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Macrobrachium rosenbergii De Man NODAVIRUS RECOMBINANT CAPSID PROTEIN PRODUCTION AND ITS INTERACTIONS WITH RNA

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

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Chairman : Professor Tan Wen Siang, PhD
Faculty : Biotechnology and Biomolecular Sciences

Macrobrachium rosenbergii nodavirus (*Mr*Nv) is a causative agent of white tail disease (WTD) causing nearly 100% mortality in post-larvae of giant freshwater prawns. In 2012 the major farming areas in Malaysia were found to be infected by *Mr*Nv but till now information on the Malaysian isolate *Mr*Nv is still unavailable in the NCBI database and studies of this newly emerged member of the *Nodaviridae* family were not in depth. Studies on the other members of the *Nodaviridae* family revealed that the RNA binding site is important for virus assembly and removal of this region inhibits the virus maturation. Therefore, researches were conducted to identify the Malaysian *Mr*Nv capsid sequence, structural morphologies, nucleic acid contents and the RNA binding site of this virus. In this study, *Mr*Nv was isolated from infected prawns obtained from a prawn farm in Negeri Sembilan, Malaysia. Prawn muscle tissues were screened with PCR to detect *Mr*Nv. The nucleotide sequence of the *Mr*Nv *capsid* gene isolated from a Malaysian isolate was sequenced and later compared with those available in the NCBI database. Phylogenetic analysis of *Mr*Nv capsid protein revealed that the Malaysian isolate was closely related to the Chinese isolates. The coding region of *Mr*Nv capsid protein was cloned into pTrcHis2-TOPO expression vector and introduced into *Escherichia coli* TOP10 cells. The recombinant capsid protein of *Mr*Nv containing a His-tag was purified by using immobilized metal affinity chromatography (IMAC). The purified capsid protein was analysed using transmission electron microscopy (TEM), dynamic light scattering (DLS) and sucrose density gradient ultracentrifugation, which revealed the formation of virus-like particles (VLPs) of about 30±3 nm in diameter. RNA molecules were found to be encapsidated inside the cavity of *Mr*Nv VLPs which suggested that VLPs resembled the native virus. Amino acid sequence analysis of the *Mr*Nv capsid protein revealed that 8 out of 10 amino acids located at residues 20^{th} to 29^{th} are positively-charged suggesting RNA binding region is located in this region. Deletion mutagenesis and amino acid substitutions of the positively-charged amino acids located at the N-terminal end of the *Mr*Nv capsid protein were performed to determine the RNA binding region. A total of seven mutants were created with different deletion and point mutations starting from residues 1 to 29 of the N-terminal end of the *Mr*Nv capsid protein. All the mutants were shown to assemble into VLPs ranging from 18 to 34 nm in diameter. Mutants

with the positively-charged amino acids deleted, namely 29Δ*Mr*Nvc and 20-29Δ*Mr*Nvc did not contain RNA molecules in their VLPs. A point mutation mutant, namely K20R21R22K23R24A, showed a significantly lower amount of RNA molecules compared with that of mutant R26R27R29A, suggesting that the five positively-charged amino acids residues at positions 20 to 24 play an important role in RNA binding. This study showed the positively-charged amino acids at positions 20 to 29 of the capsid protein are the RNA binding site of *Mr*Nv. In conclusion, the nucleotide sequence of Malaysian *Mr*Nv capsid gene was determined and showed high similarity with the Chinese isolates. The recombinant *Mr*Nv capsid protein produced in bacteria was able to assemble into VLPs which resembled the native virus. The RNA binding site of the capsid protein was identified and located at position 20^{th} to 29^{th}. Removal of this region did not affect virus assembly suggesting that the presence of the assembly domain. This information is useful for the development of a vaccine against *Mr*Nv and its structural analysis.

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PENGHASILAN *Macrobrachium rosenbergii* De Man NODAVIRUS PROTEIN KAPSID REKOMBINAN DAN INTERAKSINYA DENGAN RNA

