Macrobrachium rosenbergii nodavirus (MrNV) in juvenile crustacea

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The research presented and reported in this thesis was conducted within the guidelines for research ethics outlined in the *National Statement on Ethics Conduct in Research Involving Human* (1999), the *Joint NHMRC/AVCC Statement and Guidelines on Research Practice* (1997), the *James Cook University Policy on Experimentation Ethics*. *Standard Practices and Guidelines* (2001), and the *James Cook University Statement and Guidelines on Research Practice* (2001). The proposed research methodology received clearance from the James Cook University Experimentation Ethics Review (Approval number A1382)

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ABSTRACT

In the giant freshwater prawn (*Macrobrachium rosenbergii*), white tail disease (WTD) caused by *Macrobrachium rosenbergii* nodavirus (MrNV) has been found in many countries including, French West Indies, China, Taiwan, India and Thailand. In mid 2007, the index case of WTD in Australia presented in adult broodstock *M. rosenbergii* from Flinders River in western Queensland. In order to understand the phylogenetic relationship of the Australian isolate of MrNV with other MrNV isolates, the complete sequences of MrNV (RNA1 and RNA2) including protein B2 were determined in this study. Nucleotide sequence analysis showed that the identities of MrNV (RNA1) Australian strain were 94%, 95%, 95% and 97% similar to Malay, the French West Indies, Chinese and Thai strains, respectively. Also, MrNV (RNA2) Australian sequence showed 92% similar nucleotide sequence compared to the French West Indies, Chinese and Thai strains. The phylogenetic analysis demonstrated the Australian isolate of MrNV (RNA1) is closely related to the Thai and the French West Indies isolates, while MrNV (RNA2) is most distant to the other isolates. The phylogenetic tree of different nucleic acids (317 bp) of protein B2 also highlighted the differences between various nodavirus. Protein B2 of the Australian MrNV is different from fish, *Penaeus vannamei* and insect nodaviruses but it is closely related to black beetle nodavirus. The Australian protein B2 of MrNV is still in the MrNV cluster.

WTD causes significant production losses to the *Macrobrachium* spp. industry worldwide. Therefore, cell culture and an animal model could be used to study MrNV infection. In this study, C6/36 cell line was used with different staining methods to observe cytopathic effect (CPE), count disrupted cells, and measure mitochondrial activity. Also, TaqMan real-time polymerase chain reaction (PCR) was used to detect the number of viral copies of MrNV in the cells. The typical CPE such as vacuolation and viral inclusion bodies were observed in infected C6/36 cells with Mayer's haematoxylin and eosin stain (H&E) and Giemsa staining. Acridine orange was easier to detect single stranded nucleic acid, presumptive MrNV ribonucleic acid in the infected cells. The numbers of cells with disrupted cell membrane in infected treatment stained with trypan blue were higher than control treatment and rose to the maximum of 4×10^5 cells at day 8. The absorbance reading

of neutral red staining from infected samples peaked at day 4 (O.D. = 0.6) compared to control at day 12 (O.D. = over 3). However, TaqMan real-time PCR did not confirm the replication of MrNV in the cells over 14 days in the infected samples. The mean viral copies and mean cycle times of positive samples were stable at 2.07 $x10^4$ and 24.12, respectively. TaqMan real-time PCR results from different passage times also showed the decreasing number of viral copy from 500 copies in passage 1 to less than 50 copies in passage 4. Different cell line and experimental techniques may need to be developed for MrNV in order to determine the replication of the Australian MrNV.

As the replication of the Australian isolate of MrNV in the C6/36 cell line was not confirmed by TaqMan real-time PCR, an experimental animal model for the Australian MrNV was developed for this study. *Macrobrachium* can be hard to source due to their requirement for a saltwater environment for breeding and there is no operating farm in Australia. Instead, the Australian redclaw crayfish (*Cherax quadricarinatus*) were tested as a potential experimental animal model. In this experiment, the highest mortality (35%) was in the groups injected with MrNV and the lowest mortality (0%) was in the control groups. The inoculated crayfish had the smallest size (9.96 ± 0.99 cm) and lowest weight (22.34 ± 5.76 g) when compared to the feeding and control groups. The mean length of the control and feeding groups were 10.96 ± 0.68 cm and 10.33 ± 0.98 cm, respectively. The mean weight of the control treatment was 27.17 ± 5.60 g, while feeding groups had a mean weight of 25.88 ± 7.79 g. The statistical analysis of length showed a significant difference ($P < 0.05$) between different treatments, while the weight did not indicate a significant difference ($P > 0.05$). Necrotic muscle and muscle degeneration with haemocytic infiltration were observed in infected crayfish. For the first time, a quantitative real-time polymerase chain reaction (qPCR) on clinical material was developed and it confirmed MrNV infection in infected animals. The mean viral titres (2.73 x 10^2 copies) and cycle times (Ct = 31.33) lead us to hypothesize that MrNV only poorly replicates in juvenile *C. quadricarinatus* when compared to the number of viral copies in the inoculums (10^4). However, *C. quadricarinatus* may be a less than perfect but nevertheless a useable experimental animal model for MrNV infection in the future because of clinical signs, gross lesions, histopathological

changes and qPCR titres that where present in experimentally infected *C. quadricarinatus*.

RNA interference (RNAi) is an innate immune response which is triggered by the recognition of intracellular dsRNA that subsequently leads to inhibition of sequence-specific viral RNA in the cells. Also, RNAi has been shown to work against viral infection in prawns such as yellow head virus (YHV) and white spot syndrome virus (WSSV). However, no study of RNAi has been reported against MrNV infection. In this study, RNAi was designed against protein B2 of the Australian MrNV and tested in *C. quadricarinatus*. Mortality results at 10% and 60% showed in Stealth RNAi with MrNV and Stealth control RNAi with MrNV treatment, respectively. Percentage mortality (60%) of Stealth control RNAi with MrNV treatment was significantly different to other treatments ($P < 0.05$). Redclaw crayfish in control Stealth RNAi + MrNV treatment had the smallest size (8.7 ± 0.99 cm) and lowest weight (17.69 ± 3.55 g) when compared to other treatments which averaged 10.3 cm in length and 22.90 g in weight. Moreover, length and weight analyses of Stealth control RNAi with MrNV showed significant differences ($P < 0.05$) between different treatments. Clinical signs of MrNV and histopathological lesions such as myolysis with haemocytic infiltration in the muscle were found in infected redclaw crayfish. In two out of ten redclaw crayfish inoculated with Stealth RNAi with MrNV treatment and eight out of ten in the Stealth control RNAi with MrNV treatment MrNV was detected using qPCR. The mean number of viral copies from Stealth RNAi + MrNV treatment was 1.29×10^1 and cycle time was 34.88. The mean of viral copies from control Stealth RNAi + MrNV treatment was 3.54×10^1 and cycle time was 34.83. The mean viral copies and mean cycle times of positive samples were 3.45×10^1 and 34.84, respectively. No significant differences in viral copies or cycle time from qPCR between Stealth RNAi + MrNV treatment and control Stealth RNAi + MrNV treatment were found ($P > 0.05$). However, RNAi could be an effective tool to prevent MrNV infection and decrease mortality in infected redclaw crayfish. Thus, it may be possible to use RNAi to control viral infection on crustacean farms.

After testing RNAi against protein B2 of the Australian MrMV with *C. quadricarinatus*, it can be concluded that RNAi can prevent MrNV infection and

decrease mortality. Thus, RNAi may be used to control other viral infection in crustacean farming but each has to be redesigned to be specific to the viral strains. Moreover, the complete sequences of the Australian MrNV (RNA1 and RNA2) including protein B2 were different compared to fish and insect nodaviruses. To control nodavirus infection in different species, specific-sequence RNAi should be designed related to the particular viral strains. This study is the first development of experimental animal model for MrNV and the first use of RNAi against protein B2 of the Australian MrNV using *C. quadricarinatus* to control the disease. The information of RNAi from this study will be able to be applied for other nodavirus including fish and insect nodavirus to control the disease outbreak in the future.

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LIST OF ABBREVIATIONS

°C	Degree celsius
CNS	Central nervous system
ANOVA	Analysis of variance
ATV	Antibiotic trypsin versene
BBV	Black beetle virus
bp	Base pairs
BLAST	Basic Local Alignment Search Tool
C6/36	Singh's *Aedes albopictus* cell line
cDNA	Complementary deoxyribonucleic acid
cm	Centimeter
CMV	Cucumber mosaic virus
CNS	Central nervous system
CPE	Cytopathic effect
CsCl	Caesium chloride
Ct	Cycle time
Da	Dalton
DIG	Digoxigenin
DNA	Deoxyribonucleic acid
dsBRD	Double stranded ribonucleic acid binding domain
dsRNA	Double stranded ribonucleic acid
ELISA	Enzyme Linked Immunosorbent Assay
FAO	Food and agriculture organisation
FBS	Foetal bovine serum
FHV	Flock house virus
g	Gravity
GAV	Gill associated virus
H&E	Mayer's haematoxylin and eosin
ha	Hectare
HPV	Hepatopancreatic parvovirus
IHHNV	Infectious hypodermal and haematopoietic necrosis virus
IPTG	Isopropyl-β-D- thiogalactopyranoside (C9H18O5S)
Kb	Kilobase

LAMP	Loop-mediated isothermal amplification
LB	Luria bertani
LSD	Least significant difference
mg	Milligram
ml	Millilitre
mM	Millimole
M	Molar (unit of concentration)
MEGA	Molecular evolution genetic analysis
$MgCl_2$	Magnesium chloride
miRNA	Micro ribonucleic acid
MMV	*Macrobrachium* muscle virus
mRNA	Messenger ribonucleic acid
MrNV	*Macrobrachium rosenbergii* nodavirus
ng	Nanogram
NCBI	National center for biotechnology information
nM	Nanomole
NMR	Nuclear magnetic resonance
NSW	New South Wales
O.D.	Optical density
ORF	Open reading frame
PAZ	Piwi-argonaute-zwille
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PL	Postlarvae
*Pmerg*DNV	*Penaeus merguiensis* densovirus
PSFC	Port Stephens Fisheries Centre
qPCR	Quantitative polymerase chain reaction
RdRp	RNA dependent RNA polymerase
RISC	RNA-inducing silencing complex
RPMI	Roswell Park Memorial Institute - 1640
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
RT	Reverse transcriptase
RT-PCR	Reverse transcriptase polymerase chain reaction

s	Sec
S-ELISA	Sandwich-Enzyme Linked Immunosorbent Assay
SD	Standard deviation
shRNA	Short hairpin ribonucleic acid
siRNA	Short interfering ribonucleic acid
SJNNV	Striped jack nervous necrosis virus
ssDNA	Single stranded deoxyribonucleic acid
SSN-1	Snakehead fish cell line
ssRNA	Single stranded ribonucleic acid
SPSS	Statistical package for the social sciences
TAS-ELISA	Triple antibody Enzyme Linked Immunosorbent Assay
Taq	*Thermophilus aquaticus*
V/V	Volume per volume
VER	Viral encephalopathy and retinopathy
VNN	Viral nervous necrosis
WSSV	White spot syndrome virus
WMD	White muscle disease
WTD	White tail disease
W/V	Weight per volume
X Gal	5'Bromo-4-chloro-3-indolyl-β-D- thiogalactopyranoside
XSV	Extra small virus
YHV	Yellow head virus
µl	Microliter
µm	Micrometer

LIST OF PUBLICATIONS

1. Owens, L., La Fauce, K., Juntunen, K., Hayakijkosol, O. and Zeng, C. (2009) *Macrobrachium rosenbergii* nodavirus disease (white tail disease) in Australia. *Diseases of Aquatic Organisms* **85**: 175-180

2. Hayakijkosol, O., La Fauce, K. and Owens, L. (2011) Experimental infection of redclaw crayfish (*Cherax quadricarinatus*) with *Macrobrachium rosenbergii* nodavirus, the aetiological agent of white tail disease. *Aquaculture* **319**: 25-29

3. Hayakijkosol, O., La Fauce, K. and Owens, L. (2011) Investigation into the Pathogenicity of Reovirus to Juvenile *Cherax quadricarinatus*. *Aquaculture* **316**: 1-5

4. Hayakijkosol, O. and Owens, L. (2012) B2 or not B2: RNA interference reduces *Macrobrachium rosenbergii* nodavirus replication in redclaw crayfish (*Cherax quadricarinatus*). *Aquaculture* **326-329**: 40-45

5. Hayakijkosol, O. and Owens, L. (2011) Cell culture for the Australian isolate of *Macrobrachium rosenbergii* nodavirus. (Submitted to *Journal of Fish Diseases*)

6. Hayakijkosol, O., Burgess, G., La Fauce, K. and Owens, L. (2012) The complete sequence of the Australia isolate of *Macrobrachium rosenbergii* nodavirus which causes white tail disease. (Submitted to *Journal of Fish Diseases*)

CHAPTER 1

GENERAL INTRODUCTION

The world aquaculture production is increasing every year. It grew from 133.8 million tonnes in 2004 to 145.1 million tonnes in 2009 (FAO, 2010). At the same time, the demand of aquaculture products increased due to the increasing human consumption particularly in China. In terms of trade, crustacea continues to have a high value in international traded aquaculture products. Therefore, crustacean species such as penaeids and *Macrobrachium* have become important economical aquaculture products worldwide.

The world percentage of aquaculture production from freshwater was 59.9 percent by quantity and 56 percent by value (FAO, 2010). On the other hand, 32.3 percent of world aquaculture production used marine waters and was valued at 30.7 percent. Therefore, aquaculture production from brackish water contributed only 7.7 percent by quantity but 13.3 percent by value due to high-valued crustacea and finfish production. World crustacean production in 2008 was distributed among brackish water (2.4 million tonnes, 47.7 percent), freshwater (1.9 million tonnes, 38.2 percent) and marine water (0.7 million tonnes, 14.1 percent). The largest quantity of crustacean production from freshwater was from *Penaeus vannamei* (0.5 million tonnes) produced in China.

Many freshwater prawn species are currently being cultured worldwide. The farming of a major commercial species is *Macrobrachium rosenbergii* or the giant freshwater prawn which is mostly developed in Southern and South-Eastern Asia and to a lesser extent in Northern South American and West Indies (New, 2002; New, 2005; Bonami and Sri Widada, 2011). *M. rosenbergii* is the most favoured species for faming purposes. The culture of *M. rosenbergii* began in the early 1960s by Shao Wen Ling who is a Food and Agriculture Organisation (FAO) expert (Daisy, 2007). During his time at the Anuenue Fisheries Research Center in Honolulu (Hawaii, USA), Shao Wen Ling and his team used his knowlage of *M. rosenbergii* to successfully commercialise the culture of *M. rosenbergii* in 1965 (New, 2000). In the last decade, *M. rosenbergii* production rose to 24.5 percent in value and 35 percent in

quantity and became a major contributor to global aquaculture in term of value and quality (Daisy, 2007).

The Australian prawn production rose by 12 percent ($34.1 million) in 2009-2010 following a 12 percent increase (2848 tonnes) by quantity (ABARE, 2010). However, in Australia the giant freshwater prawns (*M. rosenbergii*) have ultimately failed due to disease problems. Microsporidian infection in *M. rosenbergii* was the first recorded failure of a farm in northern Queensland (Bergin, 1986). Later, *Enterobacter aerogenes* and *Vibrio alginolyticus* affected commercial farms in Western Australia and northern Queensland (Owens and Evans, 1989). Also, white tail disease (WTD) is a new viral disease affecting the giant freshwater prawn (*M. rosenbergii*) (Owens *et al*., 2009). The first report of WTD in Australia was by Owens *et al*. in 2009.

The causative agent of WTD has been identified as *Macrobrachium rosenbergii* nodavirus (MrNV). MrNV is a small, icosahedral, non-enveloped virion, 26 to 27 nm in diameter with two positive sense RNA segments (Owens *et al*., 2009). The clinical sign of WTD which develops in postlarvae is a whitish muscle, particularly in the abdomen. Mortalities may reach 100 percent in some hatcheries (Sudhakaran *et al*., 2008). WTD was first detected on the islands of Guadeloupe and Martinique (French West Indies) in 1997 (Arcier *et al*., 1999) and then in Taiwan, China, India and Thailand. This disease has caused economic losses in hatcheries and farms with mortalities often reaching 100 percent within 2 or 3 days (Arcier *et al*., 1999; Sudhakaran *et al*., 2007a; Sudhakaran *et al*., 2008). Clinical signs are not easy to observe, particularly in the earlier stages of infection (Yoganandhan *et al*., 2005).

In France, India, Taiwan and Thailand, the characteristics and genome of MrNV and detection techniques have been studied using Enzyme Linked Immunosorbent Assay (ELISA) and viral genome detection methods such as dot blot hybridization, *in situ* hybridization and reverse transcription polymerase chain reaction (RT-PCR) including one step multiplex to detect MrNV in *M. rosenbergii* (Sudhakaran *et al*., 2007a; Sudhakaran *et al*., 2008). To understand the characteristics of the Australian isolate of MrNV, sequencing of the Australian MrNV, cell culture (C6/36 cell line), an experimental animal model (*Cherax quadricarinatus*) and RNA interference

(RNAi) were developed in this thesis in order to use this information to control MrNV outbreaks in the future.

A few complete sequences of MrNV have been published in GenBank and can be used to develop diagnostic techniques, prevention and treatment of MrNV infection. However, the Australian isolate of MrNV has not been fully sequenced and analysed compared to other isolates. In order to analyse the nucleotide sequence similarities and amino acid changes of MrNV, the complete sequences of the Australian isolate of MrNV were analysed in this study.

As *M. rosenbergii* is hard to obtain in Australia, as it has a requirement for marine water to spawn and is highly cannibalistic, an easier experimental model was sort. *C. quadricarinatus* is a robust freshwater crustacean that naturally coexists with *M. rosenbergii* and it is an easy to manipulate experimental crustacea. Therefore, experiments were set up to see if *C. quadricarinatus* could be used as a substitute for *M. rosenbergii* in order to establish a working animal model. Moreover, cell culture was also used to determine the susceptibility of C6/36 cell line to the Australian isolate of MrNV in order to use less animals in the future studies.

As there are no antiviral medicines or vaccination available for viral diseases, RNAi has been developed and it may be possible to this as protection against viral diseases in crustacea. RNAi pathway is an ancient mechanism to protect the host against viral genomes and rogue genetic elements in the cell life cycle (Li *et al*., 2002). Double strand RNA (dsRNA) is degraded to small pieces by exonucleases. However, protein B2 of nodaviruses is produced to inhibit the degradation of dsRNA in cells. Therefore, RNAi against protein B2 gene was used in a sequence-specific manner against the viruses' protective mechanism. Nevertheless, no studies of specific RNAi against protein B2 production of any nodavirus have been reported.

In order to understand the infectivity of WTD and similarities of the Australian strain of MrNV, full sequences of the Australian MrNV were analysed and compared to other isolates. Also this study aims to determine the susceptibility of C6/36 cell line and the Australian redclaw crayfish (*C. quadricarinatus*) to the Australian isolate of MrNV from the giant freshwater prawn (*M. rosenbergii*). In addition, specific RNAi

targeting protein B2 gene was used against protein B2 in order to control MrNV infection in experimental animals. When complete sequences of the Australian MrNV, an appropriated cell culture, experimental animal model and specific RNAi against protein B2 for the Australian isolate of MrNV were completed, the information from this study facilitates the development of effective diagnostic tools, monitoring and control of the Australian isolate of MrNV outbreak in the future. Also, these methodologies can be developed and applied to control other nodavirus such as fish nodaviruses.

CHAPTER 2

REVIEW OF LITERATURE

2.1. Introduction

2.1.1. *Macrobrachium rosenbergii*

Many freshwater prawn species are currently being cultured with major commercial farming, including *Macrobrachium rosenbergii*, which has been exported and imported into many tropical and subtropical countries. The giant freshwater prawn (*M. rosenbergii*) is the largest known palaemonid in the world (Daisy, 2007) and it was one of the first species to become scientifically known. *Palaemon carcinus*, *P. dacqueti* and *P. rosenbergii* have been previous names for *M. rosenbergii* and it was not until 1959 that *M. rosenbergii* became universally accepted (New, 2002). The modern aquaculture of *M. rosenbergii* has become a commercial culture since 1965 (New, 2000). *M. rosenbergii* is an economically important crustacean farmed on a large scale in many countries. Its culture is mostly developed in southern and south-eastern Asian countries including northern South American and West Indies in order to help the substantial losses from the epidemic white spot disease in marine prawn industries (Bonami and Sir Widada, 2011).

M. rosenbergii is distributed throughout the tropical and subtropical zones of the world (south and southeast Asia, northern Oceania and Western Pacific islands) (New, 2002). They are found in inland freshwater areas such as lakes, rivers, swamps, canals and ponds and require brackish water for the initial stage of their life cycle. Some taxonomists recognize a western sub-species (*Macrobrachium rosenbergii dacqueti*) (Sunier, 1925) which is found in the water of the east coast of India, Bay of Bengal, Gulf of Thailand, Malaysia and Indonesia) (New, 2002). Also, an eastern sub-species (*Macrobrachium rosenbergii rosenbergii*) (De Man, 1879) is found in Philippines, Indonesia, Papua New Guinea and northern Australia (New, 2002).

The *M. rosenbergii* industry has suffered important economic losses from two viral diseases that were reviewed by Bonami and Sir Widada in 2011. The first viral infection in *M. rosenbergii*, affecting animal digestive system, is a parvovirus which has been observed for more than 18 years (Anderson *et al.*, 1990). The second viral disease is a new viral disease called white tail disease (WTD) which was reported during larvae mortalities (Arcier *et al*., 1999). WTD was named due to the clinical signs of the disease in postlarvae which produced whitish muscles, particularly in the abdomen. In this review, limited data of the parvovirus is available; therefore this review will be focused on WTD.

Not only has the epidemiology of disease outbreaks in crustacea been based on different diseases or different species, but also on genetic divergence (Vago, 1966; De Bruyn *et al*., 2004). Huxley's line separates extensive genetic divergence between eastern and western forms of the giant freshwater prawn (*M. rosenbergii*) (Figure 2.1). The two forms of the giant freshwater prawns were considered to be monophyletic by De Man (1879). However, a recent study using RT-PCR demonstrated that the level of genetic divergence between both sites suggested different species (De Bruyn *et al*., 2004).

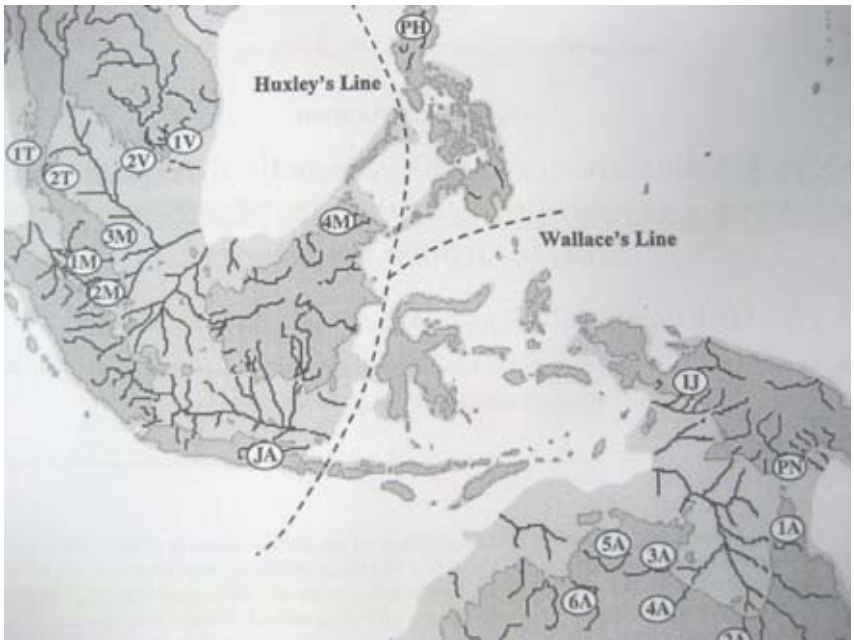

Figure 2.1: Location samples of *Macrobrachium rosenbergii* using Huxley's line to describe genetic divergence between eastern and western forms (De Bruyn *et al*., 2004)

2.1.2. *Cherax quadricarinatus*

Not only is the giant freshwater prawn (*M. rosenbergii*) an economically important crustacean but also the redclaw crayfish (*C. quadricarinatus*) are cultured on a large scale worldwide in South Africa, China, Taiwan, America and Australia (Tan and Owens, 2000 ; La Fauce and Owens, 2007). *C. quadricarinatus* has a natural range along Northern Australia (Northern Territory and northern Queensland), Papua New Guinea, the Gulf of Carpentaria and the Timor Sea (Merrick and Lambert, 1991; Macaranas *et al*., 1995). The farming of *C. quadricarinatus* is an increasingly important species in the aquaculture industry because of their economic significance. Redclaw aquaculture in Australia is poised for significant expansion because of suitable land and water in northern Australia (Jones, 1998). These conditions could support aquaculture production of several thousand tonnes.

However, serious disease-causing organisms including bacteria, protozoans and viruses have been identified in *C. quadricarinatus*. Many viral diseases such as bacilliform viruses, parvoviruses and reoviruses have been detected in *C. quadricarinatus* (Anderson and Prior, 1992; Groff *et al*., 1993; Owens and McElnea, 2000; Edgerton *et al*., 2000; Bowater *et al.*, 2002). These diseases caused the reduction of production and profit in *C. quadricarinatus* industries (Sahul Hameed *et al*., 2004). However, no evidence of WTD in wild *C. quadricarinatus* has been reported.

This review of the literature will summarise our current understanding of WTD, diagnostic techniques for WTD and antiviral immune response to WTD. The literature review will introduce RNA interference (RNAi) against the B2 protein of WTD and focus on the mechanism and strategies of RNAi.

2.2. Nodavirus

2.2.1. History of nodavirus

There are many different varieties in the family *Nodaviridae.* Nodavirus affects newborn and juvenile animals such as fish, insects and crustaceans (Sahul Hameed *et al*., 2004). The clinical signs in fish are disoriented swimming, swimming in spirals, tail chasing or floating belly up. This disease is a massive problem for aquaculture industries that can face up to 100% mortality. Nodavirus has been detected in fish brain, eyes and spinal cord and in muscles of crustacea. It can be seriously dangerous in juvenile animals but has not yet been found in older animals; these animals may still be infected but display no signs of the disease. Therefore, older animals can be carriers of this disease and infect other animals.

A new Nodaviridae virus-borne disease affecting the giant freshwater prawn (*M. rosenbergii*) has been called white tail disease (WTD) (Sudhakaran *et al*., 2007b). WTD was first detected on the island of Guadeloupe and Martinique (French West Indies) in 1997 (Arcier *et al*., 1999) and then in Taiwan, China, India and Thailand. This disease has caused economic losses in hatcheries and farms where mortalities often reach 100% within 2 or 3 days (Arcier *et al*., 1999; Sudhakaran *et al*., 2007a; Sudhakaran *et al*., 2008). The whitish colouration of the tail and affected muscles in the cephalothoracic and abdominal parts are clinical signs of WTD. However, those clinical signs were not easy to establish, particularly in the earlier stages of infection (Yoganandhan *et al*., 2005).

2.2.2. Taxonomy of the *Nodaviridae* family

The family of *Nodaviridae* is small, nonenveloped, icosahedral viruses with diameters between 25 and 30 nm, and bipartite, comprising two positive-sense single strand polarity RNA genomes (Figure 2.2) (Ball, 1997; Totland *et al*., 1999). Virions are roughly spherical in shape and electron microscopy reveals no distinct surface structure (Murphy *et al*., 1983). The structure of the virus describes the relationship between picorna-like and flavi-like groups, and may represent a link between animal and plant positive sense RNA virus (Maramorosch and McIntosh, 1994).

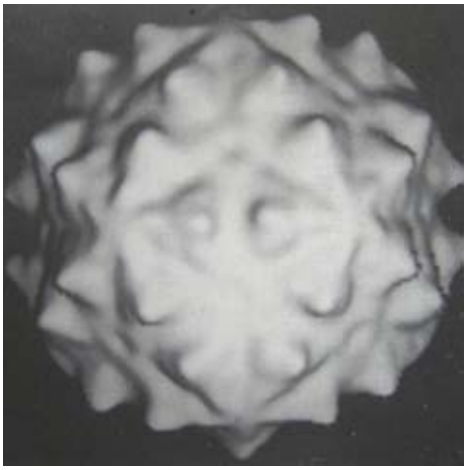

Figure 2.2: Image reconstruction of nodavirus (Murphy *et al*., 1983)

Nodaviruses are divided into two genera provisionally named alphanodaviruses and betanodaviruses. The alphanodaviruses infect insects and invertebrates and the betanodaviruses have been isolated from many species of fish (Ball, 1997). Their genomes consist of two to three segments of messenger RNA (RNA1, RNA2 and RNA3) in the same virion (Skliris *et al*., 2001). RNA1 encodes a non-structural protein of approximately 3-3.2 kb, while RNA2 encodes 1.3-1.4 kb coat protein precursor (Johnson *et al*., 2004). RNA1 and RNA2 are used in the viral replication. Also, RNA3 is a sub-genomic of RNA1 and encodes 0.4 kb. The 5' end of the genome has a methylated nucleotide cap on each segment and no poly (A) tract on the 3' terminus. The molecular mass of virions is 9 x 10^6 Da (NCBI). Virions have a buoyant density in CsCl of 1.3 to 1.37 g cm^{-3}. The sedimentation coefficient of the fastest or only component is 134 to 140 S_{20w}.

Viral replication occurs in the cytoplasm where protein A, protein B and three RNAs (RNA1, RNA2 and RNA3) can be detected (Maramorosch and McIntosh, 1994). RNA1 consists of two open reading frames (ORF) to encode the small proteins (protein A and B) (Figure 2.3). RNA2 has one ORF and encodes the coat protein precursor α which is cleaved into the coat protein β and γ after the precursor assembles into provirions. Protein β and γ are viral capsid proteins presented on the icosahedral surface lattice. However, messenger RNA3 is a subgenomic RNA product derived from the 3’ end of RNA1, and RNA3 is a messenger RNA for

protein B. Moreover, RNA1 and RNA2 are capable of prolonged autonomous replication accompanied by synthesis of RNA3.

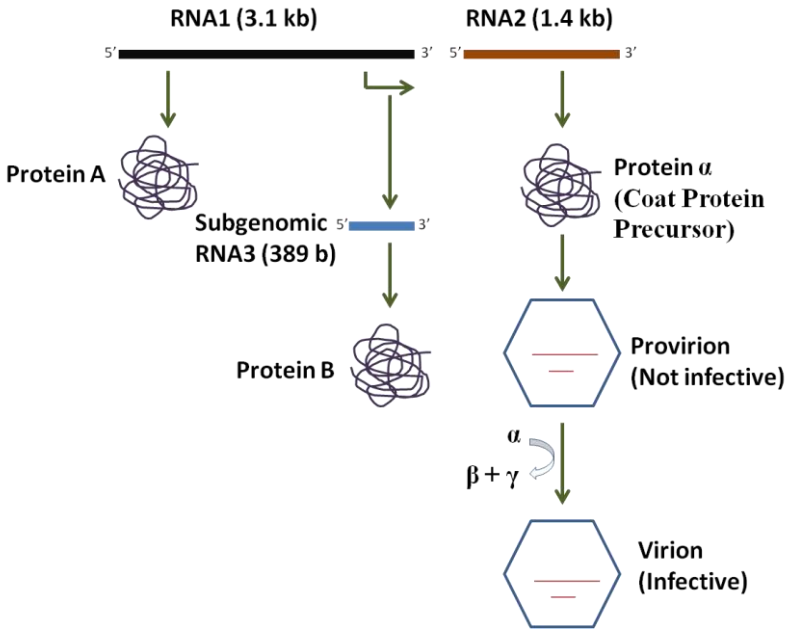

Figure 2.3: Genomic organization and strategy of replication of flock house nodavirus (modified Murphy *et al*., 1983)

RNA1 and RNA2 extracted from infected cells directs the synthesis of protein A, B and α in a cell-free protein synthesizing system (Friesen and Rueckert, 1982). The messenger RNA for protein A and protein α cosedimented with RNA1 (22S) and RNA2 (15S) when polysomal RNA was fractionated on a sucrose density gradient. RNA3 (9S) synthesized the messenger RNA for protein B and was not found in purified virions. Intracellular synthesis of RNA3 was not affected by the drug actinomycin D at concentrations which blocked the synthesis of host cell RNA. Therefore, RNA3 is a specific subgenomic viral RNA to encode protein B.

The molecular weight of proteins of nodaviruses has been reported by Friesen and Rueckert (1982) who found that RNA1 translated and directed the synthesis of a 104,000 Dalton polypeptide (protein A) in a cell-free protein synthesizing system, while RNA2 directed the synthesis of coat protein α (47,000 Dalton polypeptide). Protein α is a cleavage precursor of virion coat protein β and γ (43,000 and

5,000 Dalton polypeptide, respectively). Protein B has a molecular weight of about 10,000 Dalton and protein B has been detected in infected cells.

2.2.3. Disease outbreak and epidemiology of nodavirus

The outbreaks of nodavirus in invertebrates and fish have been found in the past 50 years. The first report of viral diseases in invertebrates such as insects (*Macropipus depurator*) was recorded by Vago (1963). This disease showed a slow development of paralysis and a slight darkening of the eyes in infected animals. Nodaviruses have also been found in insects but also they have been found in crustacea. Therefore, fish, insects and crustacean species are also susceptible to a nodavirus.

2.2.3.1. Nodavirus in fish

Fish nodaviruses have been reported from all parts of the world. Thirty two species of marine fish have been affected by nodavirus (Hegde *et al.*, 2003). Fish nodaviruses are neuropathogenic and the disease has been called viral nervous necrosis (VNN), fish encephalitis or viral encephalopathy and retinopathy (VER). The lesions in the acute stage are the degeneration and vacuolation of cells in the central nervous system (CNS) and retina of infected fish (Grotmol *et al*, 1997). Infection was observed in neurons, astrocytes, oligodendrocytes, microglia, macrophages, lymphocytes, vascular endothelium, endocardial endothelium, myocardial myocytes and the mesothelium of epicardium.

Nodaviruses in fish are the causative pathogens of viral necrosis and encephalitis that induce high mortalities in hatchery and juvenile fish in Asia, Europe, Singapore and Australia (Nishizawa *et al*., 1997; Lim *et al*., 1997; Delsert, 1997). In 1995, acute high mortality occurred in two large commercial hatcheries and juvenile rearing facilities for Atlantic halibut in Norway (Grotmal *et al*., 1997). A vacuolative encephalopathy and retinopathy were found in the brain, neurons and retina. Isometric and non-enveloped viral particles with capsid diameters of 25 nm were detected by transmission electron microscopy. The viral particles were fish nodavirus.

In Australia, nodavirus caused high mortalities in ornamental marine fish and fish farms including barramundi industries. Nodavirus outbreaks have occurred in different hatcheries in New South Wales (NSW) (NSW Department of Primary Industries, 2005). The first detected incidence of nodavirus occurred in Australian bass at the Port Stephens Fisheries Centre (PSFC) Marine Fish Hatchery in 2004. This was the first time nodavirus was found in Australian bass species and caused losses of Australian bass fingerlings. Therefore, monitoring and safeguarding the aquatic environment from the potential transmission of nodavirus was needed to control the disease outbreak using viral isolation and PCR techniques (NSW Department of Primary Industries, 2005).

