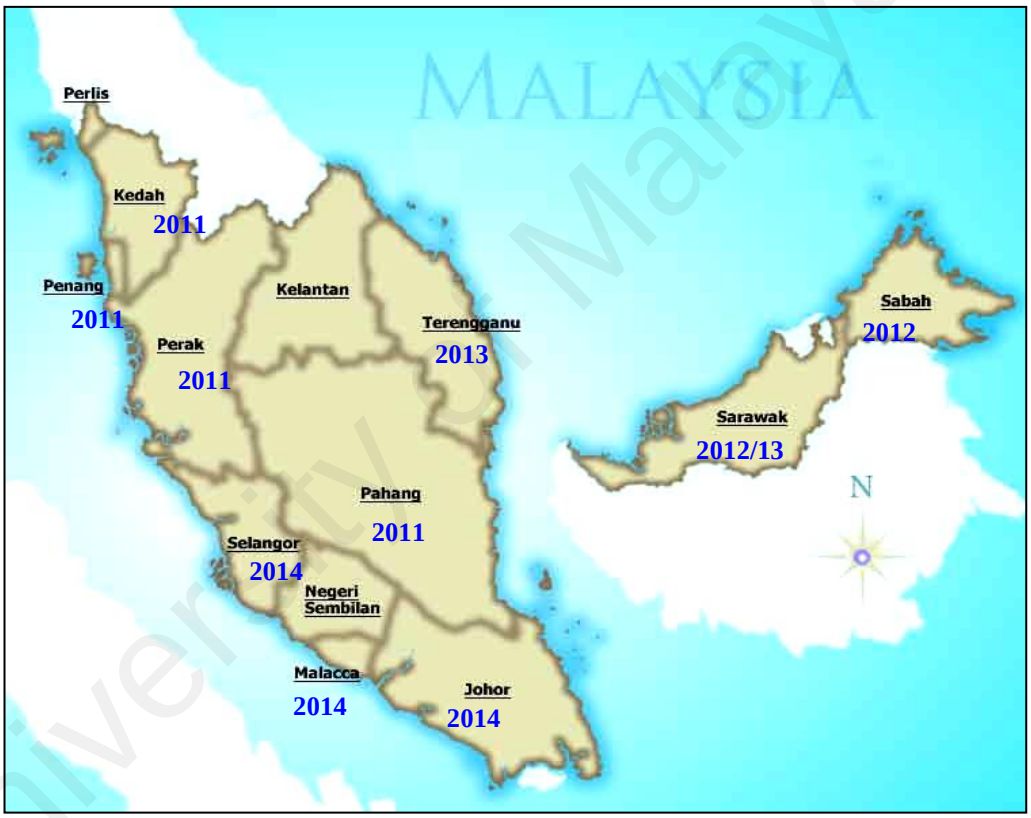

Figure 5.1: Chronological order (year) of Acute Hepatopancreatic Necrosis Disease (AHPND) occurrences in Malaysia confirmed by histopathology (Chu et al., 2016).

Being halophilic in nature, it is common if *V. parahaemolyticus* can be found in estuarine and in brackish water. Though *Macrobrachium rosenbergii* is a freshwater prawn, they can also survive in brackish environment (FAO, 2002), thus there is a possibility to be infected by *V. parahaemolyticus*. Furthermore, *V. parahaemolyticus*

has spread widely in Malaysia since 2011. Therefore, we have conducted the screening of *V. parahaemolyticus* in the samples collected from the farm.

In this study, all the samples collected from the farm did not show the presence of *V. parahaemolyticus* by performing PCR with primers AP3 (Sirikharin et al., 2014). The prevalence of samples positive with *V. parahaemolyticus* is 0% (Table 4.7). We suggested that the pond water environment is not suitable for the survival of *V. parahaemolyticus*. *V. parahaemolyticus* can be found mainly in inshore marine waters throughout the world, and it is is mostly cannot be found in estuarine marine water. The optimal temperature for the growth of *V. parahaemolyticus* is raging from 35 °C to 39 °C (Jackson, 1974). The doubling time of *V. parahaemolyticus* is as short as 5 minutes (Barrow & Miller, 1974), however, under optimal condition, it has a generation time of less than 20 minutes. During summer day, *V. parahaemolyticus* can cause food borne disease outbreaks as it is a mesophilism bacterium (Daniels et al., 2000). Even though the number of *V. parahaemolyticus* in freshly harvested seafood is comparable lower than the number of infection predicted (Sanyal & Sen, 1974), but its rapid multiplying ability under suitable temperatures can cause a disease, as people consume the contaminated food.