Oleh

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Macrobrachium rosenbergii nodavirus (*Mr*Nv) adalah agen penyebab penyakit ekor putih (WTD) dan ia menyebabkan hampir 100% kematian di pos-larva udang galah. Pada tahun 2012, udang galah di kawasan perternakan utama di Malaysia telah dijangkiti *Mr*Nv tetapi sehingga kini tiada maklumat yang boleh didapati mengenai *Mr*Nv Malaysia di pangkalan data NCBI dan kajian terhadap virus yang baru muncul ini masih tidak mendalam. Kajian terhadap ahli keluarga *Nodaviridae* yang lain mendedahkan kepentingan tapak pengikatan RNA dalam perkumpulan virus dan penyingkiran tapak ini akan menghalang penyatuan virus. Oleh itu, penyelidikan telah dilakukan untuk mengenalpasti urutan nukleotida kapsid *Mr*Nv Malaysia, ciri-ciri dan tapak pengikatan RNA virus ini. Dalam kajian ini, *Mr*Nv telah diasingkan daripada udang terjangkit virus yang diperolehi daripada ladang udang di Negeri Sembilan, Malaysia. Tisu otot udang telah dianalisis dengan PCR untuk mengesan *Mr*Nv. Urutan nukleotida *Mr*Nv capsid gen diasingkan daripada udang galah Malaysia telah dibandingkan dengan yang terdapat di pangkalan data NCBI. Analisa filogenetik daripada *Mr*Nv capsid protein mendedahkan bahawa *Mr*Nv Malaysia berkait rapat dengan *Mr*Nv China. DNA yang mengekodkan *Mr*Nv capsid protein telah diklon ke vektor pTrcHis2-TOPO dan diperkenalkan ke dalam sel-sel *Escherichia coli* TOP10. Protein kapsid rekombinan daripada *Mr*Nv mengandungi His-tag telah ditulenkan dengan menggunakan kromatografi logam kekal afiniti (IMAC). Protein kapsid ditulenkan dan dianalisa dengan menggunakan mikroskop elektron transmisi (TEM), penyerakan cahaya dinamik (DLS) dan sukrosa kecerunan ketumpatan ultra-pengemparan. Diameter partikel menyerupai virus (VLPs) adalah kira-kira 30 $\pm$ 3 nm. Molekul RNA telah dikesan di dalam ruang dalam VLPs mencadangkan VLPs menyerupai virus asli. Analisasi jujukan asid amino protein *Mr*Nv capsid mendedahkan bahawa 8 daripada 10 asid amino terletak di kedudukan ke-20 hingga 29 adalah bercas positif mencadangkan bahawa RNA mengikat di bahagian ini. Mutagenesis penghapusan bes dan penggantian asid amino pada asid amino yang bercas positif yang terletak di hujung-N protein *Mr*Nv kapsid telah dijalankan untuk menentukan kawasan ini mengikat RNA. Sebanyak 7 mutan telah dihasilkan dengan penghapusan dan titik mutasi yang berbeza bermula dari kedudukan 1 hingga 29 pada hujung-N protein *Mr*Nv kapsid. Semua mutan telah ditunjuk membentukan VLP antara

18-34 nm diameter. Mutan dengan asid amino yang bercas positif dimansuhkan, iaitu 29Δ*Mr*Nvc dan 20-29Δ*Mr*Nvc tidak mengandungi molekul RNA dalam ruang dalam VLP mereka. Satu mutan mutasi titik, iaitu K20R21R22K23R24A, menunjukkan jumlah RNA yang jauh lebih rendah berbanding dengan mutan R26R27R29A, mencadangkan lima asid amino bercas positif di kedudukan ke-20 hingga ke-24 memainkan peranan yang penting dalam pengikatan molekul RNA. Kajian ini menunjukan bahawa asid amino yang bercas positif di kedudukan ke-20 hingga 29 daripada protein kapsid adalah tapak mengikat RNA *Mr*Nv. Kesimpulannya, jujukan nukleotida gen *Mr*Nv kapsid dari Malaysia telah ditentukan dan ianya serupa dengan *Mr*Nv Cina. Protein *Mr*Nv kapsid rekombinan boleh dihasilkan dalam bakteria dan VLPs telah dikesan. Tapak pengikatan molekul RNA di kapsid *Mr*Nv telah dikenalpasti dan tapak ini terletak di kedudukan 20^{th} ke 29^{th}. Penyingkiran tapak pengikatan molekul RNA tidak akan mempengaruhi pembentukan virus mencadangkan kehadiran tapak pemasangan bagi virus ini. Maklumat ini adalah berguna untuk pembangunan vaksin terhadap *Mr*Nv dan analisa struktur.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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Δ	deletion
6 × His	six residues of histidine
a	centrifugal force
A_{260}	Optical Density at absorbance 260 nm
A_{280}	Optical Density at Absorbance 280 nm
AAV2	adeno-associated virus serotype 2
AHNV	Atlantic halibut *Hippoglossus hippoglossus* nodavirus
AK-1	Arginine kinase-1
AMV	alfafa mosaic virus
AMV	avian myeloblastosis virus
ANTXR2	Anthrax toxin receptor 2
APC	antigen-presenting cell
APS	ammonium persulfate
ATP	adenosine triphosphate
Au	gold
BBV	Black beetle virus
BCIP	5-bromo-4-chloro-3'-indolyphosphate p-toluidine-salt
BFNNV	barfin flounder nervous necrosis virus
BMV	Brome Mosaic virus
bp	base pair
Ca^{2+}	calcium ion
$CaCl_2$	calcium chloride
$CaCO_3$	Calcium carbonate
Casp3c	Caspase 3c