Viral encephalopathy and retinopathy virus (VER) was previously called barramundi picorna-like virus and striped jack nervous necrosis virus (Department of Agriculture, Fisheries and Forestry; Fisheries Research and Development Corporation, 2004). This virus caused mortalities in many susceptible fish species such as Atlantic halibut, barramundi and cobia. Virulence of VER and mortality depends on the age of fish; younger fish were more susceptible to VER. The incubation time of VER in barramundi was 4 days with onset about 9 to 28 days after hatching. VER was transmitted from parent to offspring through eggs and sperm (vertical transmission), and it may also spread through the water column to the gills, mouth and skin including virus-contaminated food (horizontal transmission). However, no aerosol transmissions appeared and there are no data relating to transmission on fomites (Munday *et al*., 2002).

Nodaviruses cause abnormal swimming behaviour and nervous necrosis including lack of appetite, changes in pigmentation and mortality rates up to 100% (Johansen *et al*., 2002). Disease signs observed in the tank and pond level were erratic: uncoordinated darting, corkscrew swimming, loss of equilibrium, hyperactivity, and sporadic protrusion of head from water and mortality of larvae (Department of Agriculture, Fisheries and Forestry; Fisheries Research and Development Corporation, 2004). Clinical signs of disease in infected animals are overinflated swim bladder, blindness resulting from lesions in eyes, abrasions, emaciation, lethargy and changes of skin colour whereby barramundi became lighter, but the grouper became darker (Figure 2.4). However, information on the existence of

different phenotypes among fish nodavirus strains such as host specificity, temperature optima and virulence of fish nodavirus is essential in order to obtain a better understanding of the epidemiology of fish nodavirus (Totland *et al*., 1999).

Figure 2.4: Viral encephalopathy and retinopathy virus (VER) in seven - banded grouper (*Epinephelus septemfasciatus*). Infected fish (dark fish) compared to normal fish (light fish) (Department of Agriculture, Fisheries and Forestry; Fisheries Research and Development Corporation, 2004).

2.2.3.2. Nodavirus in insects

Different nodaviruses such as Nodamura virus, black beetle virus (BBV), flock house virus (FHV), gypsy moth virus and others have been found in Diptera, Coleoptera and Lepidoptera (Maramorosch and McIntosh, 1994). These viruses were isolated from plaques on *Drosophila* cell monolayers. In addition, nodamura virus was identified from *Culex triaeniorhynchus* mosquitoes. This virus caused paralysis and was transmitted by *Aedes aegypti* which suck blood from mice. The viruses multiplied and caused death in adult *Aedes albopictus* and *Toxorhynchites amboinensis* mosquitoes but did not affect *Culex triaeniorhynchus* mosquitoes. This virus also killed adult honey bees and wax moth larvae. Nodamura viruses replicated in *Aedes albopictus* and *Aedes aegypti* cell cultures. Not only has nodavirus been found in insects, but also in plants. FHV has been isolated from

whole plants such as cowpea and tobacco. *Drosophila* cells can be used to grow and detect this virus. Moreover, nodamura virus, BBV and Gypsy moth virus including *Macrobrachium rosenbergii* nodavirus (MrNV) and extra small virus (XSV) have been isolated from four different species of aquatic insects: *Belostoma* sp., *Aesohna* sp., *Cybister* sp. and *Notonecta* sp. (Sudhakaran *et al*., 2008).

2.2.3.3. Nodavirus in crustacea

China is the most important producer of the giant freshwater prawn *M. rosenbergii* with approximately 33,000 ha of culture and produced over 100,000 metric tonnes in 2001 (Qian *et al*., 2003). MrNV and XSV have spread throughout the main prawn areas such as Guangdong provinces, Guangxi, Zhejiang, Shanghai and Jiangsu. However, the giant freshwater prawn industries have suffered major economic losses because of disease outbreaks.

WTD has caused high mortalities and huge economic losses (Wang *et al*., 2008). White tail disease presented similar gross lesions to muscle necrosis which was reported in *M. rosenbergii* in Taiwan (Cheng and Chen, 1998), French West Indies (Arcier *et al*., 1999) and Thailand (Nash *et al*., 1987). Moreover, the clinical sign is not easy to identify, particularly at the earlier stages of infection. The studies of the pathogenicity and detection techniques MrNV and XSV in giant freshwater prawns have been reported by Sahul Hameed *et al.* (2004).

Live feed such as the brine shrimp, Artemia, is an important nutrient in the dietary requirement of cultivated fish and crustaceans during the larval stages (Sudhakaran *et al*., 2006). Artemia is nutritious and easily handled. However, Artemia are possible vectors for bacteria, parasites and viruses such as MrNV and XSV, either as reservoirs or mechanical carriers. Pathogenicities of MrNV and XSV were presented for different developmental stages of Artemia and there is a need to investigate the possibility that Artemia might transmit MrNV and XSV to the postlarvae of freshwater prawns.

2.2.4. *Macrobrachium rosenbergii* nodavirus (MrNV)

2.2.4.1. History

In 1994, mortalities 5% to 90% of *M. rosenbergii* were reported in Pointe Noire in Guadeloupe (French West Indies) (Sri Widada and Bonami, 2011). White post-larvae were found near the surface of the rearing tank. The presence of clinical signs was followed by sudden mortalities starting 3 days after led to subsequent economic losses. MrNV and XSV have been isolated and found to be associated with white tail disease (WTD) or white muscle disease (WMD) (Sahul Hameed *et al.*, 2004).

MrNV and XSV have been found in both brackish water and freshwater (Sahul Hameed *et al*., 2005). Both viruses have been detected in larvae, postlarvae and early juvenile stages of giant freshwater prawns and Malaysian prawns whereas the adult life stage has not been affected but may act as a carrier. MrNV and XSV outbreaks have been reported in many countries such as the French West Indies, China, Taiwan, Thailand and India. Moreover, clinical signs and mortality patterns are similar in different countries and it may be that movement of prawn populations is a cause of the worldwide distribution of the white tail disease.

2.2.4.2. Host

M. rosenbergii or the freshwater giant prawns was thought to be the main host of WTD. In 2006, Sudhakaran *et al*. (2006) have used three other species of marine prawns (*Penaeus indicus*, *P. japonicas* and *P. monodon*) to see the susceptibility of these species to MrNV that was tested by oral route and intramuscular injection. The results determined those three marine species were not susceptible to MrNV and no clinical sign and mortality were observed. However, MrNV was detected using RT-PCR in penaeid tissues from oral route and injection. This diagnostic technique suggested that *Penaeus* spp. can act as reservoir for MrNV. MrNV is still maintained within the prawns' tissues.

2.2.4.3. Clinical signs

Clinical signs of nodavirus in crustacea have been reported in the giant freshwater prawn (*M. rosenbergii*) (Arcier *et al*., 1999). The abdominal part of prawns was particularly milky and opaque (Figure 2.5). The whitish muscles of infected prawns were visible against a dark background and included abnormal molting in the tanks. The discoloration presented 2 to 3 days after infection, and these signs continued beyond 15 days. The prevalence of opaque and milky prawns often increased sharply to be as much as 90% (Arcier *et al*., 1999). The discoloration appears to start at the tail and progress towards the head. Multifocal areas of necrotic tissues were found in the striated muscles. A few postlarvae presenting those clinical signs can survive and grow normally. However, clinical signs followed by death are usual, with a variable mortality rate up to 95% (Sahul Hameed *et al.*, 2004). Mortalities reached a maximum at around the fifth day after the appearance of the first gross sign. This is significantly damaging to hatcheries and pond nurseries, causing economic loss and affecting the livelihoods of primary producers.

Figure 2.5: *Macrobrachium rosenbergii* juvenile showing milky tail of white tail disease (Owens *et al*., 2009)

2.2.4.4. Histopathology

The affected tissues were the striated muscles of the abdomen and cephalothorax including intratubular connective tissue of the hepatopancreas (Arcier *et al*., 1999). Multifocal areas of necrotic muscles stained more eosinophilic and lost their striated

appearance (Figure 2.6). Oedema in necrotic muscle fibers was also present. Fibrosis and focal to multifocal areas of haemocytic infiltration was seen within infected muscles. Histopathological changes were also characterized by pale to darkly basophilic reticulated cytoplasmic inclusions in the connective tissue cells. Cytoplasmic viral inclusions of approximately 1 to 40 µm in diameter were seen in affected muscle cells (Arcier *et al*., 1999). However, no viral inclusions were observed in hepatopancreatic tubules and mucosal epithelial cells of the gut.

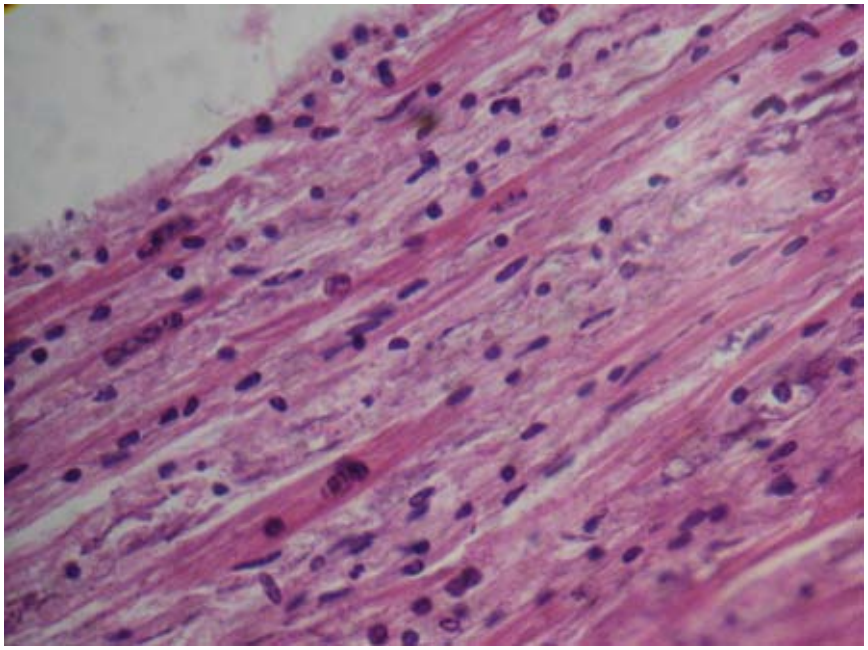

Figure 2.6: An area of necrotic muscles stained more eosinophilic and loss the banded line of muscle (Arcier *et al*., 1999).

2.2.4.5. Transmission

MrNV mainly affects juvenile *M. rosenbergii* and causes high mortalities in young prawns. Viral replication occurs in the cytoplasm of the connective tissue cells of the tail. Viruses may transmit by vertical and horizontal transmission; that is viruses can transfer from one animal to other animals by feed or from parents (Sahul Hameed *et al.*, 2004). The main route of MrNV transmission was the vertical route that was demonstrated in the experimental infection of brooders with MrNV (Sudhakaran *et al*., 2007a). Also, the positive results using RT-PCR were detected MrNV infection from ovarian tissues and fertilized eggs. Moreover, 100% mortality was

reported in pre-larvae from infected hatched eggs. However, spreading of the disease may be complex. Therefore, some of surviving larvae can grow to adults and act as carriers of MrNV.

2.2.4.6. Geographic range

The first report of WTD was in Pointe Noire in Guadeloupe (Arcier *et al*., 1999) and then in Martinique, French West Indies. Also, MrNV has been found in many countries such as Taiwan (Tung *et al*., 1999), China (Qian *et al*., 2003), India (Sahul Hameed *et al.*, 2004), Thailand (Yoganandhan *et al*., 2006) and Australia (Owens *et al*., 2009). The genomic analysis of MrNV isolated from various countries was different and closely related to the MrNV-type called MMV for *Macrobrachium* muscle virus which was observed in Taiwan (Tung *et al*., 1999).

MrNV has been isolated and some complete sequences of MrNV have been analysed to develop effective diagnostic techniques. However, few complete viral genome of MrNV have been published. Only, three complete sequences of MrNV (RNA1) (GenBank Accession number AY222839 (the French West Indies), AY231436 (China) and FJ751226 (Thailand)) have been published on GenBank. Also, only seven nucleotide complete sequence of MrNV (RNA2) (GenBank Accession number AY222840 (the French West Indies), AY231437 (China), FJ751225 (Thailand), EU150126 (Thailand), EU150127 (Thailand), EU150128 (Thailand) and EU150129 (Thailand)) have been found on GenBank. Moreover, no complete sequence of MrNV Australian isolate has been published.

2.2.4.7. Viral components and genomic segments

MrNV have been placed in the family of *Nodaviridae* based on its characteristics and genome sequence (Sahul Hameed *et al.*, 2004). The MrNV is a small icosahedral non-enveloped, 26-27 nm in diameter, exhibiting a density of 1.27-1.28 g mL^{-1} in CsCl. The virus was detected in the cytoplasm of connective tissue cells. The genome of MrNV is formed by two pieces of single stranded RNA (ssRNA) (RNA1 and RNA2) of 2.9 and 1.26 kb, respectively, having a single polypeptide of 43 kDa in the capsid (Romestand and Bonami, 2003). Qian *et al.* (2003) have reported the

occurrence of an additional virus: XSV associated with MrNV in giant freshwater prawns. XSV is a virus-like particle, of an icosahedral shape and 15 nm in diameter with a linear single stranded RNA (Sri Widada and Bonami, 2004). However, the relationship between the two viruses (MrNV and XSV) in WTD of *M. rosenbergii* remains unclear (Zhang *et al*., 2006).

In the previous information, nodaviruses are divided into two genera named the alphanodaviruses which have been isolated from invertebrates and the betanodaviruses have been found in fish (Ball, 1997). However, MrNV has not been classified into a genus and therefore is termed unclassified *Nodaviridae* in NCBI data base.

2.2.4.8. Protein B2

The nodavirus genome is divided into two different positive sense RNAs (RNA1 and RNA2) (Johnson *et al*., 2004). RNA dependent RNA polymerase (RdRp) catalyses the replication of RNA1 and RNA2 segments in the same virion. RNA1 encodes protein A, while RNA2 encodes viral capsid and precursor protein α. Protein B also catalyses the synthesis of RNA3 from RNA1 template. RNA3 encodes protein B1 and B2 in overlapping reading frame. Protein B1 is in the same reading frame as protein A, while protein B2 is in the +1 reading frame relative to protein A.

The structure of protein B2 from the flock house virus was investigated by heteronuclear nuclear magnetic resonance (NMR) methods (Lingel *et al*., 2005). Protein B2 is a homodimer in solution and contains three α helices (α 1, α2 and α3) per monomer which are arranged in a triangular manner as amino acids and C terminus of monomer are in proximity (Figure 2.7). Helices α1 and α2 are connected by an extended linker between Gly24 and Pro31. Helix α2 is very long and spans 30 residues while helix α3 is shorter.

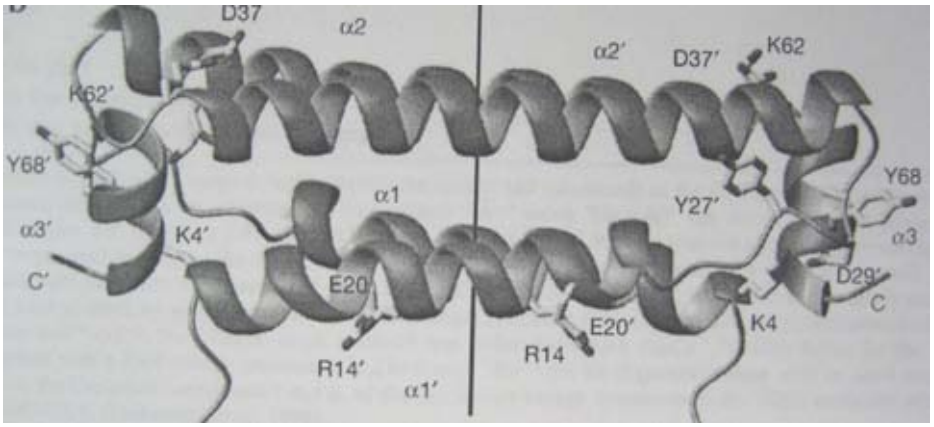

Figure 2.7: Asymmetric dimmer of three helix structure of protein B2 (Lingel *et al*., 2005)

The B2 gene is used to produce protein B2 that inhibits the gene silencing suppression activity of the host cells. Protein B2 combines with dsRNA in the host cell. The antiviral mechanism (RNA interference) is inhibited by protein B2 because RNA interference cannot degrade the dsRNA of viruses in the host cell. Therefore, protein B2 is an important protein to protect the virus in the host cell. B2 gene in animal cells and plant cells both share key features but do not show much sequence similarity (Li *et al*., 2002a).

Protein B2 has been presented by following the protocol of Lingel *et al*., 2005. Protein B2 complementary DNA was amplified by PCR using a random primed S2 cell cDNA as a template and cloned into a derivative vector. After induction by 0.5 mM isopropyl-β-D-thigalactoside, the recombinant protein was expressed in *E. coli* for 14 hr at 25°C. A better understanding of protein B2 can be used to produce the specific RNA interference against MrNV infection in the future.

2.3. Diagnostic Techniques for MrNV

Many diagnostic techniques have been used to detect nodavirus infection including MrNV in *M. rosenbergii* due to the importance of *M. rosenbergii* in aquaculture and world trade. Therefore, easy and rapid techniques are essential for early diagnosis to monitor the animal health status and restrain disease outbreaks. Antigen-based detection methods, genomic-based detection tools, hybridization and *in situ* RT-PCR

have been developed to diagnose WTD infection and they will be reviewed in this literature.

2.3.1. Antigen-based detection methods of MrNV

Antigen-based detection techniques such as Enzyme Linked Immunosorbent Assay (ELISA) were developed to be able to detected many diseases in crustacea. ELISA has been developed as a reliable diagnostic tool allowing viral detection in large numbers of samples (Romestand and Bonami, 2003). The developed protocol of ELISA and the production of specific antibodies is based on analysis of tissue extracts or the whole body of *M. rosenbergii* using a single antibody directed against MrNV. This technique was improved by an avidinperoxidase detection system, and optimized as a simple routine and inexpensive diagnostic method for specific and sensitive detection of MrNV.

These techniques include a sandwich-Enzyme Linked Immunosorbent Assay (S-ELISA) which was used to detect MrNV using polyclonal antibodies produced in Balb/C mice (Romestand and Bonami, 2003). The whole viral particle or free capsid protein was trapped on an antibody-coated plate. The antigen-antibody complex was revealed by secondary antibody to be able to see the positive results. This technique was rapid, inexpensive and presenting high specificity. However, the stocks of antibody produced by hybridoma methodology had limitations for this technique. Moreover, a triple antibody Enzyme Linked Immunosorbent Assay (TAS-ELISA) was developed in China and used to detect viral diseases such as MrNV in postlarvae hatcheries using rabbit polyclonal antibodies to trap MrNV antigen (Qian *et al*., 2006).

2.3.2. Genomic-based detection tools of MrNV

Many detection techniques, based on genomic sequences of MrNV such as hybridization, RT-PCR, quantitative real-time RT-PCR and loop-mediated isothermal amplification (LAMP) have been developed and used to identify MrNV infection.

2.3.2.1. Hybridization

Dot blot hybridization is one of genomic-based diagnostic techniques using DIG-labeled and cloned on MrNV genome as probes (Sri Widada *et al*., 2003). This technique established the animal health status of the prawn and detected MrNV in animal tissues. Ten fold dilution of total MrNV RNA extraction from infected animal was sensitive enough for Dot blot hybridization to detect MrNV. The probe used for hybridization was a DNA fragment derived from RNA of the viruses. The particular probe was labelled with digoxigenin (DIG)-labelled nucleotide by PCR using a PCR DIG labeling kit (Roche, Meylan, France).

In situ hybridization is another technique used to detect MrNV infection but requires more technical skills and expensive equipment (Bonami and Sri Widada, 2011). The tissue slides from RNA-DNA hybridization were examined under light microscopy for cell and displayed a dark to purple staining that indicated the presence of homologous viral RNA. However, it is essential to establish virus localization in animal tissues. *In situ* hybridization can determine that MrNV was found in many animal tissues such as muscle, connective tissue of the abdomen, cephalothorax and appendages. Also, MrNV was found in the cytoplasm of the infected cells. No MrNV was observed in the gills, hepatopancreas and digestive system.

2.3.2.2. RT-PCR

Polymerase chain reaction (PCR) is a diagnostic technique that can produce large amounts of specific DNA fragments in a simple enzymatic reaction (Payment and Trudel, 1993). Beginning with a single molecule of RNA or DNA, over a billion copies can be synthesized in the PCR process. Repeated cycles of denaturation, primers, the enzyme from the bacterium *Thermophilus aquaticus* (Taq) and extension of genetic materials are needed to lead to exponential increases in the target genetic sequences. As a general principle, PCR is carried out in a series of cycles with the DNA first denatured at a high temperature in order to render the specific sequence single stranded DNA. The oligonucleotides hybridize and then synthesize strands of the specific sequence using enzymatic extension. These steps are repeated 20 to 40 cycles until a sufficient amount of target DNA is produced.

The genome of MrNV is composed of ssRNA which requires a step of reverse transcriptase (RT) to convert to complementary deoxyribonucleic acid (cDNA). Different sets of primers were designed and tested specifically for MrNV (RNA1 or RNA2) (Sri Widada *et al*., 2003). The combination of the sets of primers showed the increase of the amplification efficiency. Also, the 10 fold dilutions of viral RNA extracted can be detected and are more sensitive than the dot-blot hybridization. In the routine screening, an RNA content of 0.15 - 1.5 mg of the animal tissue is enough to detect MrNV using RT-PCR technique.

An example for RT-PCR used in the detection of MrNV outbreaks is RT-PCR diagnostic test that was used to identify MrNV genomes using specific primers (Sahul Hameed *et al.*, 2004). The primers used for MrNV in RT-PCR is 5' GCG TTA TAG ATG GCA CAA GG 3' (forward) and 5' AGC TGT GAA ACT TCC ACT GG 3' (reverse) with product size of 425 base pairs (bp). Therefore, whole animals or pieces of different organs (gills, hepatopancreas, heart, muscles, intestine or hemolymph) from infected crustaceans have had RNA extracted and used in RT-PCR technique with specific primers to detect MrNV disease (Weng *et al.*, 2007; Zhang *et al.*, 2004; Hsieh *et al*., 2006).

2.3.2.3. *In situ* RT-PCR

In situ RT-PCR has been developed using RT-PCR technique together with histological method (Bonami and Sri Widada, 2011). Microtome sections of tissue samples were prepared before using RT-PCR technique to detect the virus. DIG-labeled dUTP was used in amplification process and then revealed by anti-DIG antibody in *in situ* hybridization.

2.3.2.4. Quantitative real-time RT-PCR

Real-time PCR has been used for the accurate quantification of viral cDNA copies that measure the infectious level by the increase in fluorescence during each amplification cycle (De la Vaga *et al*., 2004). This technique requires only a small amount of RNA transcribed to cDNA. In 2004 real-time PCR demonstrated that handling stress can lead to rapid increases of gill associated virus (GAV) infection

levels in *P. monodon* (De la Vaga *et al*., 2004). The real-time PCR revealed that GAV loads increased significantly in haemocytes collected. TaqMan real-time PCR has been developed and used to detect hepatopancreatic parvovirus in Australian *P. merguiensis* (La Fauce *et al*., 2007).

TaqMan real time PCR is an alternative technique which measures the number of viral copies in the tissue samples (La Fauce *et al*., 2007). Sequence specific primers and a fluorescent oligonucleotide probe designed within target DNA were needed in the TaqMan real-time PCR process to detect the specific viral sequences. Therefore, real-time PCR is successful in determining the development of viral diseases in crustacea. Also, quantitative real-time can be able to determine the number of viral copies in animal tissue samples (Hernandez-Herrera *et al*., 2007). However, quantitative real-time PCR for MrNV has been not developed in Australia.

2.3.3. Cell culture

Cell culture is the generic term used to include organ culture and cell culture (Freshney, 2005). Organ culture describes some or all of the aggregated histological features of the tissue *in vivo*. Cell culture involves a culture from dispersed cells taken from original tissue, from cell line, from primary culture or from cell strain by enzymatic, mechanical or chemical disaggregation. A three-dimensional structure with tissue density, perfusion and overgrowth of a monolayer in a flask is called histotypic culture which is used to observe cell culture. The cell proliferation and propagation of the cell line are often detected in cell cultures. Cell culture can be derived from dispersed cell suspension into the new containers. A monolayer or cell suspension is dispersed by using enzymes and simple dilution. This constitutes a passage, and the daughter culture can be used to form the beginning of a cell line. However, cell culture is a basic tool for the study of infectious disease, particularly for viruses that replicate in the host cells. Established cell lines have been used widely in the studies of viral diseases in many animal species such as fish and insects.

Recently, the molecular basis of invertebrate species has been explored (Freshney, 2005). The desire to understand diseases and control pests in agriculture and

aquaculture has also encouraged toxicity and virological studies in invertebrates as well as studies of pathogenecity and development of the diseases. A protocol of handling insect cells has developed from the protocol of mammalian cell culture and is now commercially available for insect cell lines (Freshney, 2005).

Insect cell lines have been established from many of the major species of insects (Maramorosch and McIntosh, 1994). Mosquito cell line is one such insect cell line. In general, mosquito cell lines are substrate dependent and grow as a monolayer; mostly diploid (six chromosomes) with distinct shapes, and the chromosomes are large and submetacentric or metacentric. Therefore, if the genetic background of mosquito cell lines is known, they may be suitable for cytogenetics.

Trager (1938) was the first to culture cells from mosquito tissues (*Aedes aegypti*) in 1938 (Maramorosch and McIntosh, 1994). He also used pupal head tissues to inoculate cells with Western equine encephalomyelitis virus into *Culex pipiens* cell lines, and the virus was recovered after 28 days. Therefore, *Culex pipiens* cell line was the first established insect continuous cell line. The established cell lines from mosquitoes have been used widely in the investigation of viral diseases in invertebrate species or other mosquito-borne pathogenic microorganisms.

Large scale culture of Singh's *Aedes albopictus* cell line ATC-15 (subline C6/36) was developed by Igarashi in 1978. The C6/36 mosquito cell line can grow in a suspended state in spinner bottles or flasks. The C6/36 cell line does not firmly attach to the bottom of containers; therefore it is easier to passage by way of pipetting. The C6/36 cells can multiply up to 10^6 cells per ml. A microcarrier was used in the culture system; the C6/36 cells attached to the microcarrier. The microcarrier and cells sank within a few minutes when the magnetic stirrer was stopped. This process is convenient for large scale culturing for the production of viruses in C6/36 cell lines (Maramorosch and McIntosh, 1994). In addition, the C6/36 mosquito cell line is suitable for the study of MrNV and XSV (Sudhakaran *et al*., 2007b). Nevertheless no crustacean cell lines have been established to grow and replicate MrNV and XSV over the past decade.

For the virological studies on MrNV, C6/36 cells were inoculated with 100 µl of diluted virus in cell culture flasks and incubated at 28°C (Sudhakaran *et al*., 2007b). Leibovitz L-15 medium with 5% foetal bovine serum (FBS) grow and maintain the cells and viruses. The infected cells were observed daily under phase contrast microscopy for cytopathological changes and the cell supernatant was collected. For continuation, the old supernatant or infected cells was frozen and thawed three times and filtered through a 0.22 µm membrane, and then used to re-inoculate into new cell lines. A viral pellet was prepared by ultracentrifugation for transmission electron microscopy in order to observe the structure of the viruses. The supernatant that contained viruses was used to extract the viral nucleic acids for PCR technique including RT-PCR (Hernandez-Herrera *et al*., 2007).

2.4. Protection of Crustacea Against Viral Diseases

Invertebrates lack an adaptive immunity but they rely on various innate immune responses to defend against microbial pathogens (Medzhitov and Janeway, 1997). The innate immunity is active against pathogens and environmental antigens. Also, humoral and cellular immunity are the part of the innate immune responses that contribute to the defence mechanism against pathogens. However, innate immune response has been studied against bacteria and fungi (Destoumieux-Garzon *et al*., 2000; Destoumieux-Garzon *et al*., 2001) but knowledge of invertebrates is limited regarding response directed against viral pathogens.

Invertebrates lack the protein-based adaptive immunity to prevent viral infection and also, vaccines cannot be used as a method to control or prevent viral diseases. A better understanding of crustacean immunity will help to develop or design more efficient diagnostic tools and better disease control. However, invertebrates have one mechanism of effective viral defence using RNA interference (Brennan and Anderson, 2004).

2.5. RNA Interference (RNAi)

Gene silencing, quelling and RNA interference (RNAi) are sequence specific RNA degradation mechanisms that are mechanistically related to RNA silencing processes

(Li *et al*., 2002a). The RNA silencing processes induce sequence specific degradation of complementary messenger RNA (mRNA) or by inhibiting translation in a sequence specific manner in the host cells. RNAi has been found in all eukaryotic cells from yeast to mammals (Li *et al*., 2002a). RNAi has been studied in many eukaryotic organisms such as protozoa (Bastin *et al*., 2001), fungi (Raoni and Arndt, 2003), algae (Wu-Scharf *et al*., 2000), nematode (Fire *et al*., 1998), insects (La Fauce and Owens, 2009), fish (Dang *et al*., 2008) and mammals (Caplen *et al*., 2001) including plants (Fagard and Vaucheret, 2000).

RNAi evolved as an early form of innate immunity as the recognition and silencing of potentially harmful nucleic acids of viruses (Sullivan and Ganem, 2005). RNA interference pathway as an antiviral defense system was first observed in plants (Matzke *et al.*, 1989). Many plant RNA viruses encode inhibitors of RNA interference that are required for viral growth in order to inhibit the antiviral defences. Plant viruses such as cucumber mosaic virus (CMV) in tobacco encode inhibitors that target various components of RNA interference systems (Anandalakshmi *et al.*, 1998).

Therefore, if specific RNA interference can be produced, viral infection may be controlled and eradicated. For example, RNAi has been used to protect viral infection and viral replication of prawn viruses such as yellow head virus (YHV) (Tirasophon *et al*., 2005) and white spot syndrome virus (WSSV) (Xu *et al*., 2007). Moreover, MrNV can produce protein B2 that inhibits antiviral mechanisms of the host cells. Specific RNAi against protein B2 may stop the inhibition of the antiviral mechanism and can be used to control the disease in the future.

2.5.1. RNA interference pathway

Six different types of dsRNA have been identified that trigger RNAi; short interfering RNA (siRNA), microRNA (miRNA), short hairpin RNA (shRNA), long double strand RNA (dsRNA), short interference based hairpin RNA and microRNA based hairpin RNA (Zamore *et al*., 2000; Paddison *et al*., 2002; Ambrose, *et al*., 2003). siRNA tends to produce the largest silencing and can be manufactured to a

large scale production. siRNAs are incorporated into RNAi pathway and started from long dsRNA (Zamore *et al*., 2000).

Long dsRNA serves as the initial trigger of RNA silencing which is processed by Dicer RNase into short fragments of nucleotides called short RNA (21 to 25 nucleotides). The 21 nucleotides of short RNAs composed of two 21 nucleotide strands of RNA, with 19 nucleotides of dsRNA and two unpaired nucleotides at the end (Elabashir *et al.*, 2001). Furthermore, the RNAi process can be divided into 2 steps: the initiator (Dicer) and the effector (Slice mechanism) steps (Shanker *et al*., 2005; Lee and Shinko, 2006) (Figure 2.8).

2.5.1.1. Initiator step of RNA interference pathway

Short interference RNA (siRNA) from long dsRNA or microRNA (miRNA) from endogenous hairpin RNA precursor or short hairpin RNA (shRNA) is transcripted by RNase III –like protein (Dicer) (Hannon, 2002). Dicer contains a dsRNA binding domain (dsBRD), two catalytic RNase III domain, a helicase domain and a piwi-argonaute-zwille (PAZ) interaction domain (Bernstein *et al*., 2003). Dicer unwinds and cleaves long dsRNA in to siRNA 21-23 nucleotides. The siRNA are bound into a multiprotein RNA-inducing silencing complex (RISC). RISC contains endonuclease, exonuclease, helicase and homology searching domain (Lee and Shinko, 2006). The siRNA are unwound by helicase from RISC and leave the antisense strand to guide RISC to homologous target mRNA (Dykxhoorn *et al*., 2003).

2.5.1.2. Effector step of RNA interference pathway

The effector step is the last step of the RNAi processes. siRNA are incorporated into dsRNA-induced silencing complex (RISC) to induce the cycle of RNA degradation which specifically represses the expression of target genes or genomic regions. One strand of siRNA is assembled into RISC that cleaves the target mRNA in the host cells. The target mRNA is cleaved at a single site between siRNA and mRNA (Elabashir *et al.*, 2001). Then degradation by exonucleases occurs to divide mRNA

into small pieces. The cleavage occurs only in the homologous region to siRNA. Then, RISC can be reused to repeat the RNAi mechanism.

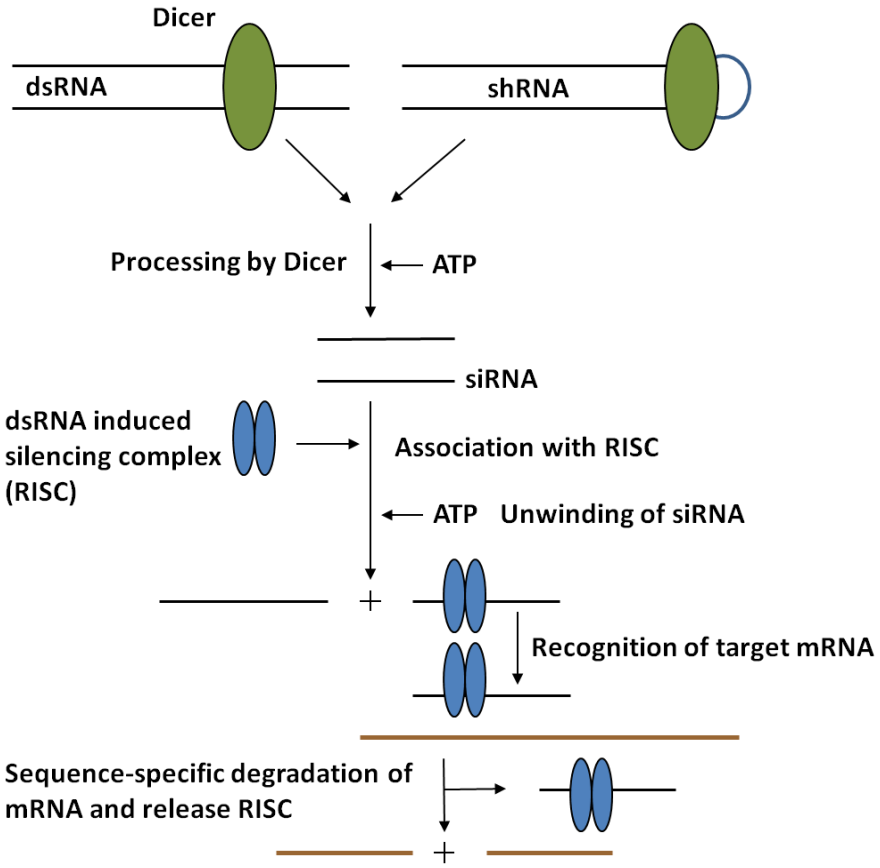

Figure 2.8: Two steps of RNA interference pathway: the initiator the effector steps. Long double-stranded RNA (dsRNA) or small hairpin RNA (shRNA) is processed by Dicer to form a short interfering RNA (siRNA), which associates with dsRNA-induced silencing protein complex (RISC) and mediates target sequence specificity for subsequent mRNA cleavage to divide mRNA into small pieces and release RISC (modified from Rutz and Scheffold, 2004).