The optimum pH range for growth of *V. parahaemolyticus* is 7.6 to 8.6 (Beuchat, 1975). Of the tested pond, the pH range was from 6.8 to 7.6 tested by API freshwater master test kit (Table 4.8 & Table 4.9), which might be a bit difficult for the survival of *V. parahaemolyticus*. Another important factor which affects the growth of *Vibrio parahaemolyticus* is salinity of water. The salinity range suitable for their growth in the marine environment is ranging from 0.8% to 3% (DePaola et al., 2000). On the other hand, Liu and the team (2016) reported that the optimal growing condition for *V. parahaemolyticus* is 37 °C and 3% NaCl concentration. We suggested that the pond

water might not be suitable for *Vibrio parahaemolyticus* growing as the salinity of pond water was 0 ppm.

5.4 Biosafety measures in farm

5.4.1 Watching for signs of problems

Continuous exchange of a small proportion of the pond water is the normal way to maintain a good water quality. However, some farmers change water more suddenly every two weeks and in much larger proportions, to make the prawns moult. The more that moult of the prawns, the less potential losses that may be caused by cannibalism. Phytoplankton cover the surface of the pond causes low dissolved oxygen content at night, thus this problem can be controlled by exchanging water. If the prawns begin to crawl out from the ponds or stay at the edges of the pond in daylight, it might indicate the dissolved oxygen content in the pond is low. If this is happened, the pond has to be flushed. Dense phytoplankton blooms in the pond may also cause high pH level of water which then leads to prawn mortalities (FAO, 2002).

5.4.2 Dealing with problems of predation

Predation is one of the major problems in aquaculture farming, including freshwater prawn farming. Predation is mainly caused by other aquatic species, birds, snakes and humans. Human predation is especially the main problem in freshwater prawn farm because of the high value of prawn products and they are easily to be caught. This problem can be minimized by using fences. Moreover, insects, carnivorous fish and birds are also serious predators in freshwater prawn farming. In the past, chemicals have been used to kill insects in prawn farm but this is not recommended because it affects the pond ecosystem. Some fish and insects can be prevented to enter the farm by using suitable screens or gravel filters, while most of the commercial prawn farms use simple

net filters. To minimize the predators in the farm, farmers can stock the prawns after each pond is filled, so that the predators have less chance to become established. High netting fences can be set up around the ponds to prevent the entry of fish, amphibians, reptiles and some mammals. During cull-harvesting of prawns, seining can remove some predators. Netting or string can be used by stretching across the top of ponds as a deterrent to prevent the entry of birds (Figure 5.2) (FAO, 2002).

Figure 5.2: Netting can be used to protect freshwater prawns from predators (FAO, 2002).

5.4.3 Dealing with disease outbreaks

An outbreak means an unexpected occurrence of mortality or disease. This may be caused by significant pathogens or water quality changes. If a serious health problem or disease affecting significant percentage of prawn stocks, a chain of emergency responses should be carried out. If a water quality is suspected, the appropriate emergency response will be carried out to minimize the impact of disease and its spread to the other sites.

A site may be officially isolated or quarantined to prevent disease outbreaks. The isolation remains until the cause and source of disease has been found and managed.

The movement of all prawns within the site will be ceased and the prawns will not be handled. In addition, equipment and personnel are not allowed to move on or off site unless special arrangements are made. Visitors or non-essential staffs are not allowed on site unless authorized by management. Disinfection and hygiene are required on site, including procedure for staff and equipment will be strictly enforced. Samples will be handled properly and transferred for lab investigation. The parameters which are useful to maintain records and from part of information or history during disease investigations includes source of prawn, period on farm, dates of disease onset, age and prawn species affected, stocking density, mortality pattern, water quality, recent handling, grading, net changes, tank transfer, etc. Continuous monitoring is required after the initial work to determine the course of outbreak and to check whether the treatment measures are being effective and useful. Sampling of prawns for laboratory analysis maybe required repeatedly (Kueh, 2009).

Yellow head virus infection was found in the samples collected from the farm. Early detection of yellow head virus can reduce the loss by emergency harvesting and also thorough disinfection. Furthermore, maintaining optimal pond environment and rearing conditions may help to minimize the implication, such as maintain consistent phytoplankton blooms at about 25-35 cm transparency by maintain the pH in the pond. After harvesting the shrimp, organic materials and sediments accumulated in the pond are removed followed by a new pond preparation. Another method to minimize the risk of yellow head virus infection is by using a semi-closed or closed system which restricts inflow of water and recirculate treated water. Host-free virus and waterborne infection is possible to be found in water up to 72 hours. Water disinfection is also a method to minimize the infection, for example, using chlorine at 20-30 ppm is an effective disinfectant (Bower et al., 1994).

5.4.4 Monitoring and record keeping

The main objective of monitoring prawn in the farm is to enable early detection of disease and surveillance for disease incursions. Second, prawn monitoring checks the efficacy of treatment and control programs in prawn farm. Third, monitoring prawn farm collects information to develop future risk mitigation measures. The information includes environmental and breeding factors that may influence disease such as stocking age and stocking density.