2.5.2. Limitation of RNA interference

RNA interference can be an extremely powerful treatment for gene silencing and antiviral medicine. However, RNAi has some limitations such as off-target effects, RNAi suppressor protein and the use of synthesized siRNA. Firstly, RNAi can silence the wrong genes or degrade closely related genes but non-targeted mRNAs that are called off-target effects (Jackson *et al*., 2003). In genome-wide monitoring

gene technology using cell culture, a match of as few as six to seven nucleotides to siRNA has been verified off-target (Jackson *et al*., 2004; Jackson *et al*., 2006). Therefore, specific siRNA-mediated gene silencing is essential for the proper design of RNAi.

Viruses can encode proteins called RNA-interference suppressor proteins that are suppressors of RNA silencing mechanism (Li and Ding, 2001). RNA interference suppressor proteins are required for viral adaption against a host antiviral response. Therefore, RNA-interference suppressor proteins could be essential for viral infection of the host cells. RNA-interference suppressor proteins have been found in many viruses such as plant viruses (Anandalakshmi *et al*., 1998; Brigneti *et al*., 1998), insect viruses (Li *et al*., 2002b) and fish viruses (Ou *et al*., 2007). For example, protein B2 of betanodaviruses from fish viruses has been studied as RNA-interference suppressor protein and used to determine RNAi activity related to antiviral mechanism (Ou *et al*., 2007). Further studies of RNA-interference suppressor proteins may be useful to understand the mechanism of RNAi activities in the host cells.

The use of synthesized short interfering RNA has a number of other factors which affect the efficiency of gene silencing involving the RNAi mechanism. Firstly, the use of siRNA is costly due to different siRNAs targeting the same gene can have different efficacies. Therefore, designed siRNAs may be unstable to unwind from 5' to 3' to be preferentially assembled into the RISC (Khvorova *et al*., 2003). Secondly, not every siRNA is efficient in gene silencing, that is its not perfectly homologous to the mRNA target because of variation of mRNA structure (Dykxhoorn *et al*., 2003). Lastly, the downregulation of gene expression has been reported in cell culture experiment (Holen *et al*., 2002; Duxbury and Whang, 2004) but siRNA only lasts long for 3 to 5 days due to dilution and degradation of siRNAs.

2.6. The Control of Viral Diseases

Effective control of viral diseases needs the application of diagnostic techniques to understand the pathogenicity, biosecurity and surveillance. Without effective farm management, cultured animals may become infected via carriers from the local wild

population. Therefore, detective techniques such as viral isolation, histopathology and serology are useful (Nash *et al.*, 1988). Not only, detection techniques can be used, but also sanitation and control protocols for viral infection can prevent massive economic loss (Sahul Hameed *et al.*, 2004).

Many diagnostic tools such as histology and PCR can detect or monitor viral infection. Pathological studies and serological techniques have classified viral infection in aquatic animals (Rangel *et al*., 1999). In addition, the transmission of white tail disease is through vertical and horizontal transmissions in crustacean species. The understanding of transmission can help control and detect the viral disease including white tail disease in aquaculture industries. RT-PCR technique is now available for commercial use, and broodstock and seed screening are strongly encouraged. The positive results of broodstock or seed testing for white tail disease should lead to discarding of animals with proper sterilization. Therefore, diagnostic techniques will be helpful for disease management in aquaculture industries. Moreover, in my opinion to control disease outbreaks of white tail disease, diagnostic tools and understanding of protein B2 and RNA interference as antiviral mechanism will be needed in the future.

2.7. Conclusion

The worldwide demand for marine and freshwater animal production has increased aquaculture industries, and many disease problems caused economic losses. Nodavirus is one of the viral diseases which affect many animal species. Nodavirus has been found in insects, fish and crustaceans in many countries. The clinical signs of the disease affect animal movement and cause high mortalities. The clinical signs and lesions in some species are not easy to observe or monitor in the earlier stages of the infection. Therefore, diagnostic tools such as PCR, serology and cell cultures are helpful to detect the earlier stages of the disease.

WTD caused by MrNV is the most specific important viral disease of *M. rosenbergii* which has massive impacts in aquaculture establishments (Bonami and Sri Widada, 2011). No specific antiviral medicines or vaccines against MrNV infection are available. Therefore, preventative monitoring and good management protocols are

the best way to restrain and control the disease. In order to develop effective diagnostic tools, many complete sequences of MrNV need to be analysed and be available to be used in genomic data bases. Also, real-time PCR has been used in the detection of fish nodavirus but no real-time PCR has been developed for MrNV.

Invertebrates including crustaceans lack a true adaptive immunity and antibodies against viral infection (Little *et al*., 2005; Yodmuang *et al*., 2006). Also, vaccination cannot be used to prevent viral infection in crustaceans. The uses of chemical and veterinary drugs not only affect aquaculture, but also affect public health due to the residues of chemicals and veterinary drugs (Lupin, 2009). In the last decade, food safety issues associated with aquaculture production have been a concern. Therefore, many researchers have studied other antiviral treatments in order to prevent and control the disease outbreaks (Tirasophon *et al*., 2005; Xu *et al*., 2007; La Fauce and Owens, 2009). RNA interference is one strategy of controlling viral infection.

RNAi may be the answer to antiviral medication for viral crustacean diseases after RNAi has been used against many crustacean viruses (Tirasophon *et al*., 2005; Yodmuang *et al*., 2006; Xu *et al*., 2007). Better understanding of how protein B2 and RNAi function should help to control and eradicate MrNV infection.

CHAPTER 3

GENERAL MATERIALS AND METHODS

The general methods and materials in this chapter involved many techniques that have been used in more than one chapter of the thesis. Methods used only in one chapter will be recorded there.

3.1 Viral Preparation

White Tail Disease (WTD) infected juveniles (*Macrobrachium rosenbergii*), with prominent signs of whitish muscle in the abdomen were prepared at James Cook University, Queensland. The source of the giant freshwater prawn (*M. rosenbergii*) was the Flinders River system near the Gulf of Carpentaria region, Queensland, Australia. These prawns were the source of *Macrobrachium rosenbergii* nodavirus (MrNV). Specimens were classified as MrNV positive by histology and reverse transcriptase polymerase chain reaction (RT-PCR) at James Cook University, Queensland. Infectious material containing MrNV was prepared according to the protocol described by Sri Widada *et al*. (2003). The inoculum was prepared from infected muscle which was homogenized in phosphate buffered saline (PBS) and filtered though a 45 μm filter. Most importantly, the inoculum was considered free from additional bacterial and fungal pathogens.

3.2 Collection and Maintenance of Experimental Animals

Juvenile freshwater redclaw crayfish (*Cherax quadricarinatus*) were used in the infectivity experiment for an experimental animal model of MrNV and redclaw crayfish were used in RNAi study. Redclaw crayfish were obtained from the breeding facility at James Cook University, Queensland, Australia (Animal Ethics Number: A1382). Redclaw crayfish were housed in aquaria 1 m in width, 3 m in length and 60 cm in height. Commercial crustacean and chicken pellets were fed daily. Water exchanges were performed daily to maintain appropriate water quality. Redclaw crayfish were screened for spawner isolated mortality virus, gill associated

virus (GAV) and infectious hypodermal and haematopoietic necrosis virus (IHHNV) by histopathology.

3.3 Infectivity Studies of *C. quadricarinatus*

During infectious studies of an experimental animal model and RNAi, clinical signs and dead redclaw crayfish in experimental aquaria were monitored daily, and any dead animals were removed and recorded. Dead redclaw crayfish were immediately prepared for histology and real time RT-PCR by splitting the cephalothorax longitudinally. The first half of the cephalothorax was split into two parts. The first part was placed in Davidson's fixation for histopathology while the second part was put in 95% ethanol for real-time PCR. Also, the abdomen of redclaw crayfish was split into two parts. The first part was split longitudinally into two parts. The first half was placed in Davidson's fixation for histopathology while the second half was put in 95% ethanol for real-time PCR. The remaining half of the abdomen was stored frozen at -20°C. At the end of the experiment period, all remaining crayfish were sacrificed and processed appropriately for screening by histological examination and real-time RT-PCR.

3.4 Histopathology

C. quadricarinatus samples collected from infectious experiments were prepared for histology and placed in Davidson's fixation for 48 hr. After 48 hr, tissues of the muscles, gills and hepatopancreas of redclaw crayfish were processed to histological slides (two slides each crayfish). Tissues were transferred to 70% ethanol and dehydrated through a series of alcohols to xylene and then embedded in paraffin wax. Sections were cut at 5 μm and stained with Mayer's haematoxylin and eosin (H&E). The sections were screened and analysed under light microscopy (Olympus E C microscope). Photographs were taken using a digital camera (QIMAGING MicroPublisher 5.0 RTV).

3.5 RT-PCR and Real-Time RT-PCR

3.5.1 Nucleic acid extraction and RT-PCR cycle

Samples from *M. rosenbergii* positive samples, infectivity studies and cell culture were used in RT-PCR to confirm MrNV infection according to the processes of Sri Widada *et al.* (2003) which had used reverse transcription and amplification in a single reaction tube. Fifty mg of infected muscle and cells were utilised for RNA extraction using SV total RNA isolation system (Promega, Madison, USA). Samples were homogenised in 175 µl RNA lysis buffer and 350 µl RNA dilution buffer were added to 175 µl of lysate. After centrifugation for 10 min at 12,000*g*, then the clear lysate was transferred to a 1.5 ml microcentrifuge tube. Two hundred µl of 95% ethanol was added to the cleared lysate and transferred to the spin column before being centrifuged at 12,000*g* for 1 min. DNase incubation mix was prepared by combining 40 µl of yellow core buffer, 5 µl of 0.09M $MgCl_2$ and 5 µl of DNase I enzyme per sample. DNase incubation mix was added to spin column and incubated for 15 min at 25°C, and then 200 µl of DNase stop solution were added to the spin column before being centrifuged at 12,000*g* for 1 min. Six hundred µl of RNA wash solution were added and centrifuged at 12,000*g* for 1 min. The column was emptied and 250 µl of RNA wash solution added before being centrifuged at 12,000*g* for 2 min. The spin basket was transferred to a new microcentrifuge tube before adding 100 µl of nuclease free water to the membrane and centrifuged at 12,000*g* for 1 min.

cDNA was amplified using ImProm-II Reverse Transcriptase System (Promega, Australia). Oligo $(dT)_{15}$ primer (0.5 µg/reaction) was added into 5 µl of RNA extraction and then preheated 70°C for 5 min. The reverse transcriptase reaction mix was prepared by combining 5.6 µl of nuclease free water, 4 µl of 5x reaction buffer, 2.4 µl of 5mM $MgCl_2$, 1 µl of dNTP mix, 1 µl of recombinant RNasin ribonuclease inhibitor and 1 µl of reverse transcriptase. Fifteen µl of reverse transcriptase reaction mix were mixed and added to RNA extraction reaction for a final reaction volume of 20 µl per reaction. Then, reactions were incubated at 25°C for 5 min and 42°C for 1 hr followed by 70°C for 15 min. The RT-PCR cycle process consisted of

incubation at 95°C for 4 min, followed by two step cycles of 40 cycles at 95°C for 30s, 55°C for 45s and 72°C for 1 min.

3.5.2 Primer design and probe

Primers used in PCR process were designed from sequence data of the MrNV and XSV obtained through the National Center for Biotechnology Information (NCBI). MrNV has been sequenced and deposited at GenBank under accession numbers AY222839, AY231436 and NC005094 for MrNV (RNA1), and also AY 222840, DQ 521575, EU150126, EU150127, EU150128, EU150129 and NC 005095 for MrNV (RNA2). Extracted RNA from XSV has been sequenced and deposited at GenBank under accession number AY247793. The particular primers for MrNV and XSV were designed using many programs: Vector NTI Explorer (Vector NIT Advance^{TM} 11, Invitrogen, USA), AlignX (Invitrogen, USA), Genedoc (Free Software Foundation, USA), Oligo version 7 (Molecular Biology Insights Inc., USA) and AlleleID version 7.5 (Primire Biosoft International, USA). All sets of primers are presented in Table 3.1. Probe and primers used in quantitative real-time PCR was designed using Oligo version 7 (Molecular Biology Insights Inc., USA). The forward and reverse primers for MrNV probe were 5'-GAC CCA AAA GTA GCG AAG GA-3' at positions 2860 bp to 2879 bp and 3'-GGC CTC TCC CTT TAG TGT T-5' at positions 3040 bp to 3058 bp respectively. The probe sequence was 5'-[6FAM] AAG CAA CCG CCT TCA ATG CC [TAM]-3' at positions 2915 bp to 2934.

Table 3.1: Sets of primers for MrNV (RNA1 and RNA2) sequencing

Number	RNA	Primer Name	Orientation	Sequence	Location	Size (bp)
1	RNA1	380R	Reverse	CTA GCT GCA TCT CGA ACC GCT CCA GAA GTT TTG TGT CCA T	340 - 380	380
2	RNA1	970F	Forward	TAC CGG ACT CGC CAT AGT GT	100 – 120	970
3	RNA1	970R	Reverse	CTA TGC TGG CTA CAA GTT TGG TG	1024 - 1047	970
4	RNA1	727F	Forward	GGC AAC ATA AAG TTT GGA ATT GG	735 – 758	727
5	RNA1	727R	Reverse	TGT TGG AAC CTT ATT ATT GGC	1420 - 1441	727
6	RNA1	615F	Forward	CTA GGC TTA AGT ACC ATG CAG	1098 - 1119	615
7	RNA1	615R	Reverse	TAA TAC TTC ATC TCG AAA GGC AA	1690 - 1713	615
8	RNA1	746F	Forward	AGT CCG CCG ATT AAT TGA AGC	1577 - 1598	746
9	RNA1	746R	Reverse	TGT TCA ACT TTC TCC ACG TT	2303 – 2323	746
10	RNA1	869F	Forward	AAG AGT ATC TGC TTG GTG TCA	1890 – 1911	869
11	RNA1	869R	Reverse	ATG GTT CCT GAT AGT CTA GCG	2717 - 2738	869
12	RNA1	800F	Forward	CTC TTG ATC GTG TCA GTG GA	2425 – 2445	800
13	RNA1	800R	Reverse	CAG GCA TTG CTT ACC ACG TT	3185 - 3205	800
14	RNA1	232F	Forward	AAC ACT AAA GGG AGA AGC CGT A	2998 – 3020	232
15	RNA2	396R	Reverse	TGT GCC ATC TAT AAC GCT CCC AAA ATT GCG ATA GAC CA	358 - 396	396
16	RNA2	954F	Forward	CCA ACT TTA ACC CCA TTG TCG	141 - 162	954
17	RNA2	954R	Reverse	CAC CCT GAT AAT CGG TCA CT	1075 – 1095	954
18	RNA2	535F	Forward	AAC AAC TAT TCC ATT GAT TG	578 – 598	535
19	RNA2	535R	Reverse	AAC AAC ACC CTG ATA ATC	1095 - 1113	535
20	RNA2	325F	Forward	CAA GCA AAC TTA TAC TCA AGA TAT TAC TGG TTT GAA GCC AA	850 - 891	325
21	XSV	507F	Reverse	GGAGAACCATGAGATCACG	1 - 19	507
22	XSV	507R	Forward	CTGCTCATTACTGTTCGGAGT	488 - 508	507

3.5.3 Quantitative real-time RT-PCR (qPCR)

3.5.3.1 Standard curve for qPCR

In order to make a standard curve for qPCR, a positive control plasmid was prepared. The specific primer for the plasmid was designed from completed sequence of MrNV (RNA1) (GenBank accession number AY222839 (the French West Indies),

AY231436 (China) and FJ751226 (Thailand)). The forward and reverse primers for MrNV were 5'- CTC TTG ATC GTG TCA GTG GA -3' at positions 2425 bp to 2445 bp and 3'- CAG GCA TTG CTT ACC ACG TT -5' at positions 3185 bp to 3205 bp respectively. The PCR products were purified using Wizard® SV Gel and PCR Clean-UP system (Promega, Australia) and directly transformed into *Escherichia coli* JM 109 High Efficiency cells using pGEM-T® Easy Vector System (Promega, Australia) for cloning, according the manufacturer's instructions. Then, plasmid was purified using Wizard® Plus SV Minipreps DNA Purification System (Promega, Australia), according the manufacturer's instructions. Plasmids with DNA inserts of MrNV were sent to Macrogen Inc (Seoul, Korea) for sequencing to confirm the quality of the sequences. The cloned DNA insert of MrNV was diluted to make serial dilutions from 1 x 10^9 to 1 x 10^0 of the plasmid standard curve for qPCR assay. Serial dilutions were made using salmon sperm DNA (2 ng/µl) (Invitrogen, Australia). In three independently generated standard curves, cycle times and target concentration were observed over ten orders of magnitude in 10 fold dilution series.

3.5.3.2 Optimisation of $MgCl_2$ concentration

Optimised the $MgCl_2$ concentration was by 0.5 mM increments of $MgCl_2$ to the mastermix (Immomix, Bioline Australia) against an aliquot of MrNV positive control plasmid. The reaction mixture comprised 10 µL of Immomix reaction, 10 µM of forward primer, 10 µM of reverse primer, 10 µM of probe, 2.5 µL of plasmid template at concentration 1 x 10^5 copies for each reaction. Four replicates of each concentration of $MgCl_2$ from 0 mM to 3.5 mM were optimized.

3.5.3.3 Optimisation of probe concentration

Four replicates of mixture containing probe concentrations of 50, 60, 70, 80, 90, 100, 200 and 400 nM were optimized in the TaqMan real-time PCR. The reaction mixtures were composed of 10 µL of Immomix reaction, 10 µM of forward primer, 10 µM of reverse primer, 0.5 mM of $MgCl_2$, 2.5 µL of plasmid template at a concentration of 1 x 10^5 copies for each reaction and nuclease-free water added to a final volume of 20 µL.

3.5.3.4 Quantitative real-time RT-PCR cycle

Extracted RNA was reversed to cDNA and amplified in PCR process using SensiMix™ Probe One–Step Kit (Bioline Australia). The cycle process of qPCR consisted of incubation at 42°C for 10 min for the reverse transcriptase step and 95°C for 10 min followed by 40 cycles at 95°C for 10 s and 60°C for 60 s. Three reactions of 20 µl were performed independently on different days to determine the specificity and reproducibility of the assay. Data analysis and acquisition were performed using Rotor-Gene 6000 (Corbett Robotics).

3.5.4 Cloning and sequencing

PCR products were purified from agarose gels using Wizard® SV Gel and PCR Clean-UP system (Promega, Australia) and directly transformed into *Escherichia coli* JM 109 High Efficiency cells using pGEM-T® Easy Vector System (Promega, Australia). One hundred µl of transformation reaction was spread plated onto duplicated Luria Bertani (LB) agar plates containing 80 µg/ml 5-bromo-4-chloro-3-indoylgalactoside (X-Gal) and 0.5 mM isopropyl-D-thiogalactopyranoside (IPTG), 100 µg/ml ampicillin and incubated at 37°C overnight. Four white colonies, putatively containing MrNV insert, were inoculated to universal vials with 15 ml LB broth supplemented with 100 µg/ml ampicillin and incubated at 37°C overnight shaking at 150 rpm. Bacteria were pelleted at 4000 rpm for 5 min using Eppendorf 5804R centrifuge (Eppendorf, Germany) and recombinant plasmids were extracted from pelleted bacteria using Wizard® Plus SV Minipreps DNA Purification System (Promega, Australia), according the manufacturer's instructions. Four replicates of plasmids with DNA inserts of MrNV were sent to Macrogen Inc. (Seoul, Korea) for sequencing using M13 universal primers. Replicate samples were sent to Macrogen Inc. independently on different. Sequences from Macrogen Inc were produced for each clone and analysed using Sequencher™ software (Gene Codes Corporation). Sequences were aligned and compared to available sequences using Basic Local Alignment Search Tool (BLAST), through the National Centre for Biotechnology Information (NCBI). Alignment of nucleotide sequences was performed using GeneDoc software version 2.6.002 developed by Nicholas, Karl B. and Nicholas,

Hugh B. in 1997. Phylogenic trees of nucleotide sequences were constructed using molecular evolutionary genetics analysis (MEGA) software version 4.0.

3.6 Spectrophotometry

The quantity of DNA in a preparation and experimental samples was measured by spectrophotometer using an Eppendorf Biophotometer (Eppendorf, Germany). Fifty µl of DNA sample was put into a sterile Eppendorf disposable UVette (Eppendorf, Germany). The light absorption was read at wavelength of 260 nm (A260) and 280 nm (A280). The ratio (A260/A280) estimated the amount of DNA of the DNA samples.

3.7 Statistical analyses

Data were analysed with Statistical Package for the Social Sciences (SPSS) version 19. Comparisons of means of results were determined by univariate analysis of variance (ANOVA) and two-way ANOVA. Comparisons between significant means for result analyses were made using least significant difference (LSD). Some data needed to be transformed to the log scale in order to improve the homogeneity of variance and normal distribution. However, even after log transformation of the data, some data did not meet the assumptions. Therefore, those data were analysed using Kruskal Wallis nonparametric test.

CHAPTER 4

THE COMPLETE SEQUENCE OF THE AUSTRALIA ISOLATE OF *MACROBRACHIUM ROSENBERGII* NODAVIRUS

4.1 Introduction

In Australia, phylogenetic analysis of representatives from 18 populations of *Macrobrachium rosenbergii* has been reported by De Bruyn *et al*., (2004). Significant DNA divergence between eastern and western *M. rosenbergii* determined *M. rosenbergii* may actually represent two distinct phylogenetic species in Australia. In the meantime, diseases in *M. rosenbergii* including MrNV have been detected (Owens *et al.*, 2009). The index case of MrNV described by Owens *et al*., (2009) was in a lineage II Eastern form of *M. rosenbergii* (De Bruyn *et al*. 2004).

MrNV has been placed in the family of *Nodaviridae* (alphanodavirus) based on its characteristics and genome sequences (Sahul Hameed *et al.*, 2004; Bonami and Sri Widada, 2011). MrNV is a small icosahedral non-enveloped virus, 26 to 27 nm in diameter and two pieces of positive sense, single stranded RNA (ssRNA): RNA1 (3.2 kb) and RNA2 (1.25 kb) (Romestand and Bonami, 2003; Bonami and Sri Widada, 2011). MrNV RNA1 contains the coding sequences of two proteins, protein A and protein B required for MrNV replication and development in the host cells. Specifically, protein B2 is important for the intracellular accumulation of viral RNA in the cells because it is a sequence-nonspecific binding protein preventing host antiviral immunity (Fenner *et al*., 2006) via RNA interference.

MrNV sequences have been analysed in order to develop effective diagnostic techniques for the detection of MrNV infection, however, few complete viral genomes of MrNV have been published. In order to analyse the nucleotide sequence similarities and amino acid changes of the Australian isolate of MrNV compared to other isolates, the complete sequences of MrNV (RNA1 and RNA2) were analysed in this study. This study aims to determine the first complete sequence of the Australian MrNV (RNA1 and RNA2) including protein B2 from the giant freshwater prawn (*M. rosenbergii*) in Australia.

4.2 Materials and Methods

4.2.1 Samples of MrNV

The giant freshwater prawn (*M. rosenbergii*) was collected from the Flinders River system near the Gulf of Carpentaria, Queensland, Australia in 2004. *M. rosenbergii* were positive for MrNV (Owens *et al.*, 2009) and became the source material of the Australian isolate of MrNV (Chapter 3.1).

4.2.2 RNA extraction and RT-PCR assay

Thirty-five mg of *M. rosenbergii* tail muscle were used for RNA extraction using SV total RNA isolation system (Promega, Australia) according to the manufacturer's instructions (Chapter 3.5.1). RNA samples were used for RT-PCR according to Owens *et al.* (2009). cDNA was produced using ImProm-II Reverse Transcriptase System (Promega, Australia) following the manufacturer's protocol before DNA amplification (Immomix, Bioline Australia). Specific primers were designed from sequence data in National Center for Biotechnology Information (NCBI), GenBank of the MrNV (Table 3.1). The cycle parameters consisted of incubation at 95°C for 4 min, followed by 35 cycles at 94°C for 30s and 55°C for 30s and 72°C for 1 min and 5 min at 72°C. PCR products were electrophoresed on 0.8% agarose gels and stained with 0.5 μg ml^{-1} ethidium bromide. Bands were cut from gels and purified for use in cloning and sequencing.

4.2.3 Oligonucleotide primers

Oligonucleotide primers were designed by the Oligo program version 7 using all published sequences of MrNV obtained through the NCBI (Table 3.1). Published MrNV sequences have been deposited at GenBank under accession number AY222839, AY231436, FJ379531, DQ146969, DQ459203, DQ459204, DQ459205, DQ459206, DQ459207, DQ459208, DQ521574, JN187416 and FJ751226 for RNA1, and AY222840, AY231437, DQ521575, EU150126, EU150127, EU150128, EU150129, HM565741, GU300102, NC005095 and FJ751225 for RNA2. The program selected primer sets with appropriate melting temperature for PCR.

4.2.4 Cloning and sequencing

PCR products were purified from agarose gels using Wizard® SV Gel and PCR Clean-UP system (Promega, Australia) and directly transformed into *Escherichia coli* JM 109 High Efficiency cells using pGEM-T® Easy Vector System (Promega, Australia) according the manufacturer's instructions (Chapter 3.5.4). Recombinant plasmids were extracted from pelleted bacteria using Wizard® Plus SV Minipreps DNA Purification System (Promega, Australia), according the manufacturer's instructions. Four replicates of plasmids with DNA inserts of MrNV were sent to Macrogen Inc (Seoul, Korea) for sequencing. Replicate samples were sent to Macrogen Inc independently on different days.

4.2.5 Sequence analysis

Three forward and three reverse sequences from Macrogen Inc were produced for each clone and analysed using Sequencher™ software (Gene Codes Corporation). Sequences were aligned and compared to available sequences using BLAST, through NCBI. Alignment of nucleotide sequences was performed using GeneDoc software version 2.6.002 developed by Nicholas, Karl B. and Nicholas, Hugh B. (1997). Nucleic acids of protein B2 at position 2725 to 3126 bp of the Australian MrNV (RNA1) were compared to other protein B2 isolates including those from MrNV and *Penaeus vannamei* nodavirus in crustaceans; Nodamura virus, Boolarra virus, Flock House Virus (FHV), Black beetle virus and Drosophila melanogaster American nodavirus in insects; Striped Jack Nervous Necrosis Virus (SJNNV) and Atlantic halibut nodavirus in fish (Table 4.1). Phylogenic trees of nucleotide sequences were constructed using molecular evolutionary genetics analysis (MEGA) software version 4.0.

Table 4.1: Comparison of protein B2 (402 bp) of crustacean, insect and fish nodaviruses.

	From Accession number	Isolate / year	Description	Similarity (%)
1	JN619369	Australia 2011	B2 protein (*Macrobrachium rosenbergii* nodavirus)	100
2	FJ751226	Thailand 2011	B2 protein (*Macrobrachium rosenbergii* nodavirus)	98
3	AY222839	French West Indies 2005	B2 protein (*Macrobrachium rosenbergii* nodavirus)	98
4	AY313773	France 2005	B2 protein (*Macrobrachium rosenbergii* nodavirus)	98
5	GU300103	India 2011	B2 protein (*Macrobrachium rosenbergii* nodavirus)	97
6	AY231436	China 2006	B2 protein (*Macrobrachium rosenbergii* nodavirus)	97
7	JN187416	Malaysia 2011	B2 protein (*Macrobrachium rosenbergii* nodavirus)	95
8	NC005094	France 2005	B2 protein (*Macrobrachium rosenbergii* nodavirus)	NA
9	NC014978	Belize 2011	B2 protein (*Penaeus vannamei* nodavirus)	NA
10	NC003448	Japan 2008	B2 protein (Striped jack nervous necrosis virus)	NA
11	AB025018	Japan 2003	B2 protein (Striped jack nervous necrosis virus)	NA
12	AB056571	Japan 2002	B2 protein (Striped jack nervous necrosis virus)	NA
13	AY962683	Norway 2006	RNA-dependent polymerase (B2) gene (Atlantic halibut nodavirus)	NA
14	GQ342965	USA 2011	B2 protein (Drosophila melanogaster American nodavirus)	NA
15	NC002690	USA 2008	B2 protein (Nodamura virus)	NA
16	M33065	1993	B2 protein (Black beetle virus)	NA
17	NC004146	USA 2008	B2 protein (Flock house virus)	NA
18	JF461541	France 2011	B2 protein (Flock house virus)	NA
19	NC004142	USA 2009	B2 protein (Boolarra virus)	NA

4.3 Results

4.3.1 Complete sequences of the Australian isolate of MrNV (RNA1 and RNA2)

Complete sequences of the Australian isolate of MrNV RNA1 (3203 bp) and RNA2 (1173 bp) were successfully sequenced (Genbank accession number JN619369 (RNA1) (APPENDIX B) and JN619370 (RNA2) (APPENDIX C)).

4.3.2 Potential open reading frames (ORF)

Potential ORFs in the Australian isolate of MrNV were determined by NCBI ORF finder (www.ncbi.nlm.nih.gov/projects/gorf). MrNV (RNA1) sequence contained two slightly overlapping ORFs. The first ORF was the largest of the ORFs indentified for MrNV (RNA1). It started at nucleotide 24 with ATG codon and terminated with TAA codon at position 3140 containing 1038 amino acids (3117 bp). This protein is speculated to encode protein A or an RNA-dependent RNA polymerase. The second ORF started at nucleotide 2725 with ATG codon and terminated with TAA codon at position 3126 containing 133 amino acids (402 bp). This protein is speculated to encode protein B2 which has an RNA-binding domain.

All small ORFs did not contain significant sequence homology to available sequences using BLAST. MrNV (RNA2) had one major ORF which started at nucleotide 38 with ATG codon and terminated with TAG codon at position 1075 containing 345 amino acids (1038 bp) which is speculated to encode the capsid protein. All small ORFs had no significant sequence similarity to other nodavirus isolates using BLAST.

4.3.3 Nucleotide sequence analysis

Nucleotide sequence analysis showed that the identities of the Australian MrNV (RNA1) were 94%, 95%, 95% and 97% similar to Malaysian (JN187416), the French West Indies (AY222839), Chinese (AY231436) and the Thai (FJ751226) isolates, respectively. Nucleotide sequence analysis showed that the identities of the Australian isolate of MrNV (RNA2) had 92% similarity when compared with the French West Indies (AY222840), Chinese (AY231437) and the Thai (FJ751225, EU150126, EU150127, EU150128 and EU150129) isolates. Nucleotide sequences of the completed Australian MrNV (RNA1 and RNA2) isolate were translated into amino acids to determine amino acid changes against other MrNV isolates (Figure 4.1 and 4.2). Many protein changes in the amino acid sequence of the Australian isolate of MrNV (RNA1) were observed (Figure 4.1); whilst the protein changes in amino acid sequence of MrNV (RNA2) were less extensive (Figure 4.2).

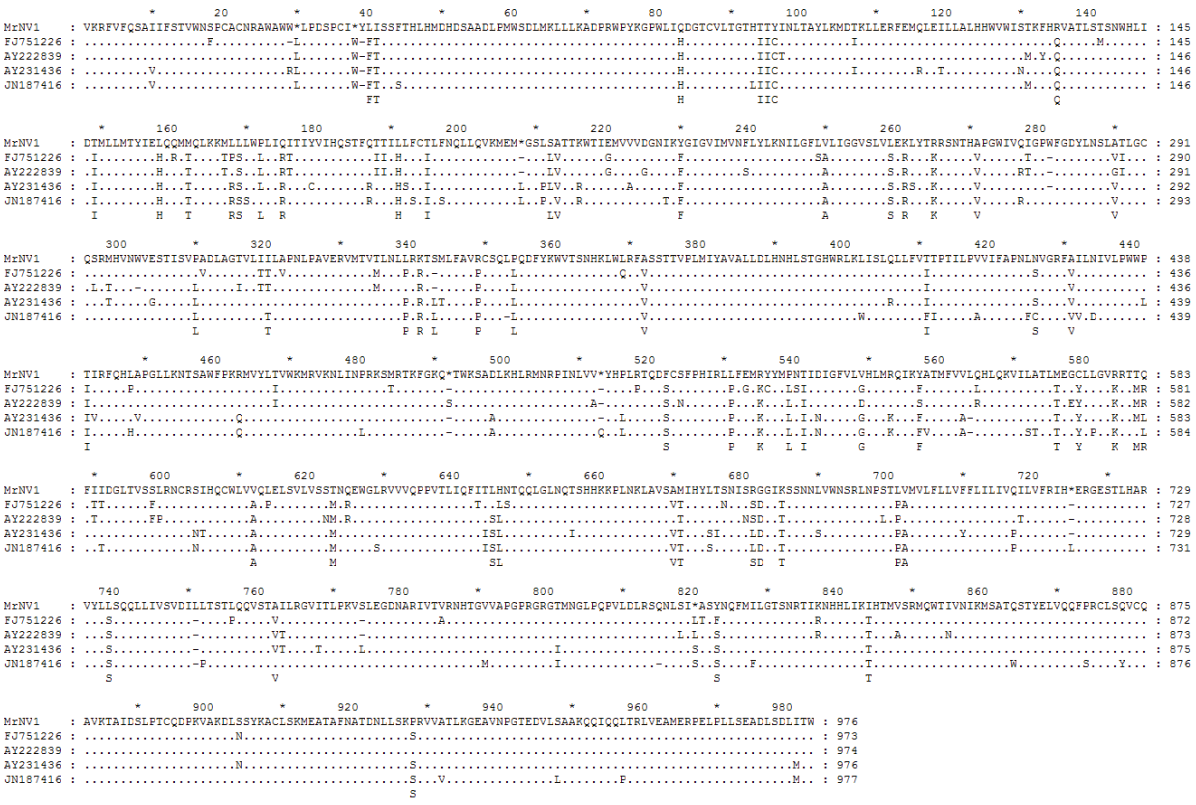

Figure 4.1: Comparison of the amino acids from the complete sequences (3126 bp) of *Macrobrachium rosenbergii* nodavirus (RNA1) from white tail disease isolates. The sequence name obtained from GenBank is given on the left and the numbering of the deduced amino acid is on the right.

Figure 4.2: Comparison of the amino acids from the complete sequences (1170 bp) of *Macrobrachium rosenbergii* nodavirus (RNA2) from white tail disease isolates. The sequence name obtained from GenBank is given on the left and the numbering of the deduced amino acid is on the right.

4.3.4 Phylogenetic comparison

The Australian isolate of MrNV (RNA1) is phylogenetically closely related to the Thai and the French West Indies isolates and are grouped into a separate clade from the Chinese and Malaysian MrNV (RNA1) isolates (Figure 4.3). The Australian, Thai and Chinese MrNV (RNA2) isolates are not phylogenetically closely related to other MrNV isolates and form separate clades whereas the French West Indies and Thai (EU150126, EU150127, EU150128 and EU150129) isolates of MrNV (RNA2) are in the same cluster (Figure 4.4).

Figure 4.3: Phylogenetic tree deduced from analysis of 3126 bp nucleotide sequences of complete MrNV (RNA1) of the Australian isolate of *Macrobrachium rosenbergii* nodavirus compared with other nodavirus isolates.

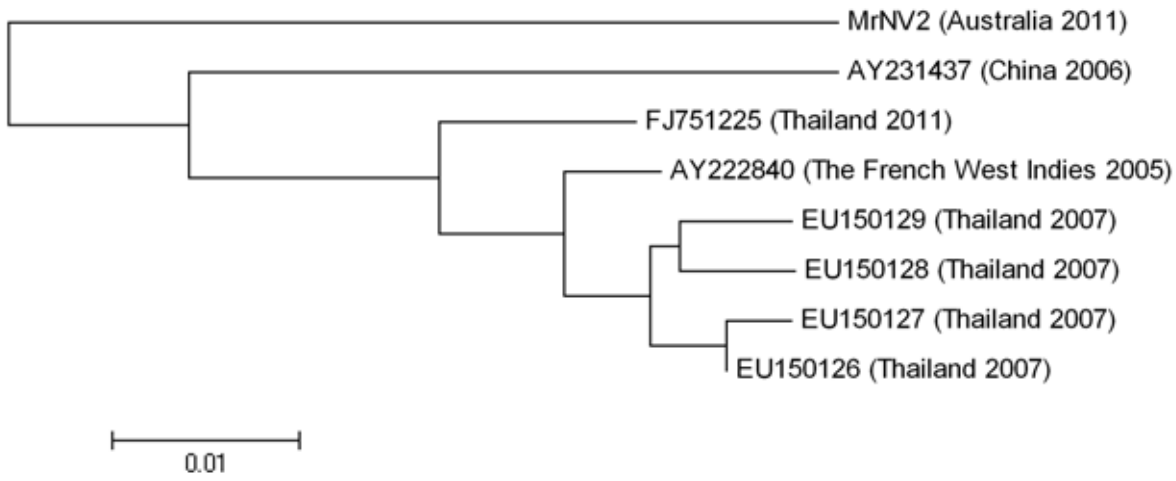

Figure 4.4: Phylogenetic tree deduced from analysis of 1170 bp nucleotide sequences of complete MrNV (RNA2) of the Australian isolate of *Macrobrachium rosenbergii* nodavirus compared with other nodavirus isolates.

The Thai, French West Indies, Taiwanese, Chinese, Malaysian, Indian and Australian isolates were phylogenetically grouped into separate clades deduced from phylogenetic analysis of the 439 bp common to all isolates (Figure 4.5) and determined that the Australian group was the second last to join the other combined group with only the joint Chinese/Malaysian group combining last. The Taiwanese/French West Indies group is phylogenetically closely related to Indian isolates while Chinese/Malaysian group is the most different phylogenetically to other MrNV isolates. Moreover, the trimmed sequences (278 bp) of the Australian MrNV (RNA2) also had a different phylogenetic relationship between the Thai, French West Indies, Taiwanese, Chinese and Indian isolates (Figure 4.6). The Thai, Indian and French West Indies isolates of MrNV (RNA2) are phylogenetically closely related whereas the Chinese group is phylogenetically closely related to Taiwanese (DQ521575)/Thai (FJ751225) group.

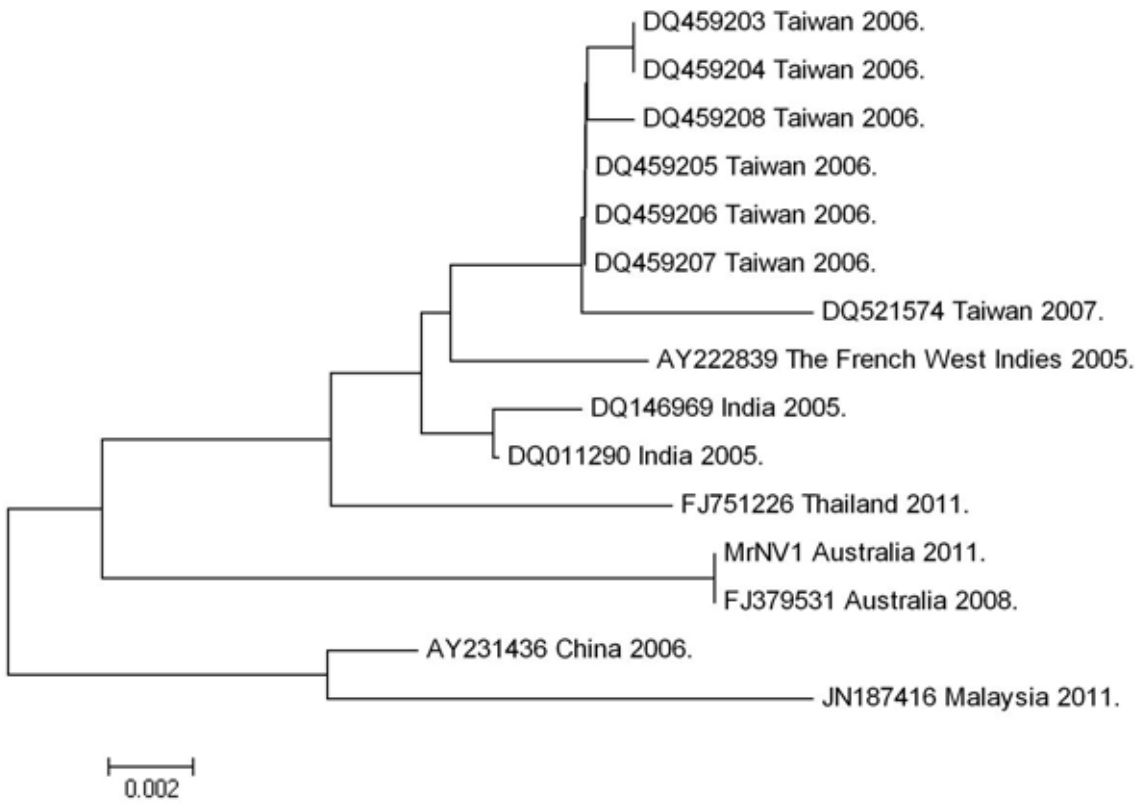

Figure 4.5: Phylogenetic tree deduced from analysis of trimmed nucleotide sequences (439 bp) of MrNV (RNA1) of the Australian isolate of *Macrobrachium rosenbergii* nodavirus compared with other nodavirus isolates.

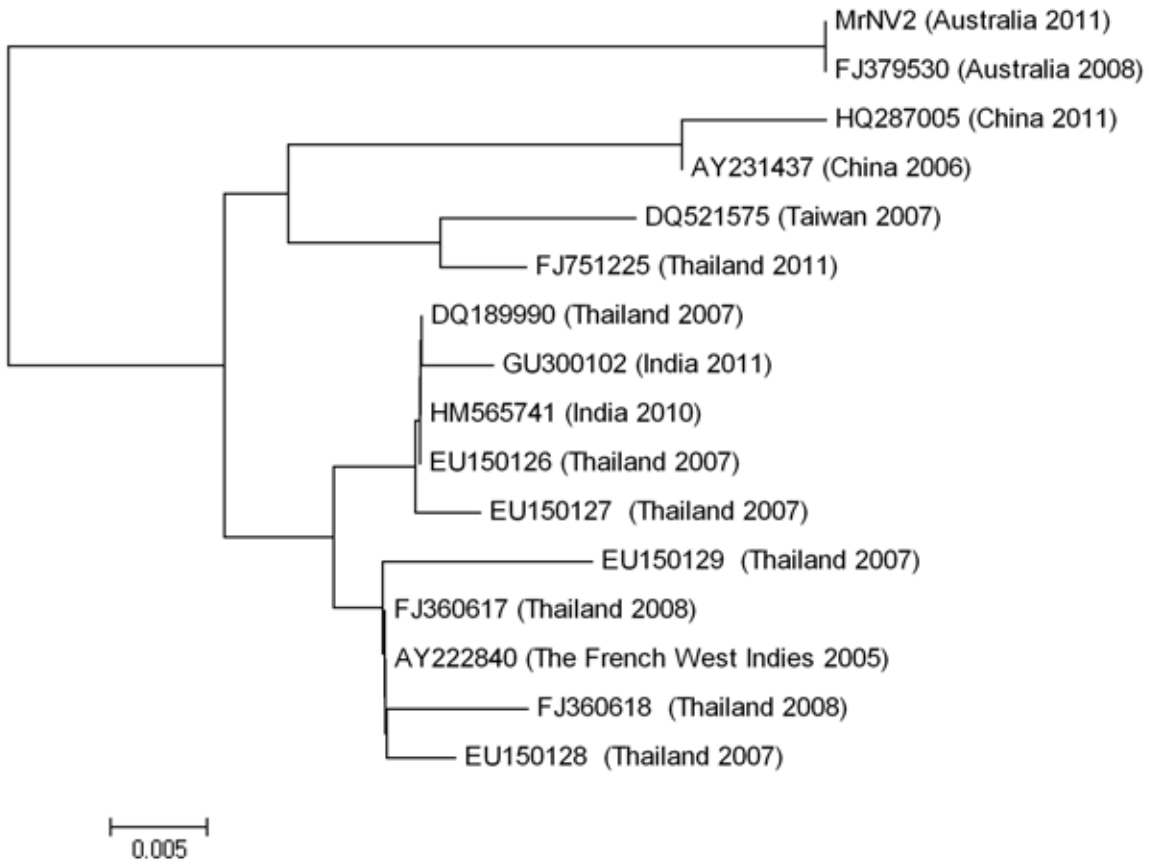

Figure 4.6: Phylogenetic tree deduced from analysis of trimmed nucleotide sequences (278 bp) of MrNV (RNA2) of the Australian isolate of *Macrobrachium rosenbergii* nodavirus compared with other nodavirus isolates.

Phylogenetic analysis protein B2 nodaviruses (317 bp) showed similarity between the insect, crustacean and fish nodaviruses and recognised two groups of nodavirus; MrNV/insect/fish nodavirus and fish/insect/*Penaeus vannamei* nodaviruses (Figure 4.7). Protein B2 of the MrNV group and black beetle virus are phylogenetically closely related and are different to fish nodaviruses. However, the incomplete sequence of *Penaeus vannamei* nodavirus from Belize is closely related to fish nodavirus at this stage.

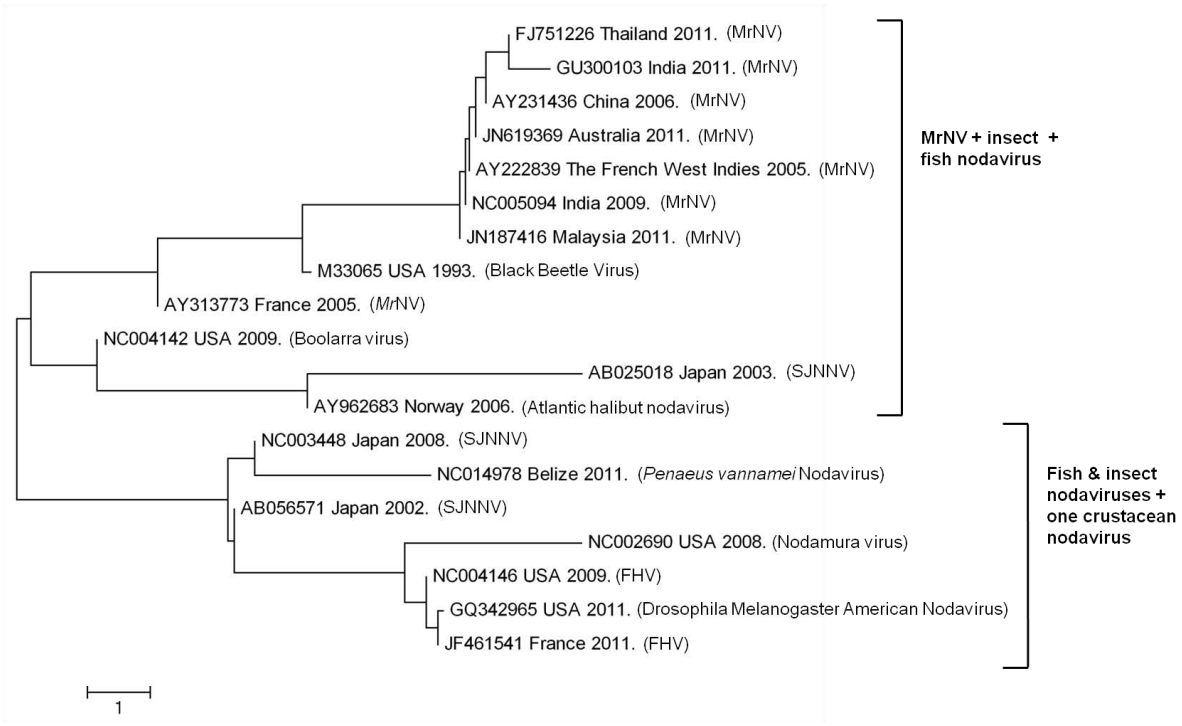

Figure 4.7: Phylogenetic tree of nucleotide (317 bp) of protein B2 of the Australian *Macrobrachium rosenbergii* nodavirus compared with fish, insect and other crustacean nodaviruses.

4.4 Discussion

Many countries in Asia have studied and monitored MrNV infection in the past 10 years. Only one paper from Australia has been published on MrNV (Owens *et al*., 2009). Owens *et al*. (2009) reported in the first study of MrNV in *M. rosenbergii* that the Australian isolate is most closely related to the Chinese isolate. However, in this study, phylogenetic analysis of the complete sequences of MrNV (RNA1) revealed the Australian isolate is phylogenetically closely related to the Thai and French West Indies isolates.

In invertebrates such as insects and crustacea, RNA interference (RNAi) is the primary response against viral infection, whereas vertebrates such as fish use combinations of host immunity such as cell-mediated immunity and interferon to defend against viral infections (Fenner *et al*., 2007; Qi *et al*., 2011). Protein B2 of alphanodavirus and betanodavirus is important for the intracellular accumulation of

viral RNA in the cells. Protein B2 is able to block the RNA silencing mechanism of the viral defence mechanism and enables completion of viral replication in the cells (Fenner *et al*., 2006). This study demonstrated different nucleic acids of protein B2 in fish, insect and crustacean nodavirus (Figure 4.7). This suggests sequences of protein B2 from different nodaviruses are not similar when comparing a 317 bp sequence and hence, RNAi targeting protein B2 has to be specific for each virus to inhibit the synthesis of protein i.e. a universal RNAi for all nodaviruses might not be achievable. The results showed mortalities and histopathological lesions decreased significantly. This current study suggests that specific RNAi against protein B2 needs to be reanalysed for fish and insect nodavirus if the technology is to be used to prevent and control the diseases.

Based on incomplete data, the sequence of protein B2 from *Penaeus vannamei* nodavirus appears to be an anomaly at this stage. It is really imperative that this virus be fully sequenced with particular attention to the area of protein B2. If this preliminary data is correct, then the implication is that the sharing of nodaviruses between insects, fish and crustaceans may have happened more than once.

In conclusion, this is the first study involving the complete sequences of the Australian MrNV (RNA1 and RNA2) and the phylogenetic relationship between existing MrNV isolates that can be used to form a better understanding of the true phylogenetic relationship of MrNV. This information will be used to develop effective diagnostic tools to detect the Australian isolate of MrNV. This study suggests sequence-specific RNAi against protein B2 can be designed to control other nodavirus infections in the future.

CHAPTER 5

C6/36 CELL CULTURE FOR THE AUSTRALIAN ISOLATE OF *MACROBRACHIUM ROSENBERGII* NODAVIRUS

5.1 Introduction

In vitro investigations using cell culture for viral detection and viral quantification have been reported. The susceptibility of the snakehead fish cell line (SSN-1) (*Ophicephalus striatus*) and Singh's *Aedes albopictus* (C6/36) cell lines to MrNV has been studied (Hernandez-Herrera *et al*., 2007; Lin *et al*., 2007; Sudhakaran *et al*., 2007b). However, the snakehead fish cell line was only semipermissible for the replication of MrNV. MrNV was detected using C6/36 cell lines and virions were seen inside of vacuoles in the infected cells (Sudhakaran *et al*., 2007b). Therefore, C3/36 cell line ATC-15 (subline C6/36) was used in this study.

This study aims to determine the susceptibility of C6/36 cell line to the Australian isolate of MrNV and to observe cytopathic effects (CPE) in order to use less experimental animals in future investigations. Alternate staining was also undertaken to understand cellular events occurring during MrNV infection. To quantify viral copies and viral replication in the infected cells, TaqMan real-time reverse transcriptase polymerase chain reaction (TaqMan real-time RT-PCR) was developed and used for the Australian isolate of MrNV (Chapter 3.5.3).

5.2 Materials and Methods

5.2.1 Cell Culture and MrNV inoculum preparation

The C6/36 cell line was prepared at James Cook University, Townsville, Australia. The RPMI (Roswell Park Memorial Institute - 1640) medium supplemented with 10% foetal bovine serum (FBS) was used to grow the cells. The 25 cm^2 flasks were incubated at 28°C and the medium was replaced every two days. When the cells formed a monolayer, the old medium was removed and the cell layer was washed

with 1x phosphate buffered saline (PBS) and antibiotic trypsin versene (ATV) twice, in order to separate cells individually and split them into two flasks with new media.

Muscle from *M. rosenbergii* was confirmed to be infected with MrNV by histopathology and PCR. The inoculum was prepared from the infected muscle which was homogenized in phosphate buffered saline (PBS) and filtered though a 45 µm filter. The experiment was performed as described in Chapter 3.1.

After the cells formed a monolayer and old medium was removed, 200 µl of MrNV inoculum was allowed to adhere to the cell monolayer and was incubated at 28°C for 1 hr. Then, RPMI with 10% FBS was added to continue growing the cells. The cells were incubated at 28°C for 14 days before staining with different dyes (see below) and cell counts were performed.

5.2.2 Staining of normal and infected C6/36 cells

After 14 days incubation, the C6/36 cells were fixed and stained (see below) with different stains: Mayer's haematoxylin and eosin stain (H&E) and Giemsa to observe the cytopathic effect under light microscopy (Olympus E C microscope with QIMAGING MicroPublisher 5.0 RTV camera). Cells were stained with trypan blue to count the dead cells with disrupted membranes. Single stranded nucleic acid of MrNV in the cells stained with acridine orange was detected using fluorescent microscopy (Leitz wetzlar Germany 2307 with Leica DFC490 camera).

5.2.2.1 Mayer's haematoxylin and eosin stain (H&E)

The cells were fixed with 95% ethanol and stained with H&E to observe cellular morphological changes.

5.2.2.2 Giemsa stain

The cells were fixed with 100% methanol before staining with Giemsa stain overnight to observe cellular morphological changes. Then the cells were rinsed with distilled water and 0.5 % aqueous acetic acid until cells were pink.

5.2.2.3 Trypan blue (0.4%)

The cells were fixed with 100% methanol and stained with trypan blue to observe the cell membranes integrity and hence determine whether cells were dead or alive.

5.2.2.4 Acridine orange

Acridine orange was used to observe nucleic acid proliferation in the cells. The cells were fixed with 95% ethanol for 30 min and then rehydrated with 80%, 70% and 50% ethanol respectively for 10 s each and then rinsed with water. One percent acetic acid was added for 6 s and then removed. The cells were stained with 0.1% acridine orange solution for 3 min and then washed with McIlavain citric acid and M/10 calcium chloride.

5.2.3 Count of dead cell with disrupted membranes

Four control flasks and four infected flasks were used in this experiment. In the presence of ATV, the flasks were shaken 30 times to break up the cells into single cell suspensions. One ml of the cell suspension was removed to a 1.5 ml microcentrifuge tube. Then, 200 µl of 0.4% trypan blue was added to 200 µl of the cell suspension. After thoroughly mixing, micropipette was used to add the suspension to both sides of a haemocytometer. The dead cells that took up the dye were counted under light microscopy. The cells were observed on days 2, 4, 6, 8, 10, 12 and 14.

5.2.4 Neutral red assay

Neutral red is used to demonstrate functional respiration of the mitochondria in cells. Four replicates of control cells and four replicates of infected cells inoculated with MrNV were prepared in 96-well cell culture plate. Fifty µl of cell culture medium (RPMI with 5% FBS) were placed into the wells and an extra 50 µl of medium were added to the control wells. Fifty µl of MrNV inoculum were added to the appropriate wells giving four replicates. Then, 50 µl of cells in suspension were added to all wells before incubating the plates at 28°C. The neutral red experiment was started on

day 0 and concluded on day 14. The results were observed and recorded on days 2, 4, 6, 8, 10, 12 and 14. Therefore, 56 wells were used to measure the absorbance using spectrophotometer on each second day.

The neutral red staining was performed following the method described by Babich and Borenfreund (1990). Growth medium was removed from the cell culture wells. Two hundred µl of neutral red dye solution were added to the cells. Neutral red dye was removed after 3 hr incubation at 25°C. The cells were fixed with 0.5% (v/v) formaldehyde and 1% (w/v) $CaCl_2$ for 1 min, and then the fixative was removed. Two hundred µl of 1% (v/v) acetic acid and 50% (v/v) ethanol were added and incubated at 25°C for 20 min in order to extract the neutral red dye from the cells. After 20 min, the extracted solution was removed and the light absorbance was read at 540 nm.

5.2.5 Passages of C6/36 cell culture with MrNV

One ml of MrNV inoculum was inoculated to four C6/36 cell flasks and incubated at 28°C for 1 hr. Then, RPMI with 10% FBS was added to continue growing the cells and passaged after 1 week incubation at 28°C. After passaging, cells and supernatants were collected for quantitative polymerase chain reaction (qPCR) using TaqMan real-time RT-PCR and one ml of cells and supernatants was placed into four new C6/36 cell flasks. Then, this process was repeated for 4 passages.

5.2.6 Isolation of nucleic acids and TaqMan real-time RT-PCR assay

Fifty µl of cell culture medium (RPMI with 5% FBS), 50 µl of cells and 50 µl of MrNV inoculum was placed into 96-well cell culture plate with four replicates before incubating the plate at 28°C. The experiment was started at 0 hr and concluded at 336 hr. Cells and suspensions were collected every 4 hr using ATV to harvest the cells for RNA extraction and TaqMan real-time RT-PCR assay.

Cells and suspensions collected from TaqMan real-time RT-PCR assay were used for RNA extraction. RNA was extracted from approximately 4 x 10^3 cells using SV total RNA isolation system (Promega, Australia) according to the manufacturer's

instructions. Extracted RNA was used for cDNA amplification. cDNA was amplified using ImProm-II Reverse Transcriptase System (Promega, Australia) following the manufacturer's protocol. The experiment was performed as described in Chapter 3.5.1.

The specific primers and probe for TaqMan real-time RT-PCR were designed from MrNV complete sequences (GenBank accession number AY222839 (the French West Indies), AY231436 (China) and FJ751226 (Thailand)) to amplify 198 bp of MrNV for TaqMan real-time RT-PCR assay (Table 5.1). The cycle of DNA amplification (Immomix, Bioline, Australia) consisted of incubation at 95°C for 10 min, followed by 40 cycles at 95°C for 10s and 60°C for 60s. Data acquisition and analysis were performed using Rotor-Gene 6000 (Corbett Robotics).

Table 5.1: Nucleotide sequences and position of primers and probe used in quantitative real-time PCR.

Primers and Probe	Primer and probe sequences (5'-3')	Product length (bp)	Nucleotide position
Forward primer	5'-GAC CCA AAA GTA GCG AAG GA-3'	198	2860 to 2879
Reverse primer	3'-GGC CTC TCC CTT TAG TGT T-5'		3040 to 3058
Probe	5'-[6FAM] AAG CAA CCG CCT TCA ATG CC [TAM]-3'	143	2915 to 2934

5.2.7 Optimisation of TaqMan real-time RT-PCR assay

According to the protocol described in Chapter 3.5.3.2 and 3.5.3.3, 20 µl of reactions were performed in real-time PCR assay using a Rotor-Gene 6000 (Corbett Robotics). The cycle of real-time PCR consisted of an initial incubation at 95°C for 10 min, followed by 40 cycles at 95°C for 10 s and 60°C for 60 s. PCR reactions were performed independently on different days in order to determine the specificity and reproducibility of the assay. Four replicates of each concentration of additional $MgCl_2$ from 0 mM to 3.5 mM in increments of 0.5 mM and TaqMan probe

concentrations of 400, 200, 100, 90, 80, 70, 60 and 50 nM were optimized using mastermix (Immomix, Bioline, Australia).

5.2.8 Generation of standard curve

A positive control plasmid was prepared for standard curve in TaqMan real-time RT-PCR assay according to Chapter 3.5.3.1. The forward and reverse primers for standard curve were 5'- CTC TTG ATC GTG TCA GTG GA -3' and 3'- CAG GCA TTG CTT ACC ACG TT -5', respectively. The PCR products were run on 1% agarose gels and purified using Wizard® SV Gel and PCR Clean-UP system (Promega, Australia) and directly cloned using pGEM-T® Easy Vector System (Promega, Australia) and plasmid was purified using Wizard® Plus SV Minipreps DNA Purification System (Promega, Australia) according the manufacturer's instructions. Plasmids with DNA inserts of MrNV were sent to Macrogen Inc (Seoul, Korea) for sequencing to confirm the quality of the sequences.

To determine the sensitivity of standard curve for TaqMan real-time RT-PCR assay, three plasmid serial dilutions from 1 x 10^9 to 10^0 copies were generated. Three standard curves were generated independently at different times in order to test the higher viral dilutions were not influenced by pipetting error or absorption of DNA to tube walls. Three replicates of each plasmid serial dilution and three no template controls were contained in each run.

5.2.9 Statistic analysis

Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) version 19. The data from neutral red assay needed to be transformed to the log scale in order to improve the homogeneity of variance and normal distribution. However, even after log transformation of the data, the data did not meet the assumptions. Therefore, the data were analysed using Kruskal Wallis nonparametric test.

5.3 Results

5.3.1 Different staining results

Infected C6/36 cells stained with H&E (Figure 5.1), Giemsa (Figure 5.2) showed typical cytopathic effect of MrNV. Vacuolation in the cytoplasm of infected C6/36 cells was observed with H&E (Figure 5.1B, 5.1C and 5.1D) and Giemsa (Figure 5.2B). On the other hand, no vacuolation of MrNV was seen in control C6/36 cells stained with Giemsa (Figure 5.2A) but some small vacuolations were observed in the control C6/36 cells with H&E staining (Figure 5.1A). Moreover, possible viral inclusion bodies of MrNV were observed in infected C6/36 cells with H&E staining (Figure 5.1B, 5.1C and 5.1D).

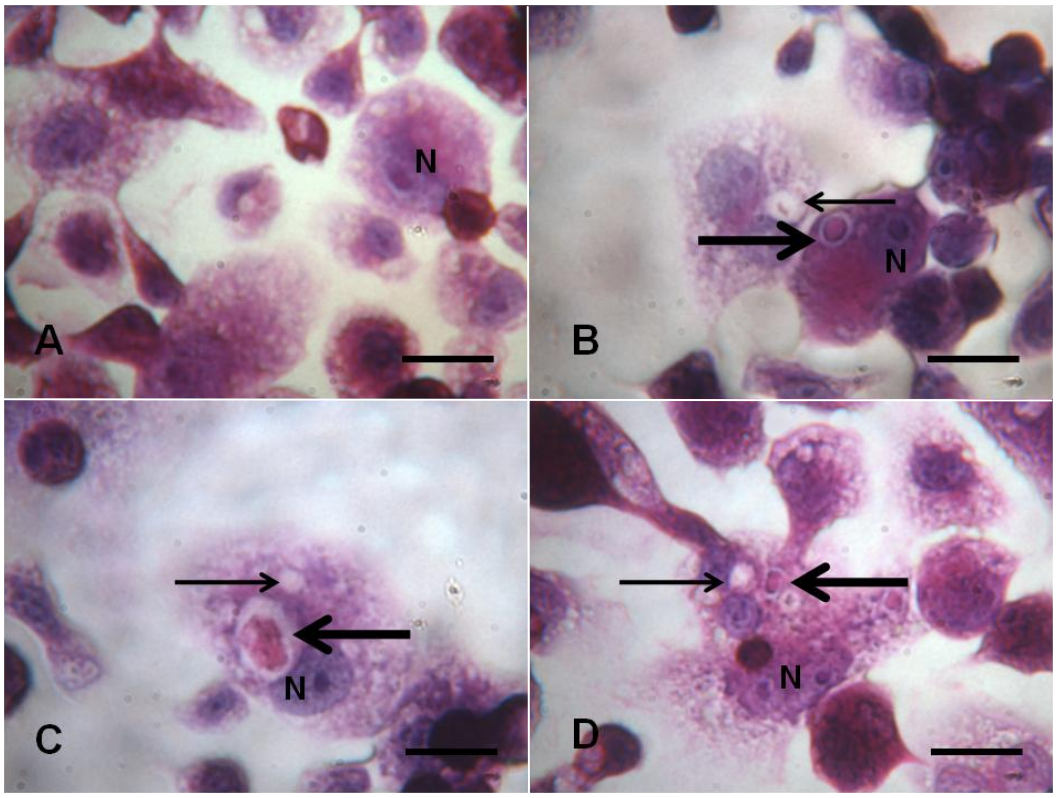

Figure 5.1: C6/36 cells stained with H&E. (A) Normal cells at 14 days. (B,C & D) MrNV infected cells at 14 days. Thicker arrows show possible MrNV inclusion bodies in the cells (bar = 10 µm) compared to cell nucleus (N). Thinner arrows show vacuolation in infected cells.

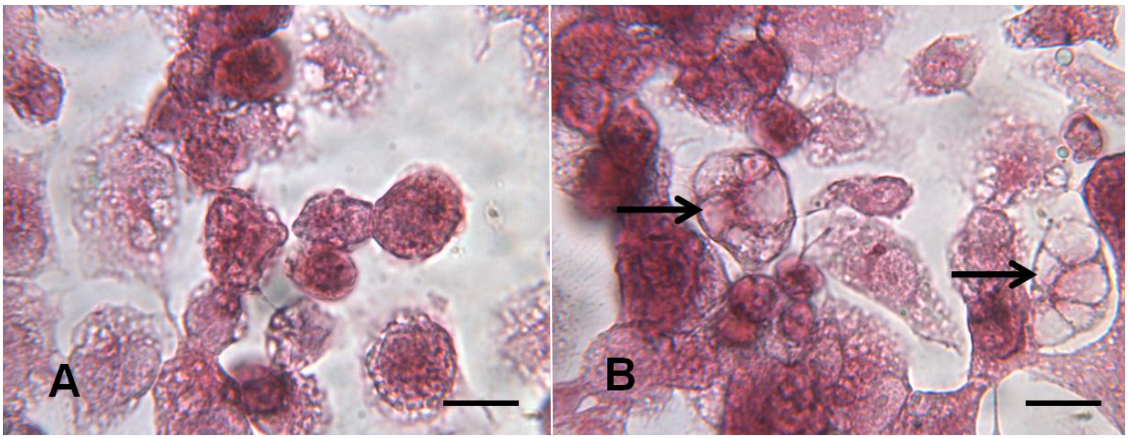

Figure 5.2: C6/36 cells stained with Giemsa. (A) Normal cells at 14 days. (B) MrNV infected cells at 14 days. Arrows show large vacuolations in the cells. Bar = 10 µm.

Acridine orange staining of control and infected C6/36 cells presented staining of nucleic acids. Limited orange colour of single stranded nucleic acid (ssRNA) of MrNV was observed in the control C6/36 cells (Figure 5.3A) and they stained predominantly green. Infected C6/36 cells showed a distinctly greater orange colour of the single stranded nucleic acid of MrNV in the cytoplasm of the cells (Figure 5.3B).

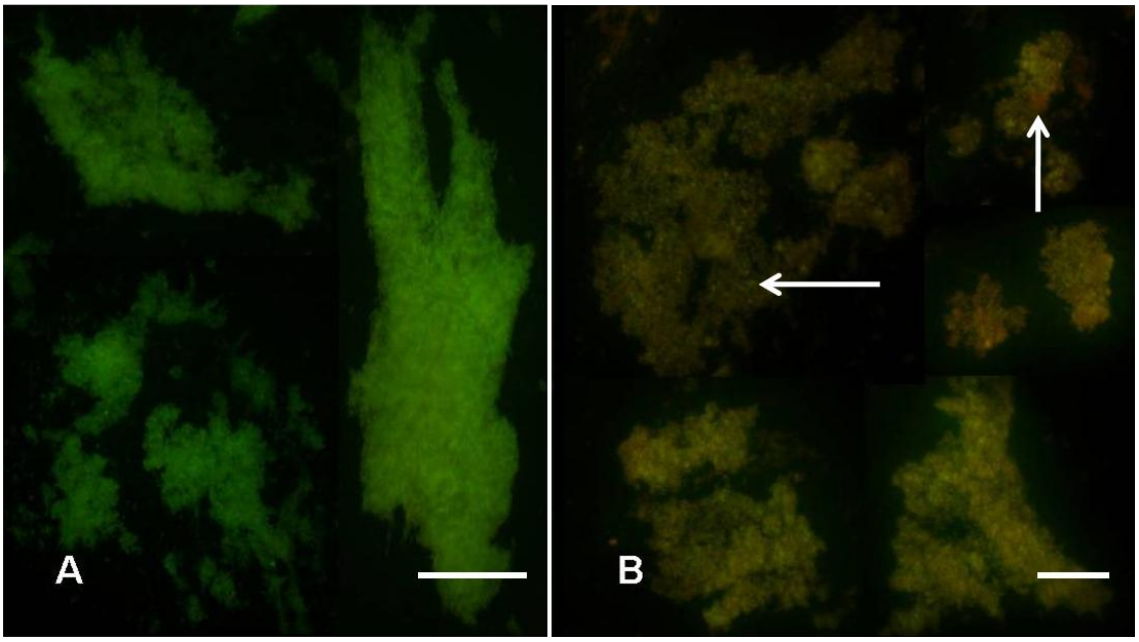

Figure 5.3: Acridine orange staining of the C6/36 cells. (A) Normal cells at 14 days (bar = 2 μm). (B) MrNV infected cells at 14 days (bar = 1 μm). Acridine orange stains MrNV ribonucleic acid in the cells (arrows) stands out in contrast to the green staining background.

5.3.2 Count of disrupted, dead cells

The number of cells with disrupted cell membranes, presumptively dead cells, were counted in a haemocytometer with trypan blue staining began to increase at day 2 in infected flasks and gradually rose to the maximum of 4×10^5 cells at day 8 (Figure 5.4). In the control flasks, counts slightly increased from day 2 to peak at day 10 but the maximum number of dead cells in control flasks (1.8×10^5) was still lower than the maximum number in the infected flasks.

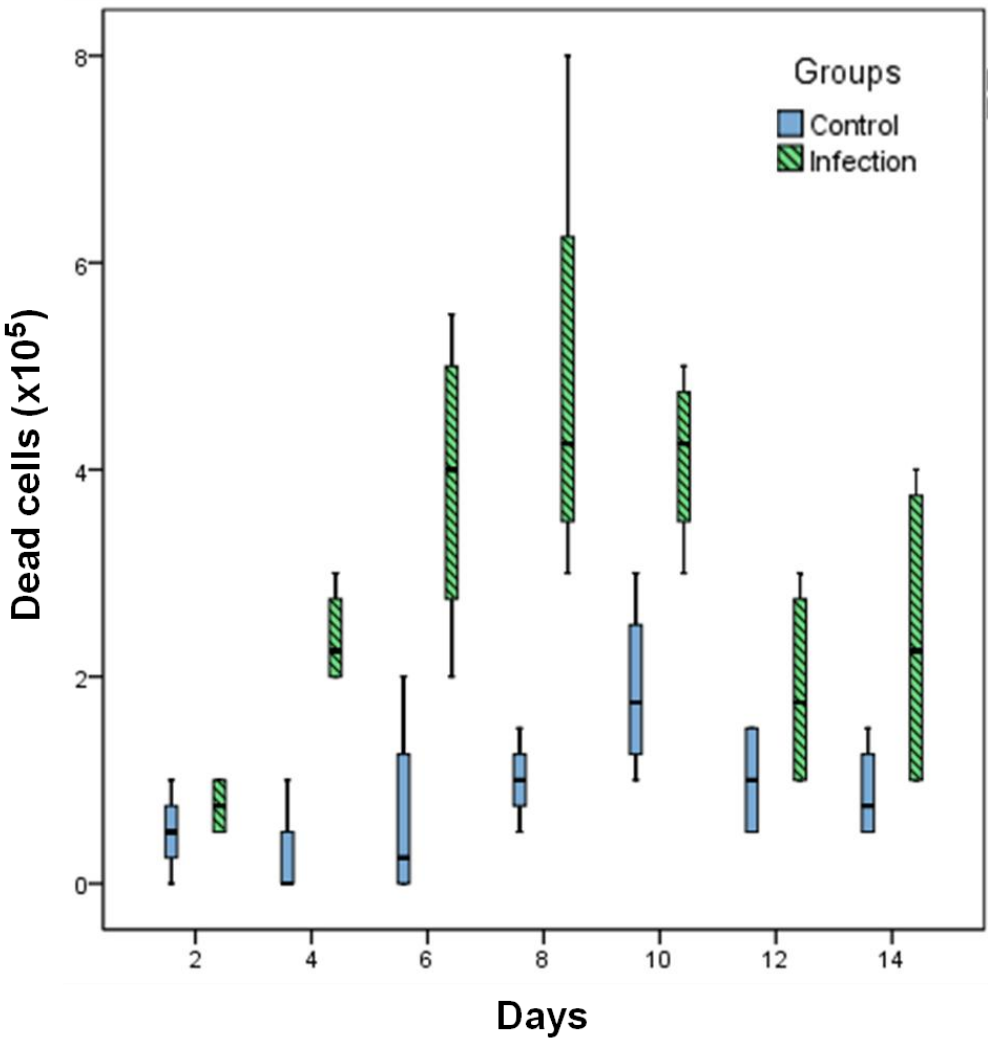

Figure 5.4: Count of damaged, dead cells from control and MrNV infected C6/36 cells using trypan blue exclusion. The number of damaged, dead cells increases from day 2 and peaks at day 8 in the infected cells, while dead cells from control groups increases slightly and peaks at day 10.