Record keeping in prawn farm is important for long term planning and comprehensive assessment of prawn health and production. The records produce data which can indicate trends and helps in events traceability. The information thereby used to improve prawn health management, identification of health issues and ensuring quality assurance of prawn produced particularly related to food safety.

5.4.5 Prawn health records

Prawns are monitored for at least once a day to record any unusual behavior, visible lesions or other symptoms of disease. Any unusual behavior and disease signs observed in prawns will be reported to farm manager. These unusual behaviors include reduced diet intake, inactivity and growth retardation, while physical changes may include white spot on shell and colour changes. The unusual behavioral changes or physical signs of prawn disease should be further investigated to avoid affecting a significant loss of prawn stock.

Feeding is one of the useful to find out any potential problems or diseases in prawn before overt clinical signs and mortality occurs. Poor feeding behavior should be reported and further investigated. In addition, mort dives is a useful method to indicate

any unusual mortalities or diseases in prawns. The samples have to be collected for further analysis by farm staff.

Weight checking and grading are important to spot any abnormalities in prawns, and the samples can be taken for further examination. Prawns with weight less than expected optimal weight are suspected to be infected by diseases. The trends may not be obvious at a point in time until comprehensive data has been collected over a period of time. The prawns with suspected diseases must be sent to laboratory for analysis. PCR detects DNA whether the disease causative agents are viable or not, so that the eradication and disinfection events can be carried out after an outbreak. However, laboratory diagnostic methods do not tell the difference between primary causative agent, these require further analysis via histology.

The prawn tissues which chosen for laboratory analysis is important to generate accurate and useful results. Sampling prawns with mild to severe clinical signs may increase the accuracy and success of diagnosis. Different disease agents tend to have different primary target organ or infection site, and these particular organs tend to have the most obvious gross lesions or spots. The targeted organs for the disease should be taken for examination to increase the success rates of disease detection (Kueh, 2009).

5.4.6 Water quality monitoring

Good water quality is vital to produce healthy prawns. The farm should maintain a regular program for monitoring and recording water quality in ponds. Water quality parameters which are important in the health of prawns are temperature, dissolved oxygen, pH, salinity, ammonia, nitrate, nitrite, hardness, alkalinity, turbidity and the levels of toxic agents. For the maintenance of good health and growth rate, the generally accepted range of the critical water quality parameters are shown as Table 5.1.

Table 5.1: The recommended range of water parameter for freshwater and marine aquatic organisms (The State of Queensland Department of Primary Industries, 2002).

Water parameter	Recommended range		Water parameter	Recommended range
	Freshwater	Marine		General aquatic
Dissolved oxygen	>4 mg/L	>4 mg/L	Arsenic	<2 mg/L
Temperature (°C)	21-32	24-33	Cadmium	<0.003 mg/L
pH	6.8-9.5	7.0-9.0	Chlorine	<0.003 mg/L
Ammonia (total)	<1.0 mg/L	<1.0 mg/L	Chromium	<0.1 mg/L
Ammonia (NH_2, unionized form)	<0.1 mg/L	<0.1 mg/L	Nickel	<0.01 mg/L in soft water, <0.04 mg/L in hard water
Nitrate (NO_3)	1-100 mg/L	1-100 mg/L	Cyanide	<0.005 mg/L
Nitrite (NO_2)	<0.1 mg/L	<1.0 mg/L	Iron	<0.5 mg/L
Salinity	0-5 ppt	15-35 ppt	Lead	<0.03 mg/L
Hardness ($CaCO_3$)	20-450 mg/L		Manganese	<0.01 mg/L
Alkalinity ($CaCO_3$)	20-400 mg/L	50-200 mg/L	Mercury	<0.00005 mg/L
Turbidity (secchi)	<80 cm	25-40 cm	Copper	<0.006 mg/L in soft water
Calcium/ Magnesium	10-160 mg/L		Tin	<0.001 mg/L
Hydrogen sulphide	<0.002 mg/L			

Measuring water chemistry is important to make sure the water quality is always optimum for the growth of prawn. If the parameter of water is drop beyond the range, actions have to be taken immediately to ensure the water quality goes back to normal range. This is because prawns are easily infected by diseases or they cannot survive if water parameter is not in optimum range. Table 5.2 shows some troubleshooting actions to optimize the water quality in prawn farm.

Table 5.2: Troubleshooting action plan to optimize the water quality in prawn farm (Pattillo, 2014).

Problems	Troubleshooting actions
Low dissolved oxygen	• Provide aeration • Provide supplemental oxygen • Perform water exchange
Super-saturated dissolved oxygen	• Provide aeration
High ammonia nitrogen	• Perform water exchange • Reduce temperature • Reduce pH • Aerate • Add beneficial bacteria • Add alkalinity
High nitrite	• Perform water exchange • Reduce temperature • Add NaCl salt (10 Cl^- : 1 NO_2^-) • Add beneficial bacteria • Add alkalinity
High chlorine	• Perform water exchange (with dechlorinated water) • Aerate • Add beneficial bacteria
High temperature	• Add cool water • Aerate • Treat for low dissolved oxygen
Low temperature	• Add warm water • Aerate

Table 5.2, continued

Problems	Troubleshooting action
High pH	• Perform water exchange • Monitor alkalinity
Low pH	• Perform water exchange • Increase alkalinity • Aerate
High solids load	• Clean filters • Perform water exchange • Treat for low dissolved oxygen • Treat for ammonia
High salinity	• Perform water exchange
Low salinity	• Add salt
Low alkalinity	• Increase alkalinity (add baking soda, limestone, etc.)
High carbon dioxide	• Aerate • Perform water exchange

5.4.7 Handling drugs and chemicals

Medicated feed for prawns should be stored separately from non-medicated feed with clearly labeling. The labels on the medicated feedbag should include some important details such as type of drugs, percentage of drug in feed, veterinarian name who prescribed the drug, effects of the drug and manufacture date. Medication to the prawns should be done according to veterinarian's instructions and medication feed should be handled according to Material Safety Data Sheet (MSDS) of incorporated drug.

Biological drugs include vaccine. These drugs are kept refrigerated and used accordingly as manufacturer's instructions. On the other hand, disinfectants are stored properly in clearly labeled containers and handled according to MSDS (Kueh, 2009). A suitable disinfecting agent is required for an effective disinfection. Disinfecting agents are chosen according to the nature of target pathogen (Table 5.3). The viruses are

basically categorized into three groups for disinfection purposes: Category A viruses have a lipid enveloped and the easiest group to inactivate since the lipid envelope is sensitive to many lipophilic compounds; category B viruses have small non lipid containing viruses and protected within a protein matrix, and they are the most difficult to inactivate; category C viruses do not have lipids but are usually larger than viruses in category B. They are intermediate to inactivate by chemical agents.

Table 5.3: Viruses and their respective disinfection category (Manual of Biosecurity and Standard Operating Procedures for Shrimp Culture, 2015).

Viruses	Disinfection category
1. White spot disease	A
2. Taura syndrome	B
3. Yellow head disease - Yellowhead virus	B
4. Infectious hypodermal and hematopoietic necrosis	B
5. Infectious myonecrosis	
6. Monodon baculovirus (MBV)	B
7. Gill-associated virus	A

CHAPTER 6: CONCLUSION

The broodstock *M. rosenbergii* from the farm are from other four states which are Kedah, Perlis, Johor and Negeri Sembilan. The source of broodstock is an important issue, as it will affect the quality of hatchery post-larvae.

On the other hand, this study had covered the screening of DNA viruses include WSSV, IHHNV, MBV, BPV and RNA viruses include YSV, TSV, IMNV, *Mr*NV infection in giant freshwater prawn in the prawn farm. The results showed that there are yellow head virus infections in freshwater prawn in Malaysia. YHV infection prevalence was high in the farm, while the other viruses were not detected based on PCR method. Future work should be done to understand the transmission and epidemiology of yellow head disease in giant freshwater prawn in Malaysia. One of the reasons for YHV infection in prawns could be contamination of broodstock. Another factor of YHV infection in prawns might be due to water supply contamination. In addition, the pond environments also could be the reason for YHV infection in prawns.

Even there are handful methods for the identification of *V. parahaemolyticus* bacteria, PCR method with AP3 primers is easy, less expensive and time saving. *V. parahaemolyticus* bacteria was not found in all the samples indicating the freshwater pond environment is not suitable for the bacteria to survive.

The outcome of the study will be useful to enhance breeding programs in the hatchery to sustain the need for industry in Malaysia to produce high quality domesticated potential broodstocks. Therefore, a health surveillance program of the broodstock is needed as a biosafety measure to minimize the risk of disease outbreaks into the farm. Moreover, the information on prevalence of the particular diseases caused by viruses and bacteria provides the breeders information on the health status of

broodstock. As *Macrobrachium rosenbergii* becoming an important source for export and consumption, this practice can be applied to produce potential specific pathogen free (SPF) broodstock generation.

Frequent monitoring of viral and bacterial disease is likely to constitute an effective method for choosing healthy broodstock from the wild environment for hatchery production and controlling the spread of the disease in Malaysia. A systemic approach of biosafety measures is developed for the prawn farm to prevent diseases, these includes prawn health surveillance, disease outbreaks investigation, monitoring or record keeping, prawn health records, water quality monitoring and handling drugs and chemicals.

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