5.3.3 Neutral red assay

There was an almost linear relationship between the day of culture and absorbance readings from control C6/36 cells (Figure 5.5). The absorbance reading of neutral red staining from control samples dramatically increased from day 2 and peaked at day 12 at an optical density (O.D.) at just over 3. In the MrNV infected samples, the absorbance reading rose slightly from day 2 to day 4 to an O.D. of 0.6 and began to decrease at day 4 and remained low until day 14. There was a significant difference (Chi-square = 41.265, df = 1, $P < 0.05$) of O.D. between treatments.

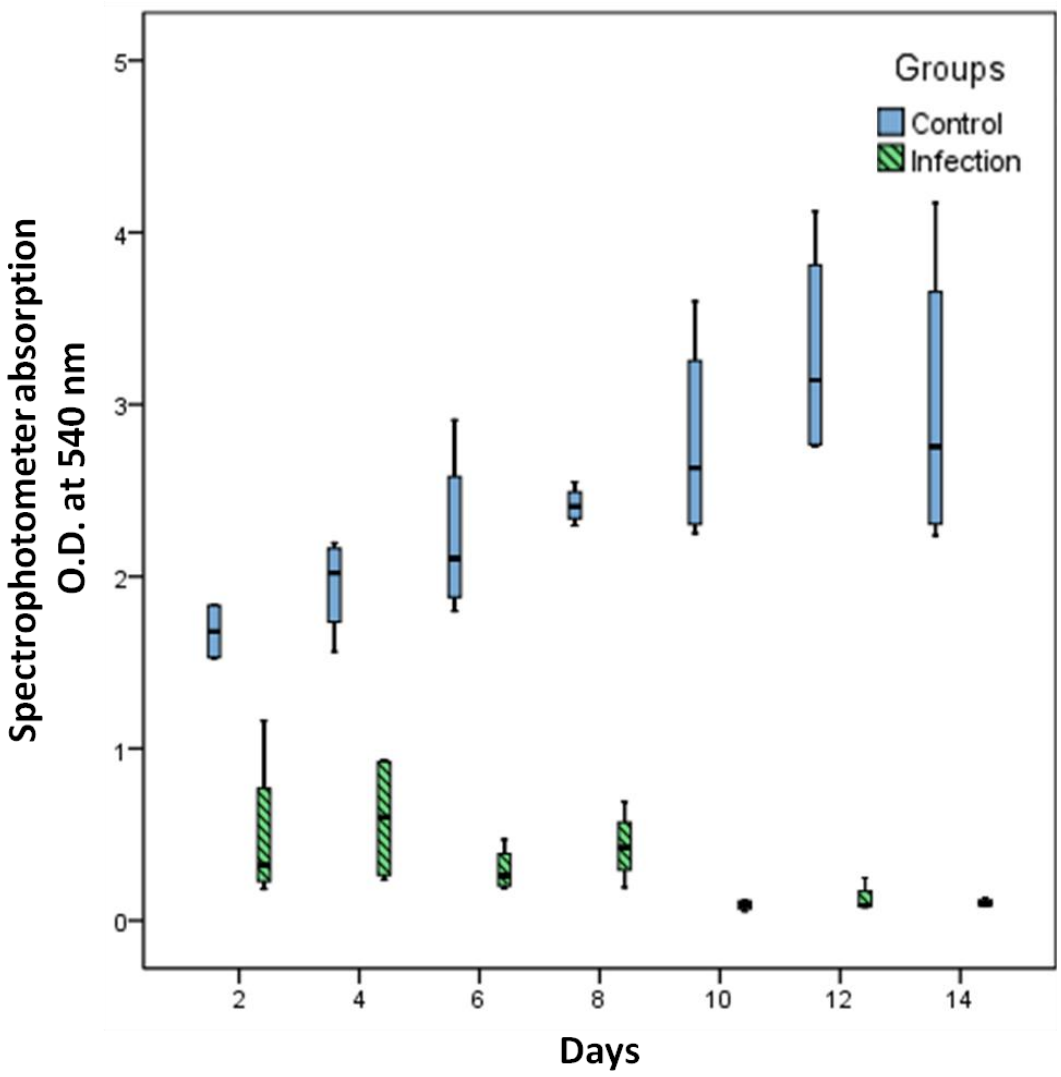

Figure 5.5: The light absorption by neutral red assay of C6/36 cells from control and infected groups in 96-well cell culture plate. A trend of the increasing light absorption begins from day 2 in the control groups, while the light absorption decreases over the same period in infected groups.

5.3.4 TaqMan real-time RT-PCR

The optimum TaqMan reaction mixture including $MgCl_2$ and probe concentrations for TaqMan real-time RT-PCR assay comprised 10 µL of Immomix reaction, 10 µM each of primer, 2.5mM of additional $MgCl_2$, 80 nM of probe, 2.5 µL of DNA template and nuclease-free water to a final volume of 20 µL (Table 5.2). The lowest limit of MrNV detection was found to be 10 copies of the plasmid. Therefore, TaqMan assay can detect the virus from 10^9 down to 10 copies. The TaqMan real-time RT-PCR results from inoculated cells found mean viral copies at

2.07 x 10^4 and mean cycle times (Ct) at 24.12. There appeared to be little increase in titre or change in Ct values over the 14 days experiment (Table 5.3). Also, TaqMan real-time RT-PCR results from 4 passages of C6/36 cell culture with MrNV showed the number of viral copies begun to decrease from 500 viral copies in passage 1 to less than 50 viral copies in passage 4 (Figure 5.6).

Table 5.2: Optimisation of $MgCl_2$ and probe concentrations for *Macrobrachium rosenbergii* nodavirus TaqMan assay. Mean cycle time (Ct) represents four replicates of each concentration.

MgCl2			**Probe**		
Concentration	**Mean Ct**	**Standard Deviation**	**Concentration**	**Mean Ct**	**Standard Deviation**
0 mM	27.56	0.24	**400 nM**	26.39	0.23
0.5 mM	27.24	0.36	**200 nM**	25.94	0.21
1.0 mM	27.20	0.12	**100 nM**	26.02	0.13
1.5 mM	27.00	0.10	**90 nM**	26.23	0.30
2.0 mM	26.32	0.10	**80 nM**	26.20	0.26
2.5 mM	26.16	0.18	**70 nM**	26.53	0.17
3.0 mM	25.79	0.27	**60 nM**	26.76	0.16
3.5 mM	26.05	0.33	**50 nM**	27.00	0.05

Table 5.3: Number of viral copies and cycle times (Ct) of *Macrobrachium rosenbergii* nodavirus for replicate assays in C6/36 cell culture hr 0, 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44, 48, 96, 144, 192, 240, 288 and 336.

Hours	Number of viral copies and cycle times of *Macrobrachium rosenbergii* nodavirus							
	Assay 1 (Copies)	Assay 1 (Ct)	Assay 2 (Copies)	Assay 2 (Ct)	Assay 3 (Copies)	Assay 3 (Ct)	Mean number of viral copies	Mean Ct
0	1.92×10^4	24.13	1.52×10^4	24.52	1.80×10^4	24.27	1.75×10^4	24.31
4	3.71×10^4	23.14	3.42×10^4	23.28	3.35×10^4	23.31	3.49×10^4	23.24
8	1.89×10^4	24.16	1.92×10^4	24.17	1.62×10^4	24.43	1.81×10^4	24.25
12	3.66×10^4	23.16	3.03×10^4	23.47	3.45×10^4	23.27	3.38×10^4	23.30
16	1.68×10^4	24.33	1.65×10^4	24.39	1.68×10^4	24.37	1.67×10^4	24.36
20	1.87×10^4	24.17	1.86×10^4	24.22	2.42×10^4	23.81	2.05×10^4	24.07
24	2.36×10^4	23.82	2.12×10^4	24.01	2.53×10^4	23.74	2.34×10^4	23.86
28	1.39×10^4	24.61	2.82×10^4	23.57	2.02×10^4	24.08	2.08×10^4	24.09
32	1.57×10^4	24.44	1.46×10^4	24.59	1.43×10^4	24.61	1.49×10^4	24.55
36	1.20×10^4	24.85	1.20×10^4	24.89	1.24×10^4	24.84	1.21×10^4	24.86
40	1.72×10^4	24.30	1.26×10^4	24.81	1.78×10^4	24.28	1.59×10^4	24.46
44	2.18×10^4	23.94	3.52×10^4	23.24	3.68×10^4	23.17	3.13×10^4	23.45
48	1.14×10^4	24.92	1.48×10^4	24.56	1.55×10^4	24.50	1.39×10^4	24.66
96	1.67×10^4	24.34	1.87×10^4	24.20	1.86×10^4	24.22	1.80×10^4	24.25
144	1.28×10^4	24.75	1.30×10^4	24.76	1.47×10^4	24.58	1.35×10^4	24.70
192	1.29×10^4	24.73	1.74×10^4	24.31	2.32×10^4	23.88	1.78×10^4	24.31
240	1.88×10^4	24.16	1.67×10^4	24.38	2.10×10^4	24.03	1.88×10^4	24.16
288	3.35×10^4	23.29	2.75×10^4	23.61	2.75×10^4	23.62	2.95×10^4	23.51
336	1.99×10^4	24.08	2.22×10^4	23.95	2.41×10^4	23.82	2.21×10^4	23.95
					Overall means		2.07×10^4	24.12

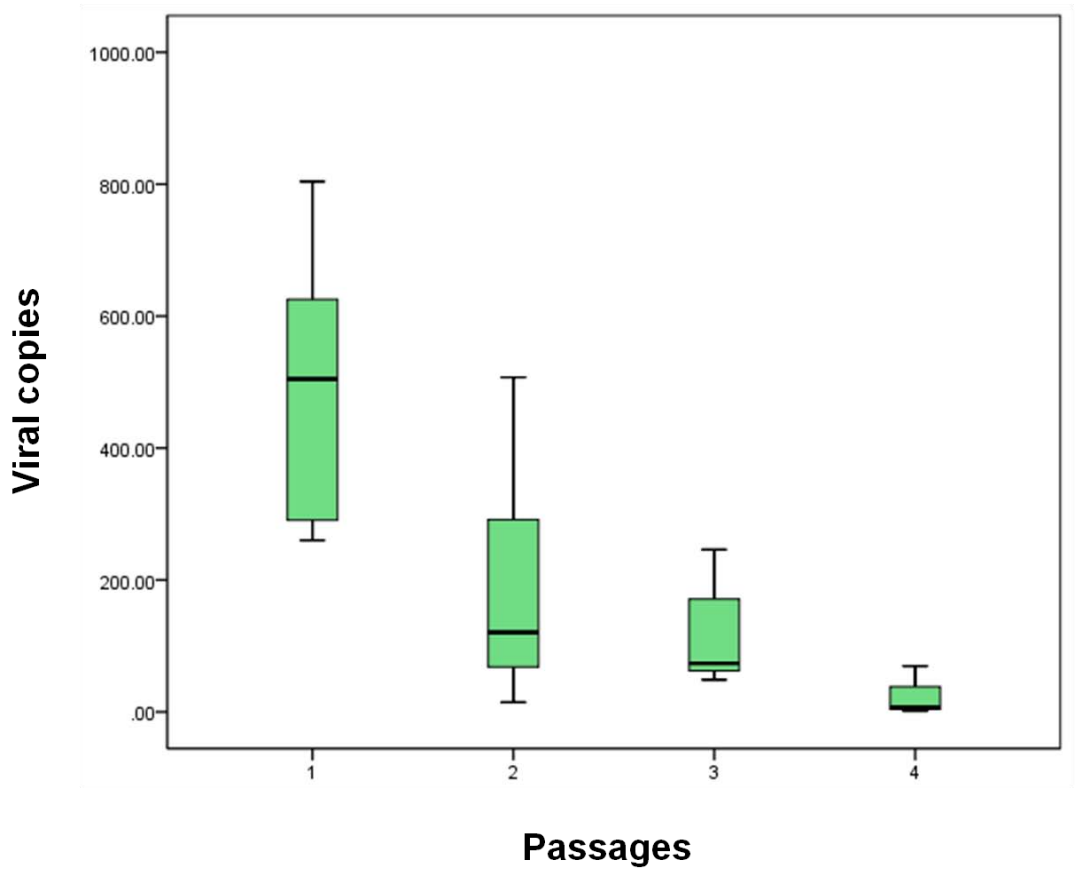

Figure 5.6: The number of viral copies using reverse transcriptase real-time PCR from passage 1 to 4 of C6/36 cell infected with MrNV. A trend of the decreasing viral copies begins from passage 1 (over 500 copies) to passage 4 (less than 50 copies).

5.4 Discussion

The lack of an established crustacean cell line was one reason why researches have worked hard on the viral pathogenesis, viral isolation and viral cultivation of crustacean viruses including MrNV in alternate cell lines (Sudhakaran *et al.*, 2007b). Therefore, SSN and C6/36 cell lines have been used to observe the susceptibility of the cells to MrNV. The partial susceptibility of SSN and C6/36 cells to MrNV has been observed by different stains and RT-PCR (Hernandez-Herrera *et al.*, 2007; Sudhakaran *et al.*, 2007b). In this study, C6/36 cell line is affected by the Australian isolate of MrNV as visualised by histology, nucleic acid proliferation, cell mortality and neutral red assay but not by TaqMan real-time RT-PCR.

Acridine orange and RT-PCR were used to observe viral nucleic acid and multiplication of MrNV in C6/36 cell line (from 1 day to 3 day post-infection) by Sudhakaran *et al*., (2007b). However, no cytopathic effect was found in the phase contrast microscopic examination but multiple vacuoles were observed in the infected cells. The aggregation of numerous MrNV particles in the cytoplasm of C6/36 infected cells was observed under electron microscopy (Sudhakaran *et al*., 2007b).

The progressive changes of C6/36 cells infected with the Australian isolate of MrNV were synthesized into Figure 5.7 using three difference techniques (neutral red assay, trypan blue staining and TaqMan real-time RT-PCR). Neutral red has been used to establish structure-activity relationships of live cells (Babich and Borenfreund, 1990). Neutral red was also used to stain golgi apparatus and lysosomes, and measure mitochondrial activity in cells in order to identify live cells (Winckler, 1974; Babich and Borenfreund, 1990). Trypan blue was used to identify C6/36 cells with disrupted cell membranes in this study. Live cells have intact cell membranes while cell membranes of dead cells are damaged. Trypan blue can enter into damaged cells and stain them and this can identify presumptively dead cells. TaqMan real-time RT-PCR was used to detect the number of viral copies during 14 days.

Figure 5.7 showed the increase of C6/36 cell activity which was observed initially and started to decrease after day 4. Also, the count of disrupted cells rose at day 2 until day 8 and dropped to the end of experiment as cells fully disintegrated. Viral titre results showed a slow decrease of viral copies in infected C6/36 cells during the first 8 days and then slightly increased to the end of study (day 14). However, a relatively low viral tire was observed in this study. Therefore, this is no confirmation of the replication of the Australian MrNV in C6/36 cells.

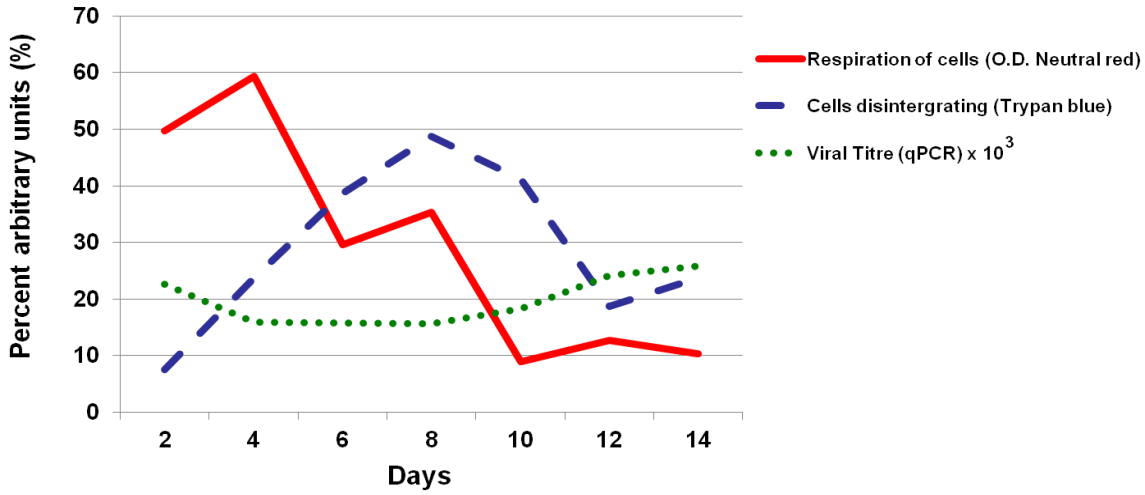

Figure 5.7: Hypothetical composite picture of C6/36 cellular events when infected with the Australian isolate of MrNV using three difference techniques (neutral red assay, trypan blue staining and TaqMan real-time RT-PCR).

TaqMan real-time RT-PCR results in this study demonstrated no increasing titre in serial passages but a drop in titre in each subsequent passage suggesting that C6/36 cell line has limited susceptibility to the Australian isolates of MrNV. Furthermore over 14 days viral titres did not increase (10^4 copies) compared to the inoculated titres at 0 hr. For comparison, hepatopancreatic parvovirus (*Pmerg*DNV) in wild caught banana prawns (*P. merguiensis*) had titres as high as 10^{13} viral copies (La Fauce *et al.*, 2007) suggesting viral replication. Therefore, there is limited evidence to suggest that the Australian isolate of MrNV is replicating efficiently in C6/36 cell line. It appears that MrNV can infect the cells (staining showing the typical cytopathic effect) but infection does not go through to patent infection. This is similar to results in SSN cells where a patent infection was not produced (Hernandez-Herrera *et al.*, 2007). Using previously infected cells with MrNV, Hernandez-Herrera *et al.* (2007) found that the second passage on healthy SSN cells was unable to reproduce the cytopathic effect. With qPCR, the number of viral copies also decreased 10-fold between day 0 to day 7 but an increase of MrNV copies was observed in supernatant. In this study of C6/36 cells with MrNV, qPCR showed a decrease of MrNV viral copies in serial passages. Brackney *et al*. (2010) suggest that C6/36 cells lack a functional antiviral RNAi mechanism against

invertebrate viruses which may be why there is partial replication in the first instance but fully patent virus is not produced as the cells are not quite compatible with the virus.

In conclusion, infected C6/36 cell line is susceptible to the Australian isolate of MrNV in that it showed the typical cytopathic effects such as vacuolations and viral inclusion bodies of MrNV infection with H&E and Giemsa. Also, decreasing light absorption in neutral red assay and high dead cell count with trypan blue from infected C6/36 cells suggested partial susceptibility of C6/36 cell line to the Australian isolates of MrNV. However, TaqMan real-time PCR did not verify replication of MrNV in the cells within 14 days. Clearly, there are effects caused by the initial infection, but the virus does not go on to replicate RNA efficiently and produce a patent infection or perhaps there is an error with the capsidation of the nucleic acid. Perhaps multiple blind serial passages might select a strain of the Australian MrNV that will replicate efficiently and produce a permissive virus/cell line combination for further research.

CHAPTER 6

EXPERIMENTAL INFECTION OF REDCLAW CRAYFISH (*CHERAX QUADRICARINATUS*) WITH MACROBRACHIUM ROSENBERGII NODAVIRUS

6.1 Introduction

The Australian redclaw crayfish (*Cherax quadricarinatus*) is a tropical crayfish that is native to northern Australia and Papua New Guinea. Redclaw crayfish farming in Australia is a developing aquaculture industry and redclaw is slowly becoming economically viable (Jones, 1998; La Fauce *et al.*, 2007). Anderson and Prior (1992) first reported a virus, a presumed baculovirus, in *C. quadricarinatus*. Since then, a number of viruses have been described from farm-reared redclaw crayfish including bacilliform viruses, parvoviruses and reoviruses (Herbert, 1987; Anderson and Prior, 1992; Groff *et al*., 1993; Owens and McElnea, 2000; Bowater *et al*., 2002; Edgerton *et al*., 2000). Redclaw crayfish (*C. quadricarinatus*) is susceptible to many viral diseases; therefore, *C. quadricarinatus* may be susceptible to MrNV. Possibly, *C. quadricarinatus* may be developed as an experiment animal model for WTD. However, no report on WTD in *C. quadricarinatus* has been published.

Ribonucleic acid interference (RNAi) is a regulatory process that can be used to control and limit the expression of some genes of the viruses. In 2009, RNAi was studied in order to control viral infection in an *in vivo* cricket model (La Fauce and Owens, 2009). In Australia, *Macrobrachium* can be hard to source due to their need for a saltwater environment for breeding and no alternative experimental animal model for MrNV has been reported. This study aimed to determine the susceptibility of the redclaw crayfish (*C. quadricarinatus*) to the Australian MrNV isolate in order to use *C. quadricarinatus* with RNAi studies in the future.

6.2 Materials and Methods

6.2.1 Experimental animals

Juvenile *C. quadricarinatus* 5 to 8 cm in length were obtained from a commercial crayfish farm in northern Queensland and the breeding facility at James Cook University. Crayfish were housed in aquaria 45 cm wide, 150 cm in length and 40 cm high and fed commercial crustacean diet once a day. Water exchanges were performed daily to maintain appropriate water quality (Chapter 3.2).

6.2.2 Preparation of inoculum

As a positive control, infected muscle from *M. rosenbergii* was confirmed by histopathology and PCR to contain MrNV. The inoculum was prepared from infected muscle which was homogenized in phosphate buffered saline (PBS) and filtered though a 45 μm filter (Chapter 3.1). Thirty μl of inoculums (10^4 viral copies/μl) were injected intramuscular into the ventral side of the first abdominal segment of crayfish, just to the side of the ventral nerve cord.

6.2.3 Infectious challenge

Sixty redclaw crayfish were randomly distributed between six experimental aquaria divided into three treatments; control, feeding and inoculated crayfish with two replicates in each treatment. After 24 hr starvation, feeding crayfish were orally challenged with MrNV only on day 0 with approximately 5% of body weight of muscles from infected *M. rosenbergii*. Prior to inoculation, crayfish were anaesthetized by being placed in chilled water. Each animal received approximately 30 μl of inoculum (intramuscular), and following injection placed immediately into the experimental aquaria; no further injections were given. Double strength PBS (2x PBS) was injected intramuscularly into the control crayfish. The experimental period began on the day of injection (day 0) and concluded on day 60.

Redclaw crayfish were monitored daily for clinical signs and dead crayfish were removed. Dead crayfish were immediately prepared for histology and quantitative

real-time polymerase chain reaction (qPCR) by splitting the cephalothorax longitudinally. The first half of the cephalothorax was split into two parts. The first part was placed in Davidson's fixation for histopathology while the second part was put in 95% ethanol for qPCR. The remaining half of the cephalothorax was stored frozen at -20°C. At the end of the experiment period, all remaining crayfish were sacrificed, their length and weight recorded and processed appropriately for screening by histological examination and qPCR (Chapter 3.3).

6.2.4 Histopathology

After 48 hr in Davidson's fixation, the cephalothorax, gills and muscles of redclaw crayfish were collected and continued the histopathological process. The histopathological process was performed as described in Chapter 3.4.

6.2.5 RNA extraction and qPCR for MrNV

RNA was extracted from approximately 30 mg of crayfish muscles in 95% ethanol for cDNA amplification using SV total RNA isolation system (Promega, Australia) according to Chapter 3.5.1. cDNA was produced using ImProm-II Reverse Transcriptase System (Promega, Australia) following the manufacturer's protocol (Chapter 3.5.1) before DNA amplification (Immomix, Bioline Australia). The specific primers and probe were designed from completed sequence of MrNV RNA1 (GenBank accession number AY222839 (the French West Indies), AY231436 (China) and FJ751226 (Thailand)) to amplify 198 bp of PCR product. Probe, forward and reverse primers for MrNV were performed as showed in Table 5.1. The cycle process consisted of incubation at 95°C for 10 min, followed by 40 cycles at 95°C for 10s and 60°C for 60s. Three reactions of 20 µl were performed independently on different days to determine the specificity and reproducibility of the assay. Data acquisition and analysis were performed using Rotor-Gene 6000 (Corbett Robotics). The experiment was performed as described in Chapter 3.5.3.4.

6.2.6 Standard curve for qPCR

In order to make a standard curve for qPCR, a positive control plasmid was prepared. The specific primer for the plasmid was designed from completed sequence of MrNV RNA1 (GenBank accession number AY222839 (the French West Indies), AY231436 (China) and FJ751226 (Thailand)) according to Chapter 3.5.3.1. The forward and reverse primers for MrNV were 5'- CTC TTG ATC GTG TCA GTG GA -3' at positions 2425 bp to 2445 bp and 3'- CAG GCA TTG CTT ACC ACG TT -5' at positions 3185 bp to 3205 bp respectively. The PCR products were purified using Wizard® SV Gel and PCR Clean-UP system (Promega, Australia) and directly transformed into *Escherichia coli* JM 109 High Efficiency cells using pGEM-T® Easy Vector System (Promega, Australia) for cloning, according to Chapter 3.5.4. Plasmids with DNA inserts of MrNV were sent to Macrogen Inc (Seoul, Korea) for sequencing to confirm the quality of the sequences.

6.2.7 Optimisation of $MgCl_2$ concentration

Optimised the $MgCl_2$ concentration was by 0.5 mM increments of $MgCl_2$ to the mastermix (Immomix, Bioline Australia) against an aliquot of MrNV positive control plasmid. The experiment was performed as described in Chapter 3.5.3.2.

6.2.8 Optimisation of probe concentration

Four replicates of mixture containing probe concentrations of 50, 60, 70, 80, 90, 100, 200 and 400 nM were optimized in the TaqMan PCR. The experiment was performed as described in Chapter 3.5.3.3.

6.2.9 Statistical analyses

Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) version 18. Mortalities found in different treatments were evaluated by a one-way univariate analysis of variance (one-way ANOVA). Length and weight of stunted animals were also statistically evaluated by one-way ANOVA. The data

needed to be transformed to a log scale in order to meet the requirements of the homogeneity of variance and to be normal distributed.

6.3 Results

6.3.1 Mortality

The first experimental treatment displayed the highest mortality rate in the injection groups at 75% and the lowest mortality at 15% in the control groups while feeding groups showed 55% mortality (Figure 6.1). The statistical analysis of mortality in this experiment showed no significantly difference ($F = 5.895$, $df = 2,5$, $P > 0.05$) between control and infected groups. This appears erroneous and perhaps there was contamination of the control groups. Therefore, the experiment was repeated and also to check all samples with real-time RT-PCR in order to confirm the infection.

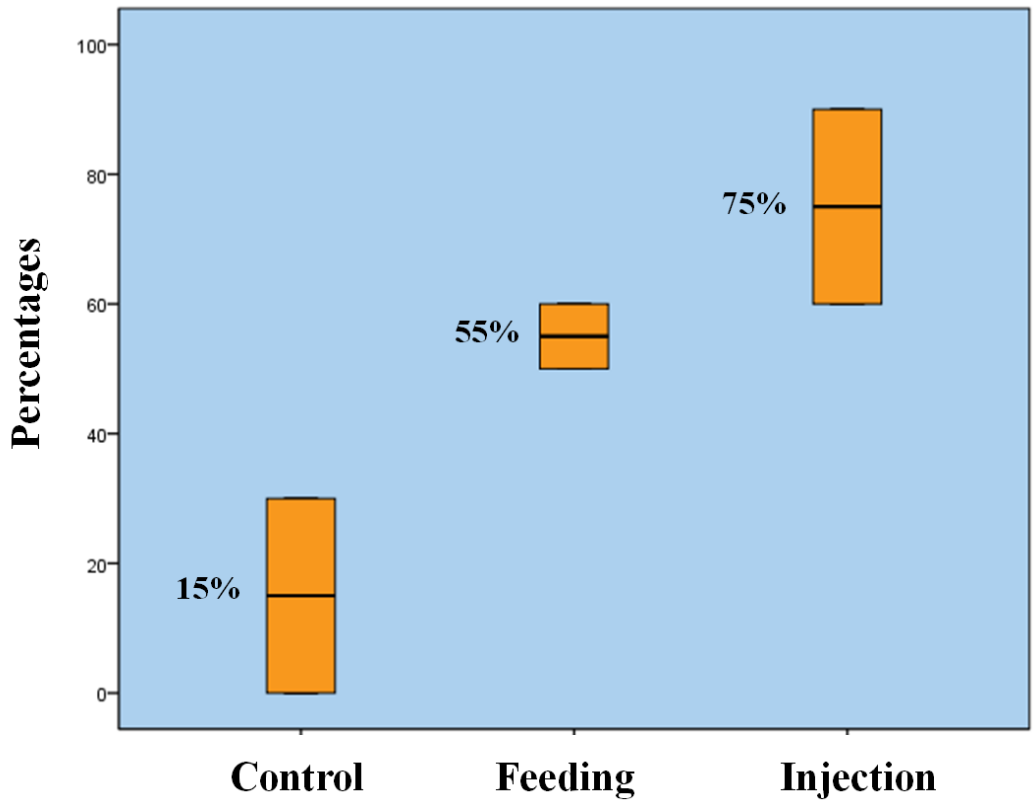

Figure 6.1: The percentage of mortalities in juvenile *Cherax quadricarinatus* over a 60 day pathogenicity trial in experiment 1. WTD were administered by feeding and inoculation

In the second experiment, the inoculated groups displayed the highest mortality rate at 35% (7/20), while the control groups had the lowest mortality at 0% (0/20) and the feeding groups showed 20% (4/20) mortality (Figure 6.2). Statistically, there was a significant difference in mortality ($F = 37$, $df = 2,5$, $P < 0.05$) between treatments. Infected *C. quadricarinatus* developed clinical signs consistent with WTD: inability to swim normally, reduced appetite and pale exoskeleton and death following clinical signs, while crayfish from control groups had normal swimming behaviour. The infected crayfish displayed muscle necrosis and myositis (Figure 6.3).

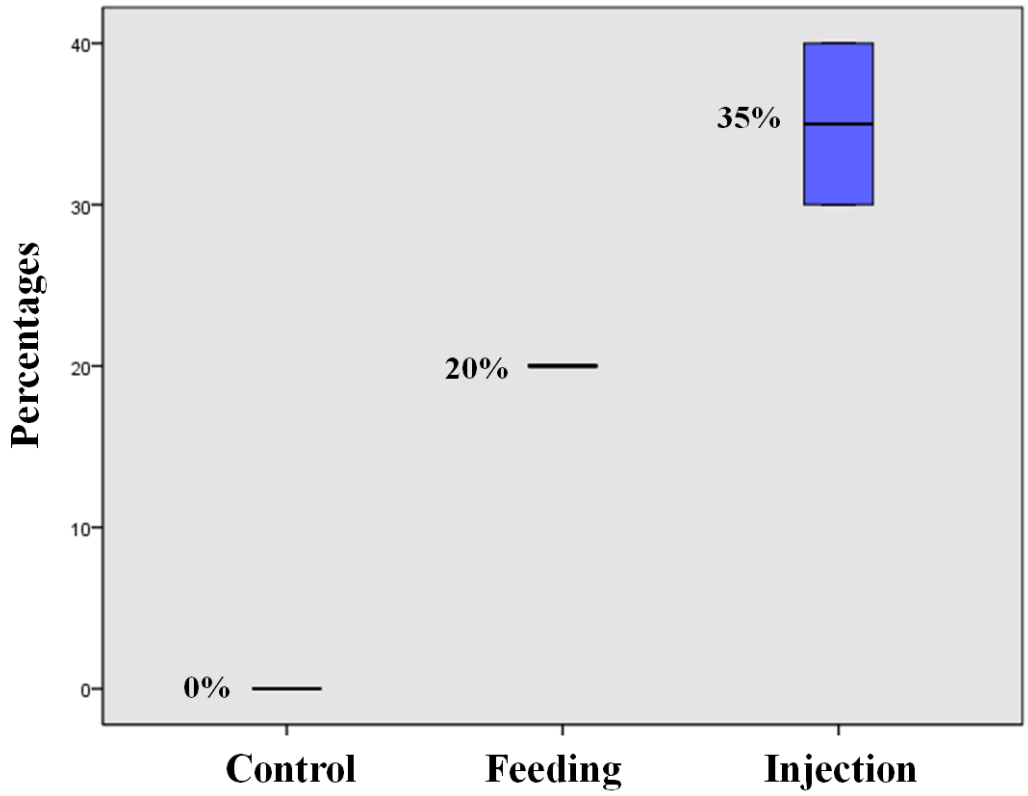

Figure 6.2: The percentage of mortalities in juvenile *Cherax quadricarinatus* over a 60 day pathogenicity trial in experiment 2. WTD were administered by feeding and inoculation

Figure 6.3: Gross lesion of white tail disease is white muscle in infected crayfish (*Cherax quadricarinatus*)

Crayfish varied in size between treatments in the second experiment. The inoculated crayfish had the smallest size (9.96 ± 0.99 cm) and lowest weight (22.34 ± 5.76 g) when compared to the feeding and control groups. The mean length of the control and feeding groups were 10.96 ± 0.68 cm and 10.33 ± 0.98 cm, respectively. The mean weight of the control treatment was 27.17 ± 5.60 g, while feeding groups had a mean weight of 25.88 ± 7.79 g. The statistical analysis of length showed a significant difference ($F = 5.509$, $df = 2,48$, $P < 0.05$) between different treatments, while the weight did not indicate a significant difference ($F = 2.264$, $df = 2,48$, $P > 0.05$). However, length and weight did not have a significantly difference ($F = 0.269$, $df = 2,30$, $P > 0.05$ and $F = 2.097$, $df = 2,30$, $P > 0.05$, respectively) in experiment 1.

6.3.2 Histopathology

6.3.2.1. Histopathological results of experiment 1

The main target of the nodavirus was in the muscle. The gross lesions included gross lesions of necrotic muscle in infected redclaw crayfish (Figure 6.3). No muscle lesion was seen in control groups. Viral necrotic lesions and reovirus inclusions were

easily observed in the hepatopancreas (Figure 6.4). Melanisation (Figure 6.5) and fungal infection (Figure 6.6) were found in the gills of infected crayfish. The summery of lesions is shown in Table 6.1.

Table 6.1: Table shows the summery of lesions which have been observed under light microscopy in experiment 1.

Groups	Myolysis (%)	Melanisation (%)	Reovirus (%)	Bacteria (%)	Fungus (%)
Control	7.14	26.19	14.29	2.38	0
Feeding	80.95	50	4.76	9.52	11.9
Injection	79.55	34.09	11.36	6.82	0

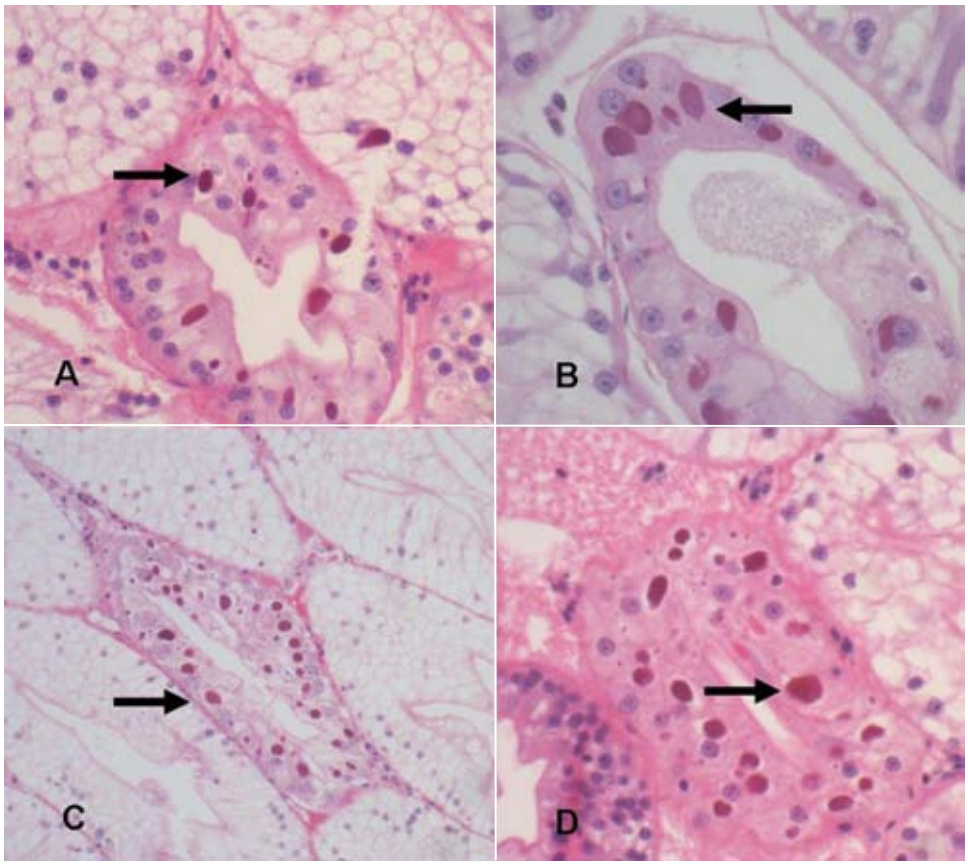

Figure 6.4: Histopathology of crayfish reovirus-infected hepatopancreatic tissues of crayfish (*Cherax quadricarinatus*). A, B, C and D: the inclusions are within cytoplasmic vacuoles and some inclusions are closely associated with the nucleus

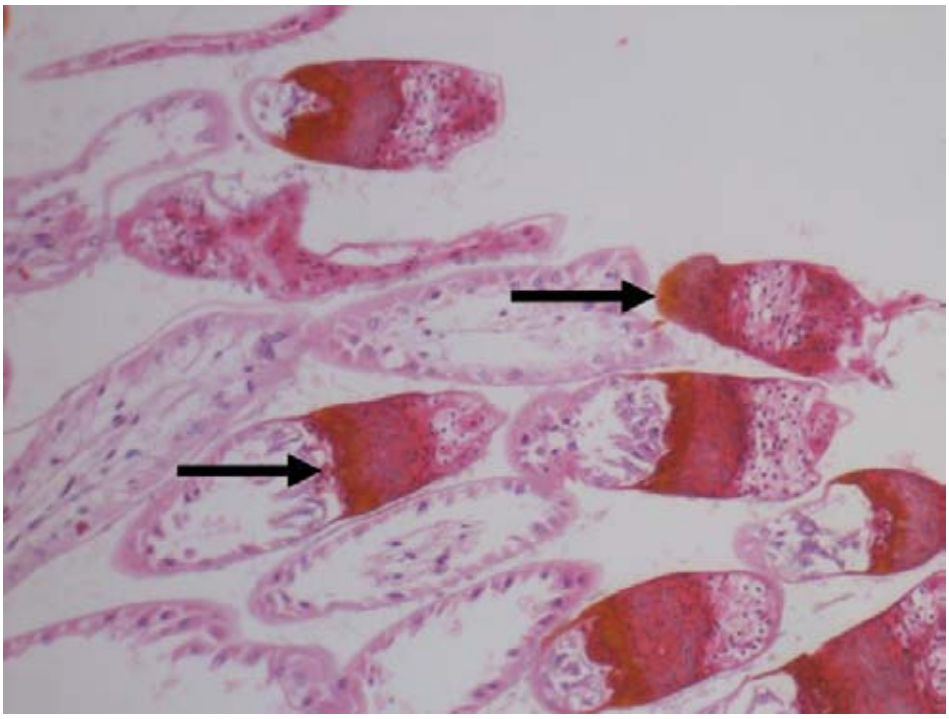

Figure 6.5: Melanisation in the gills of crayfish (*Cherax quadricarinatus*)

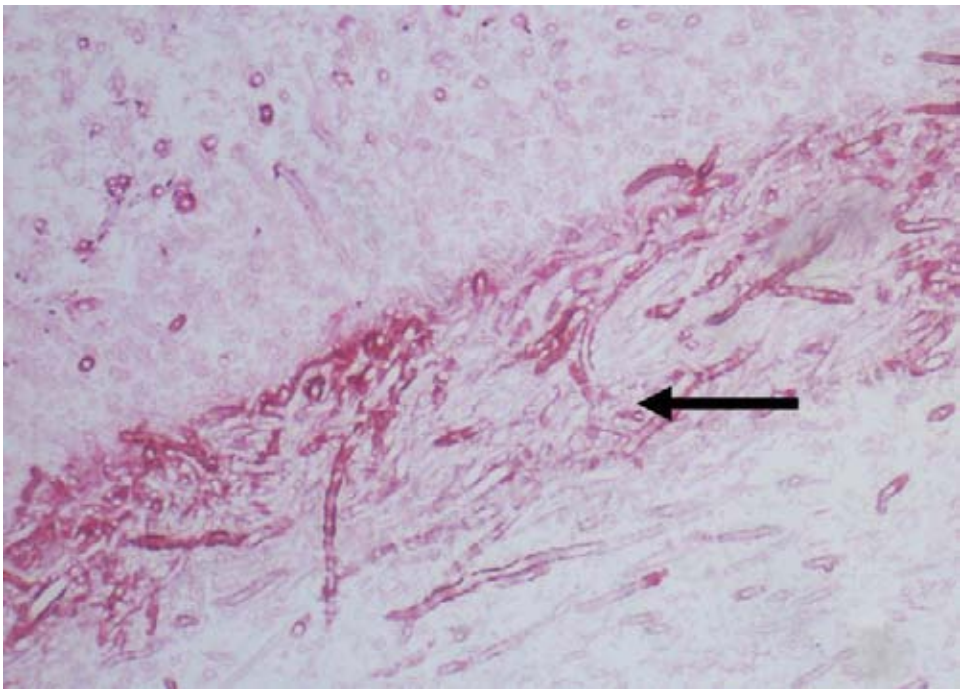

Figure 6.6: Fungal infection in the gills of crayfish (*Cherax quadricarinatus*)

6.3.2.1 Histopathological results of experiment 2

In infected crayfish, the gross lesions included necrotic muscle and muscle degeneration (Figure 6.7A and 6.7B). Inflammatory cells were observed which consisted of a massive infiltration of myonuclei and some haemocytes (myositis; Figure 6.7C and 6.7D). Some myolysis without inflammatory cells was seen in control groups (7.5%) while in the feeding and inoculated groups had 52.5% and 67.5% myolysis with inflammatory cells, respectively (Table 6.2). However, no viral inclusion bodies were observed in infected and control animals. Systemic granulomas in the hepatopancreas of infected animals were observed (Figure 6.8).

Table 6.2: Table shows the summery of lesions which have been observed under light microscopy in experiment 2.

Groups	Myolysis (%)	Melanisation (%)	Reovirus (%)	Bacteria (%)	Fungus (%)
Control	7.5	15	0	10	0
Feeding	67.5	50	0	15	0
Injection	87.5	45	0	27.5	0

The summery of myolysis and myolysis with inflammatory cells also showed in Table 6.3 that has been observed in experiment 1 and 2 that myolysis with inflammatory cells showed damaged muscle by viruses. However, no viral inclusion bodies were observed in this study.

Table 6.3: Table shows the summary of myolysis and myolysis with inflammatory cells which have been observed in experiment 1 and 2.

Groups	Experiment 1		Experiment 2	
	Myolysis (%)	(%) Myolysis with inflammatory cells	Myolysis (%)	(%) Myolysis with inflammatory cells
Control	7.14	100	7.5	0
Feeding	80.95	75	67.5	52.5
Injection	79.55	77.5	87.5	67.5

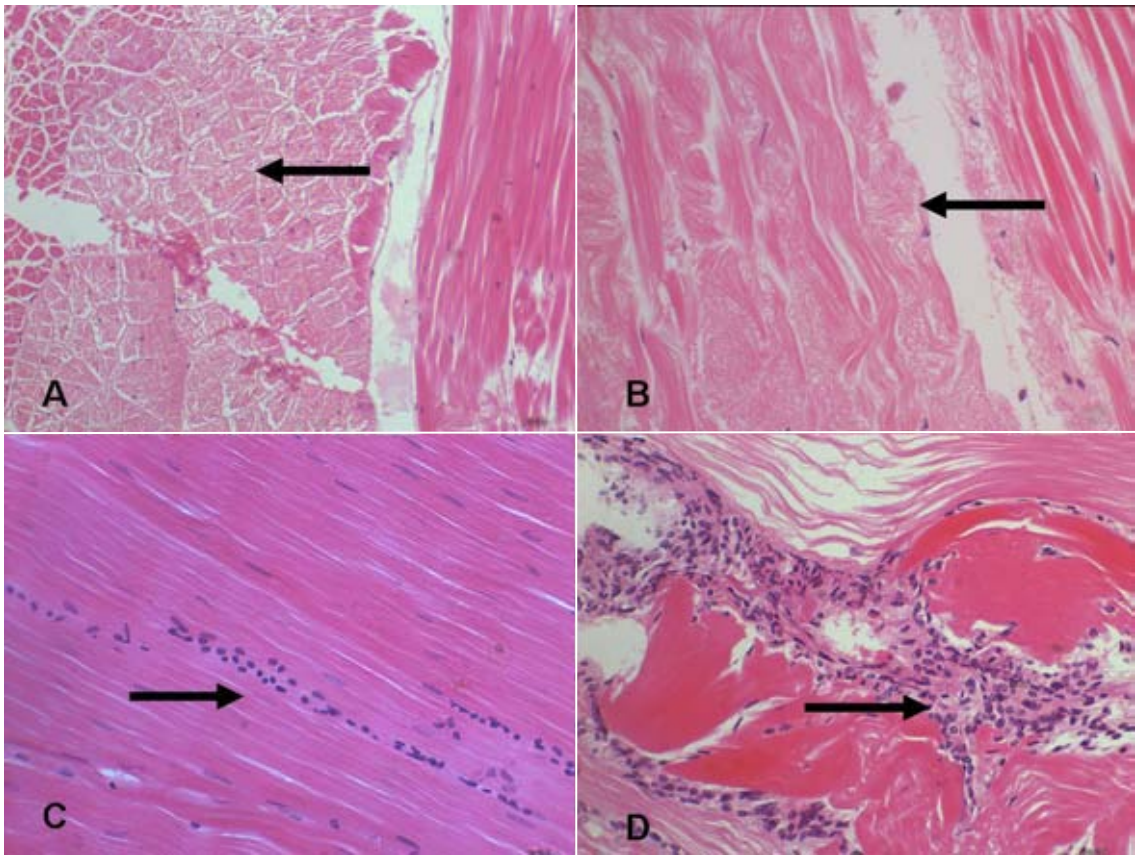

Figure 6.7: A&B Muscle degeneration (arrow) and necrotic tissues in crayfish (*C. quadricarinatus*) from feeding treatment experimentally infected with MrNV, C&D Myolysis with haemocytic infiltration (arrow) in infected crayfish muscle from inoculated treatment.

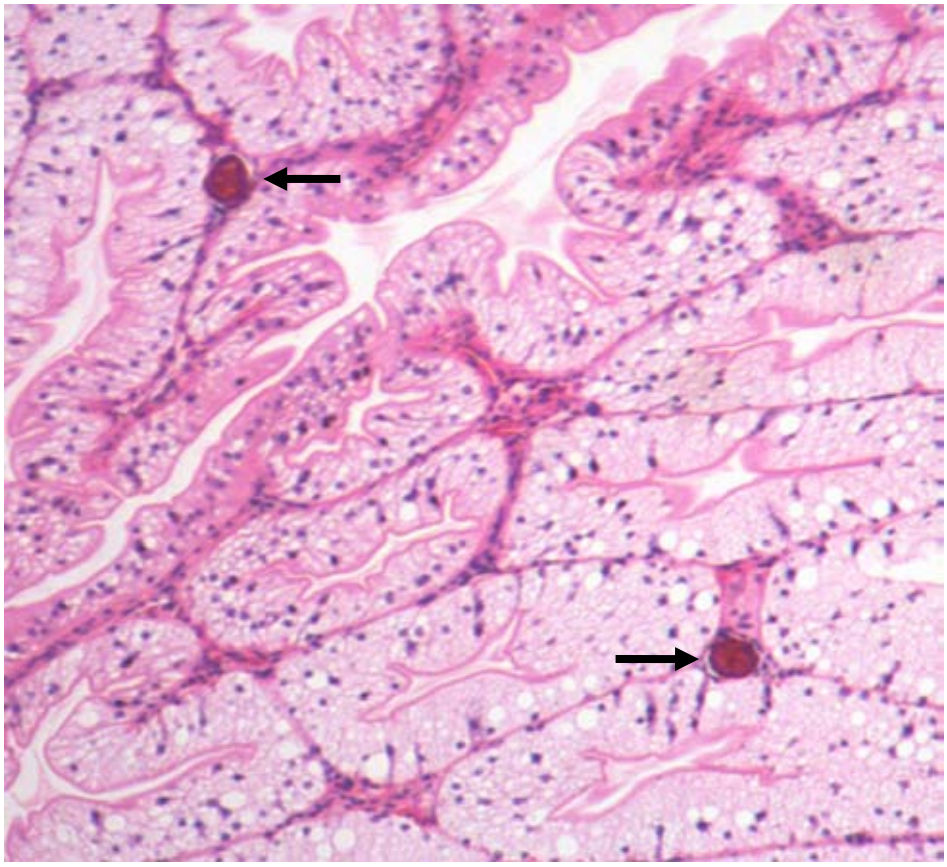

Figure 6.8: Systemic granulomas (arrows) in the hepatopancreas crayfish (*C. quadricarinatus*) from feeding treatment.

6.3.3 Quantitative real-time RT-PCR of the second experiment

Thirteen out of 40 samples from infected redclaw crayfish presented positive results in qPCR. The number of viral copies varied from 10^3 to 10 copies and cycle times varied from 25 to 36 (Table 6.4). The mean viral copies and mean cycle times of positive samples were 2.73 x 10^2 and 31.33, respectively.

Table 6.4: Number of viral copies and cycle time of *Macrobrachium rosenbergii* nodavirus for replicate assays in juvenile *Cherax quadricarinatus* that were positive. Crayfish 1 and 2 were sampled from feeding treatment while crayfish 3 to 13 were sampled from inoculated treatment. The remaining 47 animals were negative.

Table 2: Number of viral copies and cycle time of *Macrobrachium rosenbergii* nodavirus for replicate assays in juvenile *Cherax quadricarinatus* that were positive. The remaining 47 animals were negative.

Crayfish	Number of viral copies and cycle times of *Macrobrachium rosenbergii* nodavirus							
	Assay 1 (Copies)	Assay 1 (Ct)	Assay 2 (Copies)	Assay 2 (Ct)	Assay 3 (Copies)	Assay 3 (Ct)	Mean number of viral copies	Mean Ct
1	2.14×10^1	32.62	9.17×10^1	31.38	7.87×10^1	31.62	6.39×10^1	31.87
2	2.25×10^1	29.03	1.46×10^1	27.12	6.81×10^1	28.29	3.51×10^1	28.15
3	5.91×10^1	29.63	5.78×10^1	28.54	6.22×10^1	28.43	5.97×10^1	28.87
4	3.58×10^1	32.83	4.13×10^1	32.61	2.44×10^1	33.42	3.38×10^1	32.95
5	8.61×10^1	32.48	3.16×10^1	33.02	1.69×10^1	33.99	4.49×10^1	33.16
6	1.51×10^1	34.16	4.20×10^1	36.13	4.91×10^1	32.34	3.54×10^1	34.21
7	3.99×10^1	32.66	1.41×10^1	30.72	7.80×10^1	31.63	4.40×10^1	31.67
8	2.71×10^3	26.17	2.18×10^3	26.50	3.25×10^3	25.89	2.71×10^3	26.19
9	2.57×10^1	32.16	1.14×10^1	33.14	3.24×10^1	32.98	2.32×10^1	32.76
10	1.48×10^2	30.65	3.08×10^2	29.52	2.84×10^2	29.64	2.47×10^2	29.94
11	1.96×10^1	33.76	1.89×10^1	33.82	3.41×10^1	32.91	2.42×10^1	33.50
12	3.87×10^1	32.71	6.09×10^1	32.01	4.08×10^1	32.63	4.68×10^1	32.45
13	1.21×10^2	30.95	1.35×10^2	30.79	2.89×10^2	33.16	1.82×10^2	31.63
					Overall means		**2.73×10^2**	**31.33**

6.4 Discussion

This study was the first report of experimental infection of *C. quadricarinatus* with MrNV. In 2009, WTD infected *M. rosenbergii* were first reported in Australia (Owens *et al*., 2009) but no report has been published on MrNV in *C. quadricarinatus.* In this study, infected redclaw crayfish showed clinical signs such as a reduced appetite and WTD lesions like muscle degeneration (myolysis). However, because of the opaque exoskeleton, the gross lesions were not as easy to observe as in *M. rosenbergii* which has a translucent exoskeleton.

Mortality, growth changes, clinical signs, gross lesions, histopathology and qPCR associated with MrNV infection presented in juvenile *C. quadricarinatus.* This is the first time a qPCR was developed for use on clinical samples. However, the viral titres by qPCR were no higher than 10^3 copies. In contrast, hepatopancreatic parvovirus (*Pmerg*DNV) in wild caught banana prawns (*P. merguiensis*) had titres as

higher as 10^{13} viral copies (La Fauce *et al*., 2007). Therefore, there is limited evidence to suggest that MrNV is replicating efficiently in juvenile *C. quadricarinatus.*

Some myolyses were observed in control animals but no inflammatory cells were seen (Table 6.1). Also the percentages of myolysis in feeding treatment and inoculated treatment were higher than the percentage of MrNV-PCR positive animals in feeding treatment and inoculated treatment. Therefore, some other factor beside virus is probably causing lesions or perhaps in infected animals MrNV caused muscle damage but the viral copies reduced to below detection levels.

In conclusion, this study is the first report of MrNV infection and induced mortality in juvenile redclaw crayfish (*C. quadricarinatus*) in Australia. Also we were able to determine that redclaw crayfish has limited susceptibility and limited potential as a carrier of MrNV. However, *C. quadricarinatus* maybe a less-than-perfect experimental animal model for WTD because the results of mortality, clinical signs, gross lesions and histopathology showed significant changes relative to non-infected crayfish. Therefore, *C. quadricarinatus* may be used to study RNAi in order to control MrNV in the future. Further work is needed to analyse the MrNV sequences of Australian isolates in order to understand their relationship to strains in other countries.

CHAPTER 7

RNA INTERFERENCE REDUCES *MACROBRACHIUM ROSENBERGII* NODAVIRUS REPLICATION IN REDCLAW CRAYFISH (*CHERAX QUADRICARINATUS*)

7.1 Introduction

Invertebrates lack the protein-based adaptive immunity that prevents viral infection but they are still capable of effective viral defence mechanisms such as RNA interference (RNAi) (Brennan and Anderson, 2004). RNA interference is the antiviral mechanism that allows cells to control the expression of undesirable messenger RNA (mRNA) using a double-stranded RNA (dsRNA) template (Tirasophon *et al*., 2005). The recognition of intracellular dsRNA triggers RNAi to degrade the sequence specific homologous viral RNA (Hannon, 2002). RNAi has been studied in many eukaryotic organisms such as protozoa (Bastin *et al*., 2001), fungi (Raoni and Arndt, 2003), algae (Wu-Scharf *et al*., 2000), nematode (Fire *et al*., 1998), insects (La Fauce and Owens, 2009), fish (Dang *et al*., 2008), mammals (Caplen *et al*., 2001) and plants (Fagard and Vaucheret, 2000). Also, RNAi has been used to protect against viral infection and viral replication of prawn viruses such as yellow head virus (YHV) (Tirasophon *et al*., 2005), white spot syndrome virus (WSSV) (Xu *et al*., 2007) and *Penaeus merguiensis* densovirus (La Fauce and Owens, 2009). However, no report has been published on RNAi against MrNV.

A recent study (Chapter 6) demonstrated that the Australian freshwater, or redclaw crayfish (*Cherax quadricarinatus*) can be used as an experimental animal model for MrNV. MrNV protein B2 is produced to inhibit the degradation of sequence-specific viral RNA in cells. Therefore, RNAi was targeted in a sequence-specific manner against the viruses' protective mechanism to inhibit the production of protein B2; in effect an "arms race" between the virus and host cell. In this current study, we aim to investigate whether a triggered RNAi targeting protein B2 is protective, decreasing mortality and inhibiting MrNV replication in infected animals.

7.2 Materials and Methods

7.2.1 Redclaw crayfish

Juvenile *C. quadricarinatus* or redclaw crayfish (five to eight cm in length) were obtained from a commercial crayfish farm in northern Queensland. *C. quadricarinatus* were housed in aquaria 50 cm in width, 50 cm in length and 40 cm in height. *C. quadricarinatus* were fed a commercial crustacean diet once a day. Water exchanges were performed daily to maintain appropriate water quality (Chapter 3.2).

7.2.2 Preparation of inoculum

The inoculum was prepared from infected muscle of *M. rosenbergii* confirmed by histopathology and PCR to contain MrNV. This was homogenized in phosphate buffered saline (PBS) and filtered though a 45 µm filter according to Chapter 3.1. Thirty microliters of inoculum were injected intramuscularly into the ventral side of the first abdominal segment of crayfish, just to the side of the ventral nerve cord in accordance with the protocol described in Chapter 6.2.2.

7.2.3. Stealth RNAi design and Stealth RNAi assay in redclaw crayfish

Stealth RNAi and Stealth control RNAi duplexes were designed online using BLOCK-iT^{TM} RNAi Designer (Invitrogen, Australia) against protein B2 of Australian MrNV (Table 7.1). The Stealth control RNAi sequence is different from stealth RNAi to understand the specificity of RNAi controls with MrNV. Sixty reddclaw crayfish (*C. quadricarinatus*) were randomly distributed between twelve experimental aquaria divided into six treatments; (1) untouched controls, (2) placebo controls, (3) control Stealth RNAi + double strength PBS (2xPBS), (4) Stealth RNAi + 2xPBS, (5) Stealth RNAi + MrNV and (6) control Stealth RNAi + MrNV (Table 7.2) with two replicates of five redclaw crayfish in each treatment. Redclaw crayfish in each treatment received two injections: the first on day 0 and the second 24 hr later while redclaw crayfish in the untouched control treatment were not inoculated. Redclaw crayfish were anaesthetized by being placed in chilled water

(4°C) before being inoculated with 2xPBS, control Stealth RNAi, Stealth RNAi and MrNV using sterile 1 ml syringes and 26-gauge needle. Redclaw crayfish in the appropriate treatments were challenged with 30 µl of 2xPBS and 2 µg of Stealth control RNAi, 2 µg of Stealth RNAi and 10^4 virions of MrNV. The infectious challenge experiment of MrMV began on the day of injection (0 day) and concluded on day 60.

Table 7.1: Stealth RNAi and control Stealth RNAi sequences were designed using BLOCK-iT^{TM} RNAi Designer (Invitrogen, Australia) targeting protein B2 of the Australian MrNV isolate. Underlined, bold and letter 'd' in the Stealth RNAi and Stealth control RNAi sequences indicate a different base pair, an additional base pair and a deleted base pair, respectively.

Stealth RNAi	**Sequence of Stealth RNAi (5' – 3')**
Stealth RNAi (protein B2 target)	CAC<u>C</u>GACAACCU<u>A</u>CUUUCAAAGCCA
Control Stealth RNAi (Control)	CAC<u>A</u>dACAAddU<u>C</u>CUUUCAAACGGCCCA

Table 7.2: Redclaw crayfish (*Cherax quadricarinatus*) were divided into six treatments for knockdown of MrNV protein B2 using Stealth RNAi.

Treatments	**First inoculation (0 hr)**	**Second inoculation (24 hr)**
1. Untouched controls	N/A	N/A
2. Placebo controls	2xPBS	2xPBS
3. Control Stealth RNAi + 2xPBS	Control Stealth RNAi	2xPBS
4. Stealth RNAi + 2xPBS	Stealth RNAi	2xPBS
5. Stealth RNAi + MrNV	Stealth RNAi	MrNV
6. Control Stealth RNAi + MrNV	Control Stealth RNAi	MrNV

Clinical signs of MrNV infection were monitored daily and dead redclaw crayfish were removed. Dead crayfish were immediately measured for weight and length and then prepared for histopathology and quantitative real-time polymerase chain reaction (qPCR) by splitting the whole body longitudinally as described in Chapter 3.3. At the end of the experiment, all remaining crayfish were sacrificed, and

their weight and length were recorded and processed appropriately as above for screening by histopathology and qPCR.

7.2.4. Histopathology

The cephalothoraxes, gills and muscles of redclaw crayfish were collected after 48 hr in Davidson's fixation and transferred to 70% ethanol, then dehydrated through a series of alcohols to xylene and embedded in paraffin wax. The histopathological process was performed as described in Chapter 3.4. The histopathological sections (20 slides of tissues) were screened under light microscopy (Olympus E C microscope). Digital photographs of histopathological lesions were taken with a MicroPublisher 5.0 RTV camera.

7.2.5. RNA extraction and qPCR for MrNV

RNA was extracted from approximately 30 mg of redclaw crayfish muscles in 95% ethanol using SV total RNA isolation system (Promega, Australia) (Chapter 3.5.1). RNA was reversed transcribed to cDNA and DNA was amplified using SensiMix™ Probe One–Step Kit (Bioline, Australia) as described in Chapter 3.5.3.4. Data analysis and acquisition were performed using Rotor-Gene 6000 (Corbett Robotics).

7.2.6. Standard curve and optimisation for qPCR

A positive control plasmid was prepared in order to make a standard curve and optimisation of probe and $MgCl_2$ concentration for qPCR according to the protocol described in Chapter 3.5.3.2 and 3.5.3.3. The forward primer (5'- CTC TTG ATC GTG TCA GTG GA -3') and reverse primer (3'- CAG GCA TTG CTT ACC ACG TT -5') were used to make a standard curve according to Chapter 3.5.3.1. The PCR products were purified using Wizard® SV Gel and PCR Clean-UP system (Promega, Australia) and directly transformed into *Escherichia coli* JM 109 High Efficiency cells for the cloning step using pGEM-T® Easy Vector System (Promega, Australia) in accordance with Chapter 3.5.4. Plasmids with DNA inserts of MrNV were sent to Macrogen Inc (Seoul, Korea) for sequencing to confirm the quality of the sequences.

7.2.7. Statistical analyses

Statistical analyses of all data were performed using Statistical Package for the Social Sciences (SPSS) version 19. Weight and length means of redclaw crayfish were statistically evaluated by a one-way univariate analysis of variance (one-way ANOVA). Means of different treatments on viral titres from qPCR were evaluated by one-way ANOVA. Least significant difference (LSD) post-hoc test for differences between the means was used. The data that was not normally distributed needed to be transformed to a log scale in order to meet the requirements of the homogeneity of variance and to be normally distributed. The group means of viral copies and cycle time (Ct) of MrNV from positive samples were evaluated by independent sample T-test.

7.3. Results

7.3.1. Mortality in the Stealth RNAi assay

After the experiment of using Stealth RNAi which was completed at 60 days, cumulative percentage mortality of 60% (6/10) was found in control Stealth RNAi + MrNV treatment compared with 10% (1/10) mortality in Stealth RNAi + MrNV treatment (Figure 7.1). The untouched controls, placebo controls, control Stealth RNAi + 2xPBS and Stealth RNAi + 2xPBS treatments had no mortality at 0% (0/10). In control Stealth RNAi + MrNV treatment, clinical signs consistent with WTD developed: inability to swim normally, pale exoskeleton and death following clinical signs were observed in infected redclaw crayfish, while redclaw crayfish from untouched controls, placebo controls, control Stealth RNAi + 2xPBS and Stealth RNAi + 2xPBS treatments exhibited normal swimming behaviour with no pale exoskeleton.

Redclaw crayfish in the control Stealth RNAi + MrNV treatment had the smallest size (8.7 ± 0.99 cm) and lowest weight (17.69 ± 3.55 g) when compared to other treatments (Table 7.3) which averaged 10.3 cm in length and 22.90 g in weight. There was a significant difference between different treatments for length ($F = 8.106$, $df = 5, 54$, $P < 0.05$), and for weight ($F = 2.943$, $df = 5, 54$, $P < 0.05$).

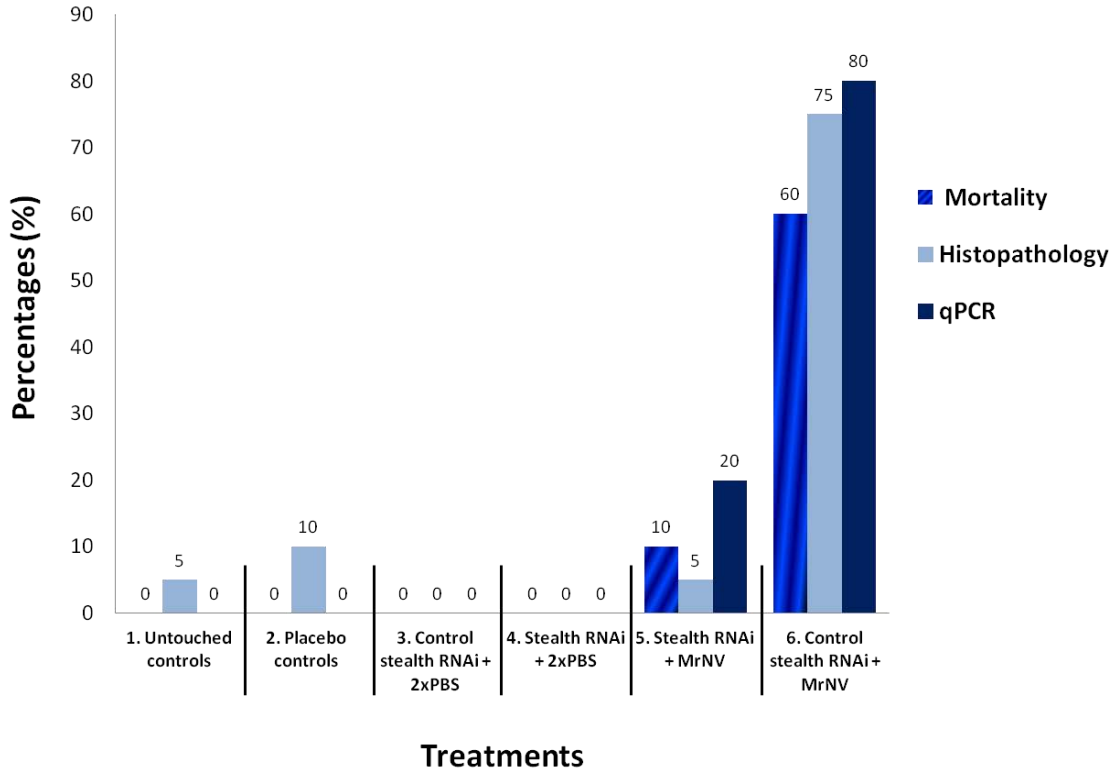

Figure 7.1: Comparison of percentages of MrNV positive results; mortality, histopathology (myolysis with inflammatory cells) and quantitative real-time PCR (qPCR) from samples of redclaw crayfish (*Cherax quadricarinatus*).

Table 7.3: Redclaw crayfish (*Cherax quadricarinatus*) length and weight means and standard deviations (SD) of six treatments in Stealth RNAi experiment after 60 days infection. Groups with the same superscript were not statistically different (P > 0.05).

Treatments	Length (mean ± SD)		Weight (mean ± SD)	
1. Untouched controls	10.46 ± 0.55[a]		22.30 ± 3.40 [a]	
2. Placebo controls	10.60 ± 0.78 [a]		23.80 ± 5.25 [a]	
3. Control Stealth RNAi + 2xPBS	10.19 ± 0.79 [a]	average 10.3 cm	23.40 ± 5.06 [a]	average 22.9 g
4. Stealth RNAi + 2xPBS	10.11 ± 0.65 [a]		21.52 ± 3.71 [a]	
5. Stealth RNAi + MrNV	10.18 ± 0.65 [a]		23.51 ± 4.07 [a]	
6. Control Stealth RNAi + MrNV	8.70 ± 0.99 [b]		17.69 ± 3.55 [b]	

7.3.2. Histopathology

The infected redclaw crayfish in control Stealth RNAi + MrNV treatment displayed muscle necrosis and myositis. Haemocytic infiltration in the muscle of infected crayfish was observed under light microscopy and consisted of a massive infiltration of myonuclei and some haemocytes (Figure 7.2). However, myolysis with inflammatory cells was observed in other treatments. The percentages of myolysis and myolysis with inflammatory cells were higher in control Stealth RNAi + MrNV treatment than other treatments (Table 7.4). On the other hand, percentage of myolysis and myolysis with inflammatory cells in Stealth RNAi + MrNV treatment were similar to other uninfected treatments. No viral inclusion bodies were observed in any treatments. Systemic granulomas (Figure 7.3) in hepatopancreas and gills including melanisation (Figure 7.4) in the gills were found in all treatments of redclaw crayfish (Table 7.4).

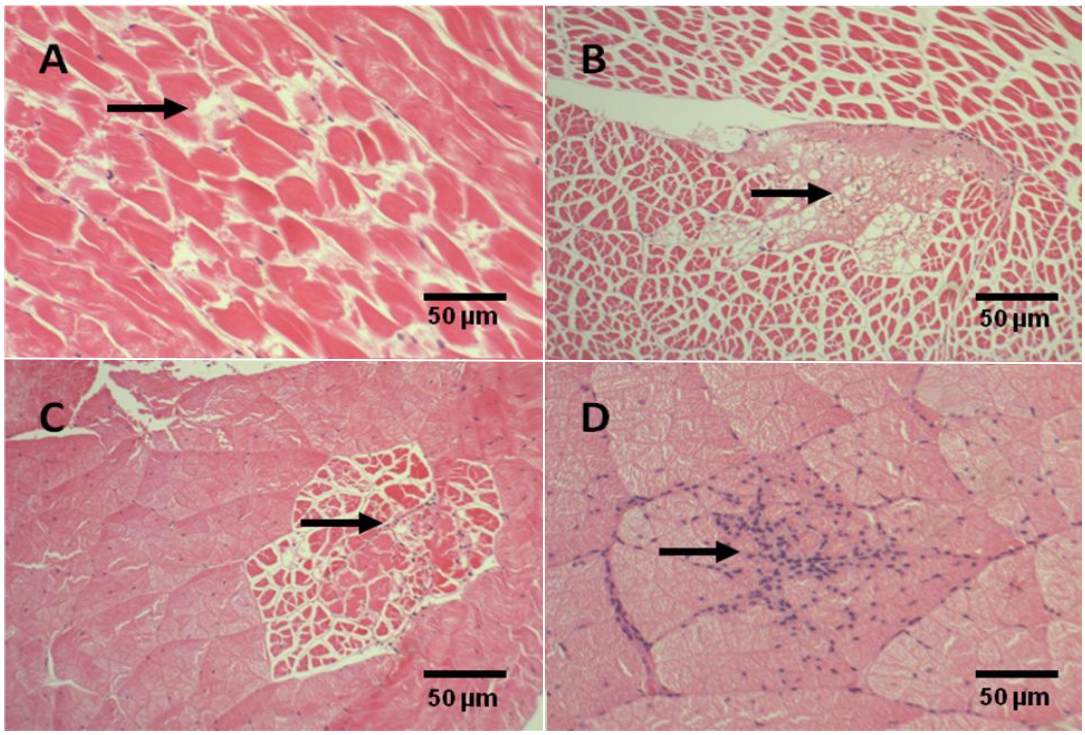

Figure7. 2: (A, B&C) Necrotic tissues (arrow), muscle degeneration (arrow) and myolysis (arrow) in redclaw crayfish (*Cherax quadricarinatus*) from control Stealth RNAi + MrNV treatment. (D) Haemocytic infiltration in the muscle of redclaw crayfish (*C. quadricarinatus*) from control Stealth RNAi + MrNV treatment.

Table 7.4: Summary of six treatments of redclaw crayfish (*Cherax quadricarinatus*) injected with Stealth RNAi and control Stealth RNAi exposed to MrNV. For each treatment, twenty samples of muscle and hepatopancreas were observed for histopathology. Also, ten samples of gills were observed under light microscopy.

Treatments	Myolysis (%)	Myolysis with inflammatory cells (%)	Gill Melanisation (%)	Systemic Granulomas (%)
1. Untouched controls	5 (1/20)	5 (1/20)	20 (2/10)	45 (9/20)
2. Placebo controls	0 (0/20)	10 (2/20)	10 (1/10)	10 (2/20)
3. Control Stealth RNAi + 2xPBS	15 (3/20)	0 (0/20)	10 (1/20)	25 (5/20)
4. Stealth RNAi + 2xPBS	10 (2/20)	0 (0/20)	10 (1/10)	15 (3/20)
5. Stealth RNAi + MrNV	15 (3/20)	5 (1/20)	30 (3/10)	10 (2/20)
6. Control Stealth RNAi + MrNV	80 (16/20)	75 (15/20)	30 (3/10)	35 (7/20)

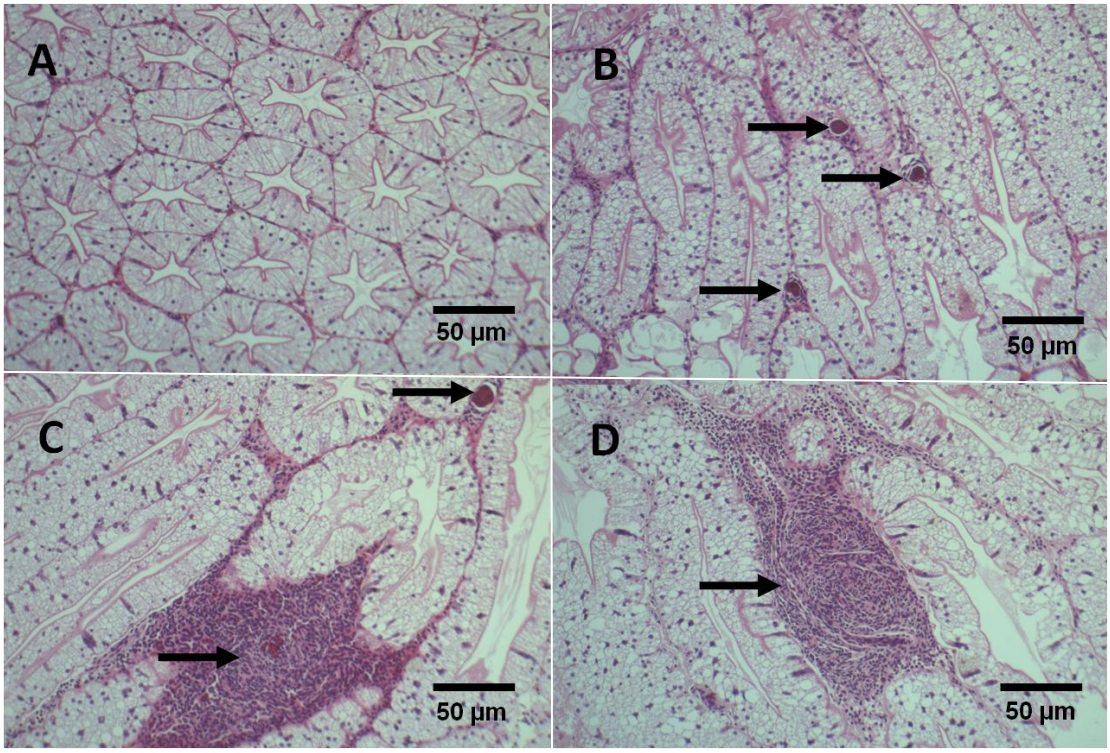

Figure 7.3: (A) Normal hepatopancreas of redclaw crayfish (*Cherax quadricarinatus*) from untouched control treatment. (B, C&D) Haemocytic infiltration in hepatopancreas redclaw crayfish (*C. quadricarinatus*) from control Stealth RNAi + MrNV treatment; systemic granulomas (arrows).

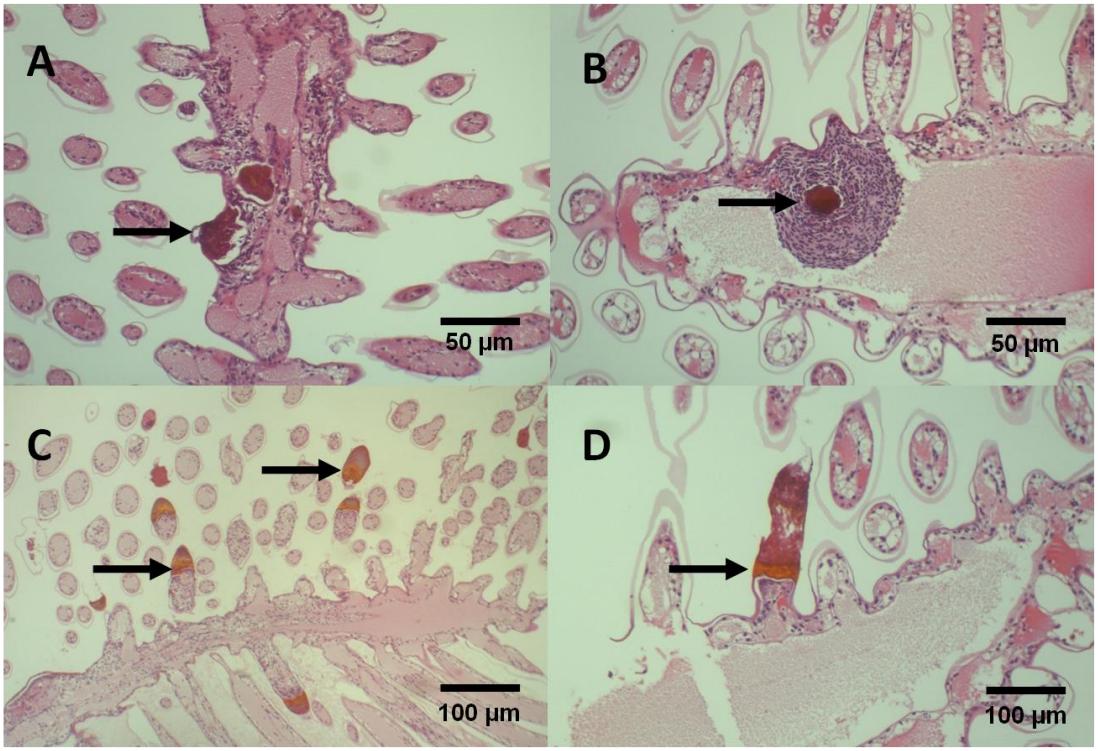

Figure 7.4: (A&B) Systemic granulomas (arrow) and haemocytic infiltration (arrow) in the gills of redclaw crayfish (*C. quadricarinatus*) from control Stealth RNAi + MrNV treatment. (C&D) Melanisation (arrows) in the gills of (*C. quadricarinatus*) from untouched control treatment and control Stealth RNAi + MrNV treatment.

7.3.3. Quantitative real-time RT-PCR

Ten out of 20 samples from infected redclaw crayfish with MrNV were positive in qPCR. Two positive samples came from Stealth RNAi + MrNV treatment and eight positive samples came from control Stealth RNAi + MrNV treatment. The mean number of viral copies from Stealth RNAi + MrNV treatment was 1.29×10^{1} and cycle time was 34.88. The mean of viral copies from control Stealth RNAi + MrNV treatment was 3.54×10^{1} and cycle time was 34.83. The mean viral copies and mean cycle times of positive samples were 3.45×10^{1} and 34.84, respectively (Table 7.5). However, no significant differences in viral copies or cycle time from qPCR between Stealth RNAi + MrNV treatment and control Stealth RNAi + MrNV treatment were found ($F = 0.263$, $df = 1, 28$, $P > 0.05$) and ($F = 0.017$, $df = 1, 28$, $P > 0.05$), respectively.

Table 7.5: Number of viral copies and cycle time (Ct) of *Macrobrachium rosenbergii* nodavirus in replicate assays of juvenile *Cherax quadricarinatus* that were positive. The remaining 50 animals were negative (below the level of detection). Groups with the same superscript were not statistically different (P > 0.05).

Number of viral copies and cycle times of *Macrobrachium rosenbergii* nodavirus											
Crayfish	Groups	Assay 1 (Copies)	Assay 1 (Ct)	Assay 2 (Copies)	Assay 2 (Ct)	Assay 3 (Copies)	Assay 3 (Ct)	Assay mean copies	Assay mean Ct	Group mean copies	Group mean Ct
1	RNAi / MrNV	2.89×10^1	34.42	3.41×10^1	35.09	1.43×10^1	35.18	2.58×10^1	34.90	a 1.29×10^1	a 34.88
2	RNAi / MrNV	1.12×10^1	35.88	2.17×10^1	35.78	7.62×10^1	32.92	3.64×10^1	34.86		
3	MrNV / Control RNAi	3.55×10^1	34.69	7.67×10^1	34.03	3.95×10^1	33.93	5.06×10^1	34.22		
4	MrNV / Control RNAi	4.21×10^1	34.43	5.89×10^1	34.44	1.37×10^1	35.57	3.82×10^1	34.81		
5	MrNV / Control RNAi	2.18×10^1	35.44	2.72×10^1	35.10	2.20×10^1	34.83	2.37×10^1	35.08		
6	MrNV / Control RNAi	3.09×10^1	35.43	1.32×10^1	35.62	2.83×10^1	34.71	2.41×10^1	35.25	a 3.54×10^1	a 34.83
7	MrNV / Control RNAi	4.14×10^1	34.45	3.69×10^1	35.16	4.05×10^1	33.89	3.96×10^1	34.50		
8	MrNV / Control RNAi	2.27×10^1	35.38	2.00×10^1	36.10	7.25×10^1	33.00	3.84×10^1	34.83		
9	MrNV / Control RNAi	5.39×10^1	34.05	3.79×10^1	35.11	3.37×10^1	34.18	4.18×10^1	34.45		
10	MrNV / Control RNAi	4.83×10^1	34.74	1.69×10^1	36.36	1.56×10^1	35.36	2.69×10^1	35.49		
						Overall means		**3.45×10^1**	**34.84**		

7.4. Discussion

A main viral defence mechanism in invertebrates including prawns is the innate immune response (Little *et al*., 2005; Yodmuang *et al*., 2006). A limited understanding of the innate immune system in prawns is particularly focused on bacterial and fungal infection and lacks information related to protection against viral disease. However, RNAi studies were reported against WSSV (Westenberg *et al*., 2005) and YHV (Yodmuang *et al*., 2006) leading to protection and inhibition of viral infection. The use of RNAi may prevent and control viral disease outbreaks worldwide.

The RNAi-induced systemic antiviral mechanism operates through two pathways: sequence-independent antiviral immunity and sequence specific antiviral immunity (Yodmuang *et al*., 2006). Inhibition of YHV replication in giant tiger prawn

(*P. monodon*) using YHV-specific dsRNA determined a potential systemic immunity against YHV infection, while nonspecific dsRNA only partially protected (Yodmuang *et al*., 2006). Similar results have been reported in hepatopancreatic parvovirus (*Pmerg*DNV) in a cricket model where unrelated RNAi also had a partial reduction of *Pmerg*DNV replication (La Fauce and Owens, 2009). In this study, only specific dsRNA to protein B2 provided a potent antiviral defence against MrNV infection.

Alphanodavirus and betanodavirus protein B2 is important for the intracellular accumulation of viral RNA in the cells (Fenner *et al*., 2006). Protein B2 is a sequence-nonspecific binding protein that binds to short dsRNA of more than 10 bp and prevents the generation of RNAi from long viral dsRNA by the enzyme Dicer. This mechanism shows that protein B2 is able to block RNA editing by the viral defence mechanism and enables completion of viral replication in the cells. Therefore, sequence-specific RNAi to protein B2 could be studied to enable control of nodavirus infection including MrNV. Injection of Stealth RNAi + MrNV in this study showed a 2.7 fold reduction in the average viral titre compared to redclaw crayfish injected with control Stealth RNAi + MrNV. In contrast, *Pmerg*DNV in the cricket model had a 10-fold reduction in *Pmerg*DNV titres (La Fauce and Owens, 2009).

Clearance of MrNV and immunological changes was reported in experimentally injected *M. rosenbergii* (Ravi *et al*., 2010). MrNV was injected intramuscularly to healthy *M. rosenbergii* and various organ samples were collected at different times to study the viral clearance using nested RT-PCR. At 25 days post-infection, the nested RT-PCR was negative for MrNV. Mortality, RT-PCR and immunological results were determined in MrNV-injected prawns: significant differences were identified in the early days of sample collection and differences became insignificant in the latter days (Ravi *et al*., 2009; Ravi *et al*., 2010). Therefore, a long-term study could be affected by the host immune response including phagocytosis, melanisation and antimicrobial substances such as superoxide and hydrogen peroxide (Johanson *et al*., 2000). RNAi does not completely block viral replication and this is why 10% of the Stealth RNAi + MrNV were positive.

In this study, a sequence specific dsRNA against protein B2 produced RNAi that was able to functionally prevent and reduce mortality in infected redclaw crayfish. RNAi is an effective tool to protect against MrNV infection and other viral diseases in the early stages of infection in crustacea. This methodology could be used against fish betanodaviruses such as VER that causes abnormal swimming behaviour, loss of appetite and death in larvae and juveniles fish (Mezeth *et al*., 2009). RNAi against protein B2 of fish nodavirus may be used to prevent and control VER or other nodavirus outbreaks. However, the stability and delivery of specific RNAi in crustacea and fish needs further study to develop effective viral control for use at the commercial scale.

CHAPTER 8

GENERAL DISCUSSION

The demand for aquaculture products continues to increase as global population increased in the past ten years. Therefore, aquaculture must be able to meets future demands (FAO, 2010). Aquaculture industries have successfully expanded in terms of area and geographical range. However, animal movement for farming is one of major concerns with regard to disease outbreaks. Therefore, biosecurity and diagnostic techniques for disease detection must be understood to better protect and control disease outbreaks.

The increase of crustacean diseases has been associated with increasing economic losses. Crustacean diseases include bacteria, fungal, parasites and viral infection in many crustacea including penaeid and *Macrobrachium* (Anderson and Prior, 1992; Groff *et al*., 1993; Owens and McElnea, 2000; Edgerton *et al*., 2000; Bowater *et al*., 2002; Owens *et al*., 2009). Nodavirus is one viral infection which causes massive damages in crustacean industries. Also, nodavirus causes disease problems in other species such as insect and fish (Vago, 1963; Nishizawa *et al*., 1997; Lim *et al*., 1997; Delsert, 1997). Therefore, this study will be useful to understand the characteristic of nodavirus and apply that information to control nodaviruses.

Complete sequences of MrNV (RNA1, RNA2 and protein B2) showed the Australian sequences have similar sizes compared to other isolates but they have different nucleotides. The Australian strain of MrNV may be endemic in Australia. In order to prevent and control MrNV outbreaks, this result can be used to develop effective diagnostic techniques for the detection of MrNV outbreak in the future. Moreover, probes and primers have been designed to detect the Australia MrNV using qPCR technology that can be used for the scanning and monitoring program for MrNV in the early stage. However, no prevalence of MrNV infection in wild prawns or aquaculture has been published. The scanning and monitoring program for MrNV should be used to determine MrNV status affected to wild and cultured prawns.

Due to the lack of crustacean cell line that can be used to study crustacean viral diseases, fish and insect cell lines have been used for crustacean viruses. In this study, C6/36 cell line was cultured and inoculated with MrNV to observe the cytopathic effect of the disease using different stainings and qPCR. Vacuolations and viral inclusion can be observed with Giemsa and H&E staining under light microscopy. Single stranded nucleic acid of MrNV with acridine orange was seen under fluorescent microscopy. The disrupted cells and mitochondria activity of cells was measured using trypan blue and neutral red stainings, respectively. Therefore, different stainings can be used in the different expectation to observe MrNV activity which was shown in this thesis. However, no evidence of complete replication of the Australian MrNV was observed in qPCR in this study. Thus, C6/36 cell line may have limited susceptibility to the Australian isolate of MrNV.

When cell culture had limited susceptibility to Australian MrNV, an experimental animal was needed for further study. However, *M. rosenbergii* is logistically hard to source for bioassay studies. Therefore, redclaw crayfish (*C. quadricarinatus*) was used as alternative bioassay species for MrNV. It may be less-than-perfect model as shown by viral replication in TaqMan real-time PCR assay. This study suggests that different cell lines or *M. rosenbergii* may be necessary to use in order to observe the viral replication of MrNV.

Viral diseases in crustacea are hard to prevent, control or eradicate by vaccination and medication because of crustacea lack an adaptive immune response against viral infection. RNAi is an evolutionary gene regulatory response that can be used to control viral infections. However, no commercial RNAi is available to prevent or control viral infection in crustacea. This study is the first to use RNAi technique against MrNV infection using *C. quadricarinatus* as an experimental animal model. The results determined that RNAi can reduce mortality in infected animals. However, the successful application of RNAi needs further studies for the stability of dsRNA in prawns at the farm level; if dsRNA can induce long term protection and if prawn immunity can be passed onto offspring. Also, an efficient technique for the delivery of RNAi needs to be developed. For example, can RNAi be delivered through food pellets for the commercial scale aquaculture.

Nodaviruses possess protein B2 which binds to and protect dsRNA against the host RNAi response (Fenner *et al*., 2007). Therefore, protein B2 is the key to understanding nodavirus infection. This study analysed the protein B2 gene and designed RNAi against protein B2 production of MrNV. RNAi is a promising strategy for control of the Australian MrNV infection in *C. quadricarinatus*. However, a similar experiment has to be repeated in *M. rosenbergii* to observe the mechanism and affectivity of RNAi against MrNV. Moreover, the methodology of RNAi in this study could be developed and used against protein B2 of other nodaviruses such as viral encephalopathy and retinopathy (VER) in fish (Mezeth *et al*., 2009). If nodavirus levels in the farms can be removed or reduced, mass production and quality of the products should be increased.

Global crustacean industries will face and deal with new diseases in the future, the development of effective and accurate diagnostic techniques that can early detect the diseases will be needed. Crustacean industries should aim at undertaking good management practices and responsible health management that can remove or minimise massive economic losses from the diseases. There is no doubt crustacean farming will continue to intensify the risk of disease including new emerging diseases. Good measurements of animal health should be maintained and effectively implemented to prevent diseases entry into the farm. In the mean time, farmers have to maintain a good sanitary procedure to eradicate diseases that would otherwise cause a major problem in the future.

In summary, this was the first investigation of MrNV infection in Australia after the original discovery. The complete sequences of the Australian isolate of MrNV including protein B2 were determined and compared to other different isolates. These complete sequences of the Australian MrNV are difference to other nodaviruses. Also, this study was the first anywhere in the world to develop *C. quadricarinatus* as an alternatively animal model for MrNV. This study was the first to determine the unapparent replication of the Australian MrNV in cell culture (C6/36) using TaqMan real-time RT-PCR. Moreover, RNAi was the first study to decrease mortality and inhibit MrNV infection in experimental animals. The results from this study suggest detection and control of MrNV can potentially increase production and prevent diseases outbreak in the future. Following the successful

experiment of RNAi against MrNV infection, the result from this study may be applied on a larger scale in order to control viral diseases in crustacean industries in the future. Also, the same technology can be used against other nodavirus such as fish nodaviruses that are known to cause substantial losses worldwide.

REFERENCES

ABARE. (2010) Australian Fisheries Statistics. Commonwealth of Australia, Canberra

Ambrose, V., Lee, R.C., Lavanway, A., Williams, P.T. and Jewell, D. (2003) MicroRNA and other tiny endogenous RNAs in *C. elegans*. *Current Biology* **13**: 807-818

Anandalakshmi, R., Pruss, G.J., Ge, X., Marathe, R., Mallory, A.C., Smith, T.H. and Vance, V.B. (1998) A viral suppressor of gene silencing in plants. *Proceedings of the Natural Academy of Sciences of the United States of America* **95**: 13079-13084

Anderson, I.G., Law, A.T., Shariff, M. and Nash, G. (1990) A parvo-like virus in the giant freshwater prawn, *Macrobrachium rosenbergii. Journal of Invertebrate Pathology* **55**: 447-449

Anderson, I.G. and Prior, H.C. (1992) Baculovirus infections in the mud crab, *Scylla serrata*, and a freshwater crayfish, *Cherax quadricarinatus*, from Australia. *Journal of Invertebrate Pathology* **60**, 265-273

Arcier, J.M., Herman, F., Lightner, D.V., Redman, R., Mari, J. and Bonami, J.R. (1999) A viral disease associated with mortalities in hatchery-reared postlarvae of the giant freshwater prawn *Macrobrachium rosenbergii. Diseases of Aquatic Organisms* **38**: 177-181

Babich, H. and Borenfreund, E. (1990) *In vitro* cytotoxicities of inorganic lead and di- and trialkyl lead compounds to fish cells. *Bulletin of Environmental Contamination and Toxicology* **44**: 456-460

Ball, L.A. (1997) Nodavirus RNA recombination. *Virology* **8**: 95-100

Bastin, P., Galvani, A. and Sperling, L. (2001) Genetic interference in protozoa. *Research in Microbiology* **302**: 316-323

Bergin, T.J. (1986) An overview of aquaculture and disease control. In: Humphrey JD, Langdon JS (eds) Proceedings on diseases of Australian fish and shellfish. Australian Fish Health Reference Laboratory, Benalla. pp: 3-9

Berntein, E., Kim, S.Y., Carmell, M.A., Murchinsor, E.P., Alcorn, H., Li, M.Z., Mills, A.A., Elledge, S.J., Anderson, K.V. and Hannon, G.J. (2003) Dicer is essential for mouse development. *Nature Genetics* **35**: 215-217

Bonami, J.R. and Sri Widada, J. (2011) Viral diseases of the giant fresh water prawns *Macrobrachium rosenbergii*: A review. *Journal of Invertebrate Pathology* **106**, 131-142

Bowater, R.O., Wingfield, M., Fisk, A., Condon, K.M.L., Reid, A., Prior, H. and Kulpa, E.C. (2002) A pravo-like virus in culture redclaw crayfish *Cherax quadricarinatus* from Queensland, Australia. *Diseases of Aquatic Organisms* **50**: 79-86

Brackney, D.E., Scott, J.C., Sagawa, F., Woodward, J.M., Miller, N.A., Schilkey, F.D., Mudge, J., Wilusz, J., Olson, K.E., Blair, C.D. and Ebel, G.D. (2010) C6/36 *Aedes albopictus* cells have a dysfunctional antiviral RNA interference response. *PLOS Neglected Tropical Diseases* **4**(10), e856

Brennan, C.A. and Anderson, K.V. (2004) *Drosophila*: the genetics of innate immune recognition and response. *Annual Review of Immunology* **22**: 457-483

Brigneti, G., Voinnet, O., Li, W.X., Ji, L.H., Ding, S.W. and Baulcombe, D.C. (1998) Viral pathogenicity determinants are suppressors of transgene silencing in *Nicotiana benthamiana*. *The EMBO Journal* **17**: 6739-6746

Caplen, N.J., Parrish, S., Imani, F., Fire, A. and Morgan, R.A. (2001) Specific inhibition of gene expression by small double-stranded RNAs in invertebrate and vertebrate systems. *Proceedings of the National Academy of Sciences of the United States of America* **98**: 9742-9747

Cheng, W. and Chen, J.C. (1998) Isolation and characterization of an *Enterococcus* like bacterium causing muscle necrosis and mortality in *Macrobrachium rosenbergii* in Taiwan. *Diseases of Aquatic Organisms* **34**: 93-101

Daisy, W. (2007) The giant freshwater prawns of the *M. rosenbergii* species group (crustacean: Decapoda: Caridea: Palaemonidae). *The Raffles Bulletin of Zoology* **55**(2): 321-336

Dang, L.T., Kondo, H., Hirono, I. and Aoki, T. (2008) Inhibition of red seabream iridovirus (RSIV) replication by small interference RNA (siRNA) in a cell culture system. *Anitiviral Research* **77**: 142-149

De Bruyn, M., Wilson, J.A. and Mather, P.B. (2004) Huxley's line demarcates extensive genetic divergence between eastern and western forms of the giant freshwater prawn, *Macrobrachium rosenbergii*. *Molecular Phylogenetics and Evolution* **30**: 251-257

De la Vaga, E., Degnan, B.M., Hall, M.R., Cowley, J.A. and Wilson, K.J. (2004) Quantitative real-time RT-PCR demonstrates that handling stress can lead to rapid increases of gill-associated virus (GAV) infection levels in *Penaeus monodon*. *Diseases of Aquatic Organisms* **59**(3): 195-203

De Man, J.G. (1879) On some species of the genus *Palaemon Fabr.* with descriptions of two new forms. *Notes from the Royal Zoological Museum of the Netherlands at Leyden* **1**(41):165-184

Delsert, C., Morin, N. and Comps, M. (1997) Fish nodavirus lytic cycle and semipermissive expression in mammalian and fish cell cultures. *Journal of Virology* **71**(7): 5673-5677

Department of Agriculture, Fisheries and Forestry, Fisheries Research and Development Corporation (2004) Aquatic animal diseases significant to Australia, identification field guide, second edition on CD Rom

Destoumieux-Garzon, D., Munoz, M. and Bachere, E. (2000) Penaeidins, a family of antimicrobial peptides from penaeid shrimp (Crustacea, Decapoda). *Cellular and Molecular Life Sciences (CMLS)* **57**: 1260-1271

Destoumieux-Garzon, D., Saulnier, D., Garnier, J., Jouffrey, C., Bulet, P. and Bachere, E. (2001) Crustacean immunity: Antifungal peptides are generated from the C terminus of shrimp hemocyanin in response to microbial challenge. *The Journal of Biological Chemistry* **276**: 47070-47077

Duxbury, M.S. and Whang, E.E. (2004) RNA interference: A practical approach. *Journal of Surgical Research* **117**: 339-344

Dykxhoorn, D.M., Novina, C.D. and Sharp, P.A. (2003) Killing the messenger: Short RNAs that silence gene expression. *Nature Reviews* **4**: 457-467

Edgerton, B.F., Webb, R., Anderson, J.G. and Kulpa, E.C. (2000) Description of a presumptive hepatopancreatic reovirus, and a putative gill parvovirus, in the freshwater crayfish *Cherax quadricarinatus*. *Diseases of Aquatic Organisms* **41**: 83-90

Elbashir, S.M., Lendeckel, W. and Tuschi, T. (2001) RNA interference is mediated by 21- and 22-nucleotides RNAs. *Genes and Development* **15**: 188-200

Fagard, M. and Vaucheret, H. (2000) (Trans) gene silencing in plants: How many mechanisms? *Annual Review of Plant Physiology and Plant Molecular Biology* **51**: 167-194

FAO. (2010) The state of world fisheries and aquaculture 2010. Food and Agriculture Organisation of the United Nations, Rome, Italy

Fenner, B.J., Goh, W. and Kwang, J. (2006) Sequestration and protection of double-stranded RNA by the betanodavirus B2 protein. *Journal of Virology* **80** (14): 6822-6833

Fenner, B.J., Goh, W. and Kwang, J. (2007) Dissection of double-stranded RNA binding protein B2 from betanodavirus. *Journal of Virology* **81**(11): 5449-5459

Fire, A., Xu, S.Q., Montgomery, M.K., Kostas, M.K., Driver, S.E. and Mello, C.C. (1998) Potent of specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **391**: 806-811

Freshney, R.I. (2005) Culture of animal cells, a manual of basic technique, Fifth edition. John Wiley & Sons, Inc., Hoboken, New Jersey, USA. pp: 1-42

Friesen, P.D. and Rueckert, R.R. (1982) Black beetle virus: messenger for protein B is a subgenomic viral RNA. *Journal of Virology* **42**(3): 986-995

Groff, J.M., McDowell, T., Friedman, C.S. and Hedrick, R.P. (1993) Detection of a nonoccluded baculovirus in the freshwater crayfish *Cherax quadricarinatus* in North America. *Journal of Aquatic Animal Health* **5**:275-279

Grotmol, S., Totland, G.K., Thorud, K. and Hjeltnes, B.K. (1997) Vacuolating encephalopathy and retinopathy associated with a nodavirus-like agent: a probable cause of mass mortality of cultured larval and juvenile Atlantic halibut *Hippoglossus hippoglossus*. *Diseases of Aquatic Organisms* **29**: 85-97

Hannon, G.J. (2002) RNA interference. *Nature* **418**: 244-251

Hayakijkosol, O. and Owens, L. (2011) Investigation into the pathogenicity of reovirus to juvenile *Cherax quadricarinatus*. *Aquaculture* **316**: 1-5

Hegde, A., Teh, H.C., Lam, T.J. and Sin, Y.M. (2003) Nodavirus infection in freshwater ornamental fish, guppy, *Poicelia reticulata* - comparative characterization and pathogenicity studies. *Archives of Virology* **148**: 575-586

Herbert, B. (1987) Notes on diseases and epibionts of *Cherax quadricarinatus* and *C. tenuimanus* (Decapoda: parastacifae). *Aquaculture* **64**: 165-173

Hernandez-Herrera, R.I., Chappe-Bonnichon, V., Roch, P., Sri Widada, J. and Bonami, J.R. (2007) Partial susceptibility of the SSN-1 fish cell line to a crustacean virus: a defective replication study. *Journal of Fish Diseases* **30**: 673-679

Holen, T., Amarzguioui, M., Wiiger, M.T., Babaie, E. and Prydz, H. (2002) Positional effects of short interfering RNAs targeting the human coagulation trigger tissue factor. *Nucleic Acids Research* **30**: 1757-1766

Hsieh, C.Y., Wu, Z.B., Tung, M.C., Tu, C., Lo, S.P., Chang, T.C., Chang, C.D., Chen, S.C., Hsieh, Y.C. and Tsai, S.S. (2006) *In situ* hybridization and RT-PCR detection of *Macrobrachium rosenbergii* nodavirus in giant freshwater prawn, *Macrobrachium rosenbergii* (de Man), in Taiwan. *Journal of Fish Diseases* **29**: 665-671

Igarashi, A. (1978). Isolation of a Singh's *Aedes albopictus* cell clone sensitive to dengue and chikungunya viruses. *Journal of Genetic Virology* **40**:531-544.

Jackson, A.L., Bartz, S.R., Schelter, J., Kobayashi, S.V., Burchard, J., Mao, M., Li, B., Cavet, G. and Linsley, P.S. (2003) Expression profiling reveals off-target gene regulation by RNAi. *Nature Biotechnology* **21**: 635-637

Jackson, A.L., Burchard, J., Schelter, J., Chau, B.N., Cleary, M., Lim, L. and Linsley, P.S. (2006) Widespread siRNA "off-target" transcript silencing mediated by seed region sequence complementarity. *RNA* **12**: 1179-1187

Jackson, A.L. and Linsley, P.S. (2004) Noise amidst the silence: off-target effects of siRNAs? *Trends in Genetics* **20**: 521-524

Johansen, R., Ranheim, T., Hansen, M.K., Taksdal, T. and Totland, G.K. (2002) Pathological changes in juvenile Atlantic halibut *Hippoglossus hippoglossus* persistently infected with nodavirus. *Diseases of Aquatic Organisms* **50**: 161-169

Johnson, K.L., Price, B.D., Eckerle, D.E. and Ball, L.A. (2004). Nodamura virus nonstructural protein B2 can enhance viral RNA accumulation in both mammalian and insect cells. *Journal of Virology* **78**(12): 6698-6704

Johanson, M.W., Keyser, P., Sritunyalucksana, K. and Soderhall, K. (2000) Crustacean hemocytes and haemotopoiesis. *Aquaculture* **191**: 45-52

Jones, C.M. (1998) The New Rural Industries: *A handbook for farmers and investors*: Redclaw crayfish. Rural Industries Research & Development Corporation pp: 1-133

Khvorova, A., Reynolds, A. and Jayasena, S.D. (2003) Functional siRNAs and miRNAs exhibit strand bias. *Cell* **115**: 209-216

La Fauce, K., Layton, R. and Owens, L. (2007) TaqMan real-time PCR for detection of hepatopancreatic parvovirus from Australia. *Journal of Virological Methods* **140**: 10-16

La Fauce, K. and Owens, L. (2007) Investigation into the pathogenicity of *Penaeus merguiensis* densovirus (*Pmerg* DNV) to juvenile *Cherax quadricarinatus*. *Aquaculture* **271**: 31-38

La Fauce, K. and Owens, L. (2009) RNA interference reduces *Pmerg*DNA expression and replication in an *in vivo* cricket model. *Journal of Invertebrate Pathology* **100**: 111-115

Lee, S.H. and Shinko, P.J. (2006) siRNA-Getting the message out. *European Journal of Pharmaceutical Sciences* **27**(5): 401-410

Li, W.H., Li, W.X. and Ding, S.W. (2002a) Induction and suppression of RNA silencing by an animal virus. *Sciences* **296**: 1319-1321

Li, W.X. and Ding, S.W. (2001) Viral suppressors of RNA silencing. *Current Opinion in Biotechnology* **12**: 150-154

Li, X., Scuderi, A., Letsou, A. and Virshup, D.M. (2002b) B56-associated protein phosphatase 2A is required for survival and protects from apoptosis in *Drosophila melanogaster*. *Molecular and Cellular Biology* **22**: 3674-3684

Lim, M.G.B., Chong, S.Y. and Kueh, S. (1997) Some observation of grouper (*Epinephelus tauvina*) and seabass (*Lates calcarifer*) nodavirus infection in Singapore. *Singapore Vet Journal* **21**: 47-51

Lin, C., Yang, C., Tu, C., Huang, C., Shih, Y., Chuang, C. and Chen, W. (2007) A novel tetraspanin C189 upregulated in C6/36 mosquito cells following dengue 2 virus infection. *Virus Research* **124**: 176-183

Lingel, A., Simon, B., Izaurralde, E. and Sattler, M. (2005) The structure of the flock house virus B2 protein, aviral suppressor of RNA interference, shows a novel mode of double stranded RNA recognition. *EMBO reports* **6**(12): 1149-1155

Little, T., Hultmark, D. and Read, A.F. (2005) Invertebrate immunity and the limits of mechanistic immunology. *Nature Immunology* **6**: 651-654

Lupin, H.M. (2009) Human health aspects of drug and chemical use in aquaculture. *Options Mediterraneennes* **86**: 95-103

Macaranas, J., Mather, P., Hoeben, P. and Capra, M. (1995) Assessment of genetic variation in wild populations of the redclaw crayfish (*Cherax quadricarinatus*, von Martens 1868) by means of allozyme and RAPD-PCR markers. *Marine Freshwater Research* **46**: 1217-1228

Maramorosch, K. and McIntosh, A.H. (1994) Arthropod cell culture systems, CRC Press, Boca Raton Ann Arbor London Tokyo pp: 1-167

Matzke, M.A., Primig, M., Trnovsky, J. and Matzke, A.J.M. (1989) Reversible methylation and inactivation of maker genes in sequentially transformed tobacco plants. *The EMBO Journal* **8**: 643-649

Medzhitov, R. and Janeway, C.A. (1997) Innate immunity: The virtues of a nonclonal system of recognition. *Cell* **91**: 295-298

Merrick, J.R. and Lambert, L.C. (1991) The yabby, marron and redclaw: production and marketing. Macarthur Press, Sydney pp: 180

Mezeth, K.B., Patel, S., Henriksen, H., Szilvay, A.M., Nerland, A.H. (2009) B2 protein from betanodavirus is expressed in recently infected but not in chronically infected fish. *Diseases of Aquatic Organisms* **83**(2): 97-103

Munday, B.L., Kwang, J. and Moody, N.J. (2002) Betanodavirus infections of teleost fish: a review. *Journal of Fish Diseases* **25**: 127-142

Murphy, F.A., Fauquet, C.M., Bishop, D.H.L., Ghabrial, S.A., Jarvis, A.W., Martelli, G.P., Mayo, M.A. and Summers, M.D. (1983) Virus taxonomy sixth report of the international committee on taxonomy of viruses, Springer-Verlag Wien New York. pp: 368-371

Nash, G., Chinabut, S. and Limsuwan, C. (1987) Idiopathic muscle necrosis in the freshwater prawn, *Macrobrachium rosenbergii*, cultured in Thailand. *Journal of Fish Diseases* **10**: 397-404

Nash, M., Nash, G., Anderson, M. and Shariff, M. (1988) A reo-like virus observed in the tiger prawn, *Penaeus monodon* Fabricius, from Malaysia. *Journal of Fish Diseases* **11**: 531-535

New, M.B. (2000) History and global status of freshwater prawn farming. In: New, M.B. & W.C. Valenti (eds.), Freshwater prawn culture: The farming of *Macrobrachium rosenbergii*. Blackwell Science, London, United Kingdom pp: 1-11

New, M.B. (2002) Farming freshwater prawns, A manual for the culture of the giant river prawn (*Macrobrachium rosenbergii*) Food and Agriculture Organization of the United Nations (FAO) Marlow, United Kingdom pp: 1-48

New, M.B. (2005) Freshwater prawn farming: global status, recent research and a glance at the future. *Aquaculture Researches* **36**: 210-230

Nishizawa, T., Furuhashi, M., Nagai, T., Nakai, T. and Muroga, K. (1997) Genomic classification of fish nodaviruses by molecular phylogenic analysis of the coat protein gene. *Applied and Environmental Microbiology* **63**(4): 1633-1636

NSW Department of primary industries (2005) Disease management in aquaculture from http://www.dpi.nsw.gov.au/fisheries/pests-diseases/animal-health/aquaculture [24/09/2008]

Ou, M.C., Chen, Y.M., Jeng, M.F., Chu,C.J., Yang, H.L. and Chen, T.Y. (2007) Identification of critical residues in nervous necrosis virus B2 for dsRNA binding and RNAi inhibiting activity through by bioinformatic analysis and mutagenesis. *Biochemical and Biophysical Research Communication* **361**: 634-640

Owens, L. and Evans, L. (1989) Common diseases of freshwater prawns (*Macrobrachium*) and crayfish (marron and yabbies) relevant to Australia. In: Postgraduate Committee in Veterinary Science (ed) Invertebrates in aquaculture. Refresher course for veterinarians. University of Sydney, Australia. Proceedings **117**: 227-240

Owens, L., La Fauce, K., Juntunen, K., Hayakijkosol, O. & Zeng, C. (2009) *Macrobrachium rosenbergii* nodavirus disease (white tail disease) in Australia. *Diseases of Aquatic Organisms* **85**, 175-180

Owens, L. and McElnea, C. (2000) Natural infection of the redclaw crayfish *Cherax quadricarinatus* with presumptive spawner-isolated mortality virus. *Diseases of Aquatic Organisms* **40**: 219-223

Paddison, P.J., Caudy, A.A., Bernstein, E., Hannon, G.J. and Conklin, D.S. (2002) Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. *Genes and Development* **16**: 948-958

Payment, P. and Trudel, M. (1993) Methods and techniques in virology, Marcel Dekker, Inc., New York. pp: 297-302

Qi, N., Cai, D., Qiu, Y., Xie, J., Wang, Z., Si, J., Zhang, J., Zhou, X. and Hu, Y. (2011) RNA binding by a novel helical fold of B2 protein from Wuhan nodavirus mediates the suppression of RNA interference and promotes B2 dimerization. *Journal of Virology* **85**(18): 9543-9554

Qian, D., Shi, Z., Zhang, S., Cao, Z., Liu, W., Li, L., Xie, Y., Cambournac, I. and Bonami, J.R. (2003) Extra small virus-like particles (XSV) and nodavirus associated with whitish muscle disease in the giant freshwater prawn *Macrobrachium rosenbergii*. *Journal of Fish Diseases* **26**: 521-527

Qian, D., Liu, W., Jianxiang, W. and Yu, L. (2006) Preparation of monoclonal antibody against *Macrobrachium rosenbergii* nodavirus and applications of TAS-ELISA for virus diagnosis in post-larvae hatcheries in East China during 2000-2004. *Aquaculture* **261**: 1144-1150

Rangel, A., Rockenmann, D.D., Hetrick, F. and Samal, S.K. (1999) Identification of grass crap haemorrhage virus as a new genogroup of aquareovirus. *Jounal of General Virology* **80**: 2399-2402

Raoni, M. and Arndt, G.M. (2003) Double-stranded RNA-mediated gene silencing in fission yeast. *Nucleic Acids Research* **31**: 4481-4489

Ravi, M., Nazeer Basha, A., Sarathi, M., Rosa Idalia, H.H. and Sri Widada, J. (2009) Studies on the occurrence of white tail disease (WTD) caused by MrNV and XSV in hatchery-reared post-larvae of *Penaeus indicus* and *P. monodon*. *Aquaculture* **292**: 117-120

Ravi, M., Nazeer Basha, A., Taju, G., Ram Kumar, R. and Sahul Hameed, A.S. (2010) Clearance of *Macrobrachium rosenbergii* nodavirus (MrNV) and extra small virus (XSV) and immunological changes in experimentally injected *Macrobrachium rosenbergii*. *Fish & Shellfish Immunology* **28**: 428-433

Romestand, B. and Bonami, J.R. (2003) A sandwich enzyme linked immunosorbent assay (S-ELISA) for detection of MrNV in the giant freshwater prawn, *Macrobrachium rosenbergii* (de Man). *Journal of Fish Diseases* **26**: 71-75

Rutz, S. and Scheffold, A. (2004) Technology review: Towards *in vivo* application of RNA interference - new toys, old problems. *Arthritis Research & Therapy* **6**(2): 78-85

Sahul Hameed, A.S., Yoganandhan, K., Sri Widada, J. and Bonami, J.R. (2004) Experimental transmission and tissue tropism of *Macrobrachium rosenbergii*

nodavirus (MrNV) and its associated extra small virus (XSV). *Diseases of Aquatic Organisms* **62**: 191-196

Shanker, P., Manjunath, N. and Lieberman, J. (2005) The prospect of silencing disease using RNA interference. *Journal of the American Medical Association* **293**: 1367-1373

Skliris, G.P., Krondiris, J.V., Sideris, D.C., Shinn, A.P., Starkey, W.G. and Richards, R.H. (2001) Phylogenetic and antigenic characterization of new fish nodavirus isolates from Europe and Asia. *Virus Research* **75**: 59-67

Sri Widada, J., Durand, S., Cambournac, I., Qian, D., Shi, Z., Dejonghe, E., Richard, V. and Bonami, J.R. (2003) Genome-based detection methods of *Macrobrachium rosenbergii* nodavirus, a pathogen of the giant freshwater prawns, *Macrobrachium rosenbergii*: dot-blot, in situ hybridization and RT-PCR. *Journal of Fish Diseases* **26**: 583-590

Sri Widada, J. and Bonami, J.R. (2004) Characteristics of the monocistronic genome of extra small virus, a virus-like particle associated with *Macrobrachium rosenbergii* nodavirus: possible candidate for a new species of satellite virus. *Journal of General Virology* **85**: 643-646

Sudhakaran, R., Ishaq Ahmed, V.P., Haribabu, P., Mukherjee, S.C., Sri Widada, J., Bonami, J.R. and Sahul Hameed, A.S. (2007a) Experimental vertical transmission of *Macrobrachium rosenbergii* nodavirus (MrNV) and extra small virus (XSV) from brooders to progeny in *Macrobrachium rosenbergii* and Artemia. *Journal of Fish Diseases* **30**: 27-35

Sudhakaran, R., Musthaq, S.S., Haribabu, P., Mukherjee, S.C., Gopal, C. and Sahul Hameed, A.S. (2006) Experimental transmission of *Macrobrachium rosenbergii* nodavirus (MrNV) and extra small virus (XSV) in three species of marine shrimp (*Penaeus indicus*, *Penaeus japonicas* and *Penaeus monodon*). *Aquaculture* **257**: 136-141

Sudhakaran, R., Parameswaran, V. and Sahul Hameed, A.S. (2007b) *In vitro* replication of *Macrobrachium rosenbergii* nodavirus and extra small virus in C6/36 mosquito cell line. *Journal of Virological Methods* **146**: 112-118

Sudhakaran, R., Syed Musthaq, S., Rajesh Kumar, S., Sarathi, M. and Sahul Hameed, A.S. (2008) Cloning and sequencing of capsid protein of Indian isolate of extra small virus from *Macrobrachium rosenbergii*. *Virus Research* **131**: 283-287

Sudhakaran, R., Yoganandhan, K., Ishaq Ahmed, V.P. and Sahul Hameed, A.S. (2006) Artemia as a possible vector for *Macrobrachium rosenbergii* nodavirus (MrNV) and extra small virus (XSV) to *Macrobrachium rosenbergii* post larvae. *Diseases of Aquatic Organisms* **70**: 161-166

Sullivan, C.S. and Ganem, D. (2005) A virus encoded inhibitor that blocks RNA interference in mammalian cells. *Journal of Virology* **79**(12): 7371-7379

Sunier, A.L.J. (1925) Two communications concerning Palaemonidae. *Tijdschrift der Nederlandsche Dierkundige Vereeniging (ser. 2)* **19**: CXV

Tan, C.K. and Owens, L. (2000) Infectivity, transmission and 16S rRNA sequencing of a rickettsia, *Coxiella cheraxi* sp. nov., from the freshwater crayfish *Cherax quadricarinatus*. *Diseases of Aquatic Organisms* **41**: 115-122

Tirasophon, W., Roshorm, Y. and Panyim, S. (2005) Silencing of yellow head virus replication in penaeid shrimp cells by dsRNA. *Biochemical and Biophysical Research Communications* **334**: 102-107

Totland, G.K., Grotmol, S., Morita, Y., Nishioka, T. and Nakai, T. (1999) Pathogenicity of nodavirus strains from striped jack *Pseudocaranx dentex* and

Atlantic halibut *Hippoglossus hippoglossus*, studied by waterborne challenge of yolk-sac larvae of both teleost species. *Diseases of Aquatic Organisms* **38**: 169-175

Tung, C.W., Wang, C.S. and Chen, S.N. (1999) Histological and electron microscopy study on *Macrobrachium* muscle virus (MMV) infection in the giant freshwater prawn, *Macrobrachium rosenbergii* (de Man), cultured in Taiwan. *Journal of Fish Diseases* **22**: 1-5

Vago, C. (1963) A new type of insect virus. *Journal of Insect Pathology* **5**: 275-276

Vago, C. (1966) A virus diseases in crustacea. *Nature* **209**: 1290

Wang, C.S., Chang, J.S., Shih, H.H. and Chen, S.N. (2007) RT-PCR amplification and sequence analysis of extra small virus associated with white tail disease of *Macrobrachium rosenbergii* (de Man) cultured in Taiwan. *Journal of Fish Diseases* **30**: 127-132

Wang, C.S., Chang, J.S., Shih, H.H. and Chen, S.N. (2008) *Macrobrachium rosenbergii* nodavirus infection in *M. rosenbergii* (de Man) with white tail disease cultured in Taiwan. *Journal of Fish Diseases* **31**: 415-422

Weng, S.P., Guo, Z.X., Sun, J.J., Chan, S.M. and He, J.G. (2007) A reovirus disease in cultured mud crab (*Scylla serrata*) in southern China. *Journal of Fish Diseases* **30**: 133-139

Westenberg, M., Heinhuis, B., Zuidema, D. and Vlak, J.M. (2005) siRNA injection induces sequence-independent protection in *Penaeus monodon* against white spot syndrome virus. *Virus Research* **114**: 133-139

Winckler, J. (1974) Vital staining of lysosomes and other cell organelles of the rat with neutral Red. *Progress in Histochemistry and Cytochemistry* **6:** 1-89

Wu-Scharf, D., Jeong, B., Chang, C. and Cerutti, H. (2000) Transgene and transposon silencing in *Chlamydomonas reinhardtii* by a DEAH-box RNA helicase. *Science* **290**: 1159-1162

Xu, J., Han, F. and Zhang, X. (2007) Silencing shrimp white spot syndrome virus (WSSV) genes by siRNA. *Antiviral Research* **73**: 126-131

Yodmuang, S., Tirasophon, W., Roshorm, Y., Chinnirunvong, W. and Panyim, S. (2006) YHV-protease dsRNA inhibits YHV replication in *Penaeus monodon* and prevents mortality. *Biochemical and Biophysical Research Communications* **341**: 351-356

Yoganandhan, K., Leartvibhas, M., Sriwongpuk, S. and Limsuwan, C. (2006) White tail disease of the giant freshwater prawn *Macrobrachium rosenbergii* in Thailand. *Diseases of Aquatic Organisms* **69**: 255-258

Yoganandhan, K., Sri Widada, J., Bonami, J.R. and Sahul Hameed, A.S. (2005) Simultaneous detection of *Macrobrachium rosenbergii* nodavirus and extra small virus by a single tube, one step multiplex RT-PCR assay. *Journal of Fish Diseases* **28**: 65-69

Zamore, P.D., Tuschi, T., Sharp, P.A. and Bartel, D.P. (2000) RNAi: Double-strand RNA directs the ATP-dependent cleavage of mRNA at 21-23 nucleotide intervals. *Cell* **101**: 25-33

Zhang, H., Wang, J., Yuan, J., Li, L., Zhang, J., Bonami, J.R. and Shi, Z. (2006) Quantitive relationship of two viruses (MrNV and XSV) in white tail disease of *Macrobrachium rosenbergii*. *Diseases of Aquatic Organisms* **71**(1): 11-17

Zhang, S., Shi, Z., Zhang, J. and Bonami, J.R. (2004) Purification and characterization of a new reovirus from the Chinese mitten crab, *Eriocheir sinensis*. *Journal of Fish Disease* **27**: 687-692

APPENDIX A

Stainings and Buffers

1. Haematoxylin and Eosin (H&E)

1.	Xylene	2 min
2.	Xylene	2 min
3.	Ethanol	2 min
4.	Ethanol	1 min
5.	Ethanol	1 min
6.	Water wash	1 min
7.	Mayer's haematoxylin	8 min
8.	Water wash	30 sec
9.	Scott's Tap water substitute	30 sec
10.	Water wash	2 min
11.	Eosin (Young's)	4 min
12.	Differentiate Eosin by washing tap water until cells appear red-purple approximately	1 min
13.	Ethanol	10 dips
14.	Ethanol	10 dips
15.	Ethanol	1 min
16.	Xylene	1 min
17.	Xylene	2 min
18.	Xylene	1 min
19.	Mount with DPX	
20.	Coverslipped	

2. Giemsa

1.	Take section to water	
2.	Place in 10% Giemsa stain (Gurr's improved R66 with tap water) approximately	5 min
3.	Rinse in tap water	
4.	Decolourise with 2% Acetic acid, greatly diluted	
5.	Air dry	

6. Mount with DPX
7. Coverslipped

3. Trypan Blue 0.4% 2 min

4. Acridine Orange

1. Fix cells in 50:50 Ether:95%Ethanol 30 min
   (Formalin cannot be used)
2. Hydrate through 80% Ethanol 10 sec
3. Hydrate through 70% Ethanol 10 sec
4. Hydrate through 50% Ethanol 10 sec
5. Rinse with distilled water
6. Treat with 1% Acetic acid 6 sec
7. Stain in 0.1% Acridine orange in McIlvaine's Citric acid Buffer 3 min
8. Wash in McIlvaine's Citric acid Buffer, pH 3.8
9. Differentiate in M/10 Calcium chloride 30 sec
10. Mount with DPX
11. Coverslipped

5. McIlvaine's Citric acid Buffer

1. Sodium phosphate dibasic (Na_2HPO_4) 10.08g
2. Citric acid monohydrate ($C_6H_8O_7.H_2O$) 3.55g
3. Distilled water 1 liter

6. M/10 Calcium chloride

1. Calcium chloride 1.109g
2. Distilled water 100ml

7. Phosphate Buffered Saline (PBS) pH 7.6 (1000 ml)

1. Sodium chloride (NaCl) 8.0g
2. Sodium phosphate dibasic (Na_2HPO_4) 0.64g
3. Potassium chloride (KCl) 0.2g
4. Potassium diphosphate (KH_2PO_4) 0.16g

APPENDIX B

Complete Nucleotide Sequence of *Macrobrachium rosenbergii* Nodavirus (MrNV) RNA1 from the Giant Freshwater Prawn *Macrobrachium rosenbergii* (Genbank accession number: JN619369)

GTTAAACGTTTTGTTTTCTAGCAATGAAGTGCAATAATCTTCTCTACGGTGTGGAACTCC
CCGTGTGCTTGCAATCGAGCATGGGCATGGTGGTAGTTACCGGACTCGCCATAGTGTATT
TGATATCTTATATCCTCATTTACTCATTTACACATGGACCACGACTCAGCCGCAGATTAG
CTTCCTATGTGGTAATCGGACCTTATGAAGCTATTGCTAAAAGCCGACCCGCGATGGCCC
TACAAAGGGCCATGGTTGATTTGACAAGACGGGACGTGCGTACTGACTGGTACCCATTGA
ACAACTTATTGATGAATAAACCTCACCGCTTATCTGAAAATGGACACAAAACTTCTGGAG
CGGTTCGAGATGCAGCTAGAAATTTAATTACTAGCGCTATAACATCACTGGGTATGGATA
AGTACGAAATTTCACCGGGTGGCCACACTGTCGACGAGCAATTGGCATCTCATCGACACT
ATGCTGTTAATGACTTACATCGAGCTTCAGCAGATGATGCAGTTAAAGAAAATGCTGTTA
TTGTGGCCATTGATACAGATTACTATCTACGTGATCCATCAATCTACTTTTCAAACAACA
ATCCTTTTATTCTGCACACTTTTCAACCAATAACTGTTGCAGGTAAAGATGGAGATGTGA
GGTTCACTATCAGCGACAACCAAGTGGACTATAGAGTAGATGGTGGTGGTAGATGGCAAC
ATAAAGTATGGAATTGGTGTGATTATGGTGAATTTCTTATATTTAAAGAACATCCTAGGA
TTCTTAGTATTAATTGGTGGCTGAGTTTCCTTGGTATTAGAAAAGTTATATACCAGAAGA
TCCAACACGCACGCCCCTGGGTGGATTGTCCAAATCGGGCCTTGGTTTGGGGATTACCTC
AATTCACTAGCTACATGATAACTTGGTTGCCAATCGAGATGAATGCACGTGAATTGGGTA
GAGTCAACTATCAGTGTGCCAGCAGACCTGGCTGGAACTGTCTAGTTGATCATACTAGCA
CCAAACTTGTAGCCAGCATAGGTCGAGAGGGTAATGACTGTCACGTTGAACTTGCTAAGG
AAGACCTCGATGTTGTTCTAGGCTTAAGTACGATGCAGTCAGTTACCTCAAGACTTTTAC
AAATGGGTTACAAGCAACCACAAACTTTGGCTACGGTTTGCCAGTTCTACAACAGTGCCG
CTTATGATATACTAAGCTGTAGCATAGTTGCTAGACCTTCACAACCACCTGTCCACTGGC
CATTGGCGTCTGAAATTGATCAGCCTACAACTTCTTTTCGTAACTACTCCAACAATATTG
TGACCTGTGGTAATCTTTGCCCCCAACTTAAACGTTGGGAGGTTTTAAGCAATTCTCTAG
AACATCGTGTTACCATGGTGGCCAACAATAAGGTTCCAACACCTCGCATAGCCAGGTTTG
CTGAAGAATACGTCCGCCTGGTAGTTCCCGAAGCGAATGTAGGTGTACCTTACAGTTTGG
AAGATGCGCGTAAAGAACTTGATAAACCCACGCAAGTCAATGCGGTAAACCAAATTTGGG
AAACAGTAGACATGGAAGTCCGCCGATTAATTGAAGCATTTGTGAAGAATGAACCGACCA

ATAAATCTGGTCGTATAATATCATCCTTTGCGGACTCAAGATTTTTGTTGAAGTTTTCCA

CATATACGCTTGCTTTTCGAGATGAGGTATTACATGCCGAACACAATCGACATTGGTTTT

GTCCTGGTTTGACACCTAATGAGATAGCAGATAAAGTATGCGACTATGTTCGTGGTGTTG

CAACACCTGCAGAAGGTGATTTTAGCAACTTTGATGGAAGGGTGTCTGCTTGGTGTCAGG

AGAACGTGATGAACGCAGTTTATCATAGATGGTTTAACCGTAAGTTCTCTAAGGAATTGC

AGAAGTATACATCAATGTTGGTTAGTTGTCCAGCTCGAGCTAAGCGTTTTGGTTTCCAGT

ACGAACCAGGAGTGGGGGTTAAGAGTGGTAGTCCAACCACCTGTGACCTTAATTCAGTTC

TAAATAACTTTACACAATACGCAGCAGTTAGGCTGACTAAACCAGACCTCTCACCACAAG

AAGCCTTTGAACAAACTGGCTTAAGTTTCGGCGATGATTCACTATTTGACAAGCAATATC

AGTCGAGGTGGAATCAAGTCGTCGAACAACTTGGTATGGAACTCAAGGTTGAACCCTTCG

ACCCTAGTAATGGTGTGACTTTTCTTGCTCGTGTTTTTCCTGATCCTTATAGTACAAATA

CTAGTTTTCAGGATCCACTAAGAACGTGGAGAAAGTTGAACATGACTTCACGCACGTGTG

TACCTGTTGAGTCAGCAGCTCTTGATCGTGTCAGTGGATATTTAGTTACTGACAAGTACT

CTCCAGTGACAAGTGAGTACTGCCATATGATTGAGAGGTGTTATATGAACACTGCCGAAA

GTGTCACTCGAAGGAGACAACGCAAGGATTGTGACCGTGAGAAACCATACTGGTTAGTGA

GTGGTGGCGCCTGGCCCCAGAGGGAGGGGGACTATGAATTGATGATGAGGGTTACCGCAG

CCCGTACTGGATTTGAGGAGTCAAAACTTATCAATTTGATAAGCCAGTTACAATCAGTTC

ATGATCCTTGGGACATCAAACCGCTAGACTATCAAGAACCATCACCTTATAAAGATACAT

TAGACATAGATGGTCAGCCGGTAGATGCAGTGGACGATCGTCAATATCAAAATGAGCGCA

ACACAGTCAACTTACGAGCTGGTGCAACAATTTCCCAGATGCCTGTCCCAAGTGTGCCAG

GCGGTGAAGACTGCAATCGACAGTCTGCCGACGTGCCAGGACCCAAAAGTAGCGAAGGAT

CTGAGCAGCTACAAGGCGTGCCTGAGCAAGATGGAAGCAACCGCCTTCAATGCCACCGAC

AACCTACTTTCAAAGCCAAGGGTGGTAGCAACACTAAAGGGAGAGGCCGTAAATCCCGGA

ACGGAGGACGTTCTATCAGCAGCAAAACAGCAAATCCAACAACTCACGAGGTTGGTGGAG

GCAATGGAAAGGCCAGAGTTACCATTGCTAAGCGAAGCAGACCTAAGCGACCTCATAACG

TGGTAAGCAATGCGGTGTAAACATGACAACCGATAAACTCTCTCTCTCTCTCTCTCACTC

TCTCTCTCTCTCTCTCTCTCT

Figure B.1: Complete nucleotide sequence of the 3203 bp amplified from the genome of *Macrobrachium rosenbergii* nodavirus (MrNV) RNA1 from the giant freshwater prawn *Macrobrachium rosenbergii.*

APPENDIX C

Complete Nucleotide Sequence of *Macrobrachium rosenbergii* Nodavirus (MrNV) RNA2 from the Giant Freshwater Prawn *Macrobrachium rosenbergii* (Genbank accession number: JN619370)

AAAGGATATTCGATATTCTATCATCTTTAACATCAAGATGGCTAGAGGTAAACAAAATTC

TAATCAGACTCAAAATAATAATAACGCAAACGGCAAGCGCCGTAAGCGTAATCGAAGGAA

TCGTAATCCGCAGACGATTCCTAACTTCAATCCCATTGTCGCAAGGCCGACTATTGCCCC

ACTTCAAACTAACATTAGAAGTGCTAGGAGTGATGTTAATGCCATCACTGTTTTAAATGG

CAGTGATTTTCTTACAACTGTCAAGGTCCGAGGTTCTAACAATTTGACTGACTCCAAGTC

TAGAATCTTGGTAAAGCAACCAATTTCTGCGAGTTCTTTTCTTGGTACCAGAATTTCTGG

TTTATCGCAATTTTGGGAGCGTTATAGATGGCATAAGGCTGCAGTTAGATACGTTCCTGC

AGTACCTAATACATTGGCTTGCCAACTTATTGGCTACATCGATACAGATCCACTAGATGA

CCCTGACGTTATCCTCGATGTTGACCAGTTACTTAGGCAGGCTACGTCACAGGTGGGTGC

CCGACAATGGAACTTCTCTGATACAACAACTATTCCATTGATTGTCAGGCGTGATGATCA

ATTGTATTATACTGGCCAAGACAAAGAGAACGTTCGGTTCTCCCAACAGGGTGTATTTTA

CCTCTTGCAAGTGACTACACTACTTAATATTAGTGGTGAAGCTATTACAGATGATTTAAT

TTCTGGTTCATTATATTTGGATTGGGTCTGTGGATTTTCCATGCCACAAATTAATCCTAC

ACCAGTGGAATTTTCACAGCTAATCTACAATGCGGATACAATTGGCGATTGGGTCCCACC

TCAGGAACTCAAACAAACTTACACTCAAGATATCACTGGTTTGAAGCCAAAATCCAAATT

TATTATTGTACCCTATATGGATAGACAAGTTCCGATGTACTACAGAAGTGCACAATCAC

TTGTAATGAGATTGATTCAACTGGTTCAATCTCATATTTTGATACTAACGACATCAAATG

TGATGGGTATATATTATTTCAGGCTAACAGTATTGGTGAAGCAAATTTCACCCTAGTGAC

CGATTATCAGGGTGTTGTTGAACCTAAACCCTACCAGTATAGGATTATCAGAGCTATCGT

CGGCAATAATTAGGTTGTCATATCTAGCACATGA

Figure C.1: Complete nucleotide sequence of the 1173 bp amplified from the genome of *Macrobrachium rosenbergii* nodavirus (MrNV) RNA1 from the giant freshwater prawn *Macrobrachium rosenbergii*.

APPENDIX D

Animal Ethics Approval

JAMES COOK UNIVERSITY
Townsville Qld 4811 Australia

Noema Patterson, Animal Ethics Officer, Research Office. Ph: 07 4781 4484; Fax: 07 4781 5521

ETHICS REVIEW COMMITTEE
Animal Ethics Committee
APPROVAL FOR ANIMAL BASED RESEARCH OR TEACHING

PRINCIPAL INVESTIGATOR	**Mr Orachun Hayakijkosol**					
OTHER INVESTIGATORS	Associate Professor Leigh Owens					
SCHOOL	Veterinary & Biomedical Sciences					
PROJECT TITLE	**Investigation into pathogenicity of macrobrachium rosenbergii nodavirus (MrNV) and extra small virus (XSV) to juvenile crustacea**					
APPROVAL DATE	13 Feb 09	**EXPIRY DATE**	31 Dec 11		**CATEGORY**	**3**

This project has been allocated Ethics Approval Number with the following conditions: **A 1382**

1. All subsequent records and correspondence relating to this project must refer to this number.
2. That there is NO departure from the approved protocols unless prior approval has been sought from the Animal Ethics Committee.

3. The Principal Investigator is to advise the responsible Ethics Monitor appointed by the Ethics Review Committee:

   - within 48 hours of any adverse effects suffered by any animal;

   and the continued ethical

   ADMINISTRATIVE DOCUMENTATION HAS BEEN REMOVED

   any reason.

4. In compliance with the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes*, and the *Queensland Animal Care and Protection Act 2001*, it is **MANDATORY** that you provide an annual report on the progress of your project. This report must also detail animal usage, and any unexpected event or serious adverse effect that may have occurred during the study.

APPENDIX E

Statistical Results

Table E.1: Table shows the statistic analysis of percentage mortality in experiment 1

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3733.333	2	1866.667	5.895	.091
Within Groups	950.000	3	316.667		
Total	4683.333	5			

Table E.2: Table shows the statistic analysis of percentage mortality in experiment 2

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1233.333	2	616.667	37.000	.008
Within Groups	50.000	3	16.667		
Total	1283.333	5			

Table E.3: Table shows the statistic analysis of length and weight of experiment animals after 60 days in experiment 1

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Length	**Between Groups**	.611	2	.305	.269	.766
	Within Groups	31.733	28	1.133		
	Total	32.344	30			
Weight	**Between Groups**	295.164	2	147.582	2.097	.142
	Within Groups	1970.209	28	70.365		
	Total	2265.373	30			

Table E.4: Table shows the statistic analysis of length and weight of experiment animals after 60 days in experiment 2

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Length	**Between Groups**	8.420	2	4.210	5.509	.007
	Within Groups	35.156	46	.764		
	Total	43.577	48			
Weight	**Between Groups**	187.598	2	93.799	2.264	.115
	Within Groups	1905.907	46	41.433		
	Total	2093.506	48			

Table E.5: Table shows the statistic analysis of spectrophotometer absorption from neutral red in cell culture experiment

Kruskal-Wallis Test

Ranks

	Groups	N	Mean Rank
Spectro	Control	28	42.50
	Infected	28	14.50
	Total	56	

Test Statistics[a,b]

	Spectro
Chi-Square	41.265
df	1
Asymp. Sig.	.000

a. Kruskal Wallis Test

b. Grouping Variable: Groups

Table E.6: Table shows the statistic analysis of percentage mortality in RNA interference experiment

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1441.667	5	288.333	6.920	.018
Within Groups	250.000	6	41.667		
Total	1691.667	11			

Table E.7: Table shows the statistic analysis of length and weight of experiment animals after 60 days in RNA interference experiment

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Length	**Between Groups**	23.326	5	4.665	8.106	.000
	Within Groups	31.078	54	.576		
	Total	54.404	59			
Weight	**Between Groups**	264.216	5	52.843	2.943	.020
	Within Groups	969.578	54	17.955		
	Total	1233.794	59			

Table E.8: Table shows the statistic analysis of viral copies from qPCR in RNA interference experiment

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.910	1	.910	.263	.612
Within Groups	96.915	28	3.461		
Total	97.825	29			

Table E.9: Table shows the statistic analysis of cycle times from qPCR in RNA interference experiment

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.012	1	.012	.017	.898
Within Groups	19.925	28	.712		
Total	19.937	29			

APPENDIX F

List of Presentations, Posters and Workshops

Presentations

2010 "Development of an Experimental Model with crayfish (*Cherax quadricarinatus)* for *Macrobrachium rosenbergii* nodavirus (White Tail Disease)", Townsville Festival of Life Sciences 2010, Townsville, Queensland

2011 "Experimental Infection of Redclaw Crayfish (*Cherax quadricarinatus*) with *Macrobrachium rosenbergii* Nodavirus, the Aetiological Agent of White Tail Disease", First fisheries research and development corporation (FRDC) Australian Scientific international Conference on Aquatic Animal Health, Cairns, Queensland

2011 "Experimental studies of *Macrobrachium rosenbergii* nodavirus (White Tail Disease) Australian isolate", North Queensland Festival of Life Sciences 2011, Townsville, Queensland

Posters

2011 "*Macrobrachium rosenbergii* nodavirus (White Tail Disease) Australian isolate", North Queensland Festival of Life Sciences 2011, Townsville, Queensland

2010 "Development of an Experimental Model with crayfish (*Cherax quadricarinatus)* for *Macrobrachium rosenbergii* Nodavirus (White Tail Disease)", Townsville Festival of Life Sciences 2010, Townsville, Queensland

Poster was presented and won recognition award.

2009 “Development of a crayfish experimental model for white tail disease (nodavirus)”, Townsville Festival of Life Sciences 2009, Townsville, Queensland

Workshops

2008 Biosafety Course, School of Veterinary and Biomedical Science, James Cook University, Townsville, Queensland, Australia

2010 Aquatic Animal Health Technicians Forum Workshop, AAHL (Geelong), CSIRO, funded by the Fishing Industries Research Corporation
Activities: visit aquatic animal laboratory and PC3 laboratory and discuss about molecular diagnostic techniques

Owens, L., La Fauce, K., Juntunen, K., Hayakijkosol, O. and Zeng, C. (2009)*Macrobrachium rosenbergii* nodavirus disease (white tail disease) in Australia. *Diseases of Aquatic Organisms* **85**: 175-180.

Hayakijkosol, O., La Fauce, K. and Owens, L. (2011) Experimental infection of redclaw crayfish (*Cherax quadricarinatus*) with *Macrobrachium rosenbergii* nodavirus, the aetiological agent of white tail disease. *Aquaculture* **319**: 25-29.

Hayakijkosol, O. and Owens, L. (2012) B2 or not B2: RNA interference reduces *Macrobrachium rosenbergii* nodavirus replication in redclaw crayfish (*Cherax quadricarinatus*). *Aquaculture* **326-329**: 40-45.