CBB G-250	*coomassie brilliant blue G-250*
CBB R-250	Coomassie brilliant blue R-250
CCMV	Cowpea chlorotic mottle virions
cDNA	complementary deoxyribonucleic acid
CHIKV	Chikungunya virus
CMV	cucumber mosaic virus
CoV	coronavirus
CPV	canine parvovirus
cryo-EM	cryo-electron microscopy
CTL	cytotoxic T lymphocytes
Cu	copper
D	diffusion coefficient
ddH_2O	double distilled water
DENV-2	Dengue virus 2
DGNNV	dragon grouper nervous necrosis virus
DIG	digoxigenin
DLS	dynamic light scattering
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
DOF	Department of Fisheries
DOX	doxorubicin
dsDNA	double-stranded DNA
dsRNA	double-stranded RNA
DTT	Dithiothreitol

ECV	endosomal carrier vesicles
EDTA	ethylenediaminetetraacetic acid
EGFP	Enhance green fluorescence protein
EM	electron microscope
env	envelope protein
FAO	Food and Agriculture Organisation of the United Nations
FFRC	Freshwater Fisheries Research Centre
fg	femto-gram
FHV	Flock House virus
g	gravity force
GnRH	gonadotropin releasing hormone
GP	glycoprotein
h	hour
HBcAg	Hepatitis B core antigen
HBsAg	hepatitis B surface antigen
HBV	hepatitis B virus
HCl	Hydrochloric acid
HCV	hepatitis C virus
HDAg	hepatitis delta antigen
HEV	hepatitis E virus
HIV	human immunodeficiency virus
HPV	human papillomavirus
Hz	Hertz
ICM-MS	Intact cell MALDI-TOF mass spectrometry
ICTV	International Committee on Taxonomy of Viruses

IgG	immunoglobulin type G
IHHNV	infectious hematopoietic and hypodermal necrosis virus
IMAC	immobilized metal affinity chromatography
IMN	idiopathic muscle necrosis
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IRDR-1	interferon related developmental regulator-1
ITC	Isothermal titration calorimetry
JE	Japanese Encephalitis
JGMV	Johnsongrass mosaic virus
k	Boltzmann's constant
K	Kelvin
kb	kilobase pair
kcal/mo l	kilocalorie per mole
KCl	potassium chloride
kDa	kilo Dalton
kg m^{-1} s^{-1}	kilogram per meter per second
KLH	Keyhole limpet hemocyanin
LA	lactobionic acid
LB	Luria Bertani
M	Molar
M1	matrix protein
mA	milli Ampere
MALDI-TOF	Matrix-assisted laser desorption/ionization-time of flight mass spectrometer
MALS	multi-angle light scattering

MCS	multiple cloning site
µg/ml	microgram per mili litre
$MgSO_4$	magnesium sulphate
MEGA	Molecular Evolutionary Genetics Analysis software
miRNA	micro-RNA
µm	micro meter
µM	micro Molar
mM	milli Molar
MMV	*Macrobrachium* muscle virus
MoNv	Mosinovirus
MRFV	Maze rayado fino virus
Mr	*Macrobrachium rosenbergii*
*Mr*Nv	*Macrobrachium rosenbergii* nodavirus
*Mr*Nvc	*Macrobrachium rosenbergii* nodavirus capsid
mt	metric ton
mW	mili Watt
n	number
N	Normality
Ƞ	viscosity of the solvent
NaCl	sodium chloride
NAGE	native agarose gel electrophoresis
NaOH	sodium hydroxide
NaPi	sodium phosphate
NBM	nucleotide binding motif
NBT	nitro-blue tetrazolium chloride

NDV	Newcastle disease virus
NFκBI-α	NF kappa B inhibitor alpha
$(NH_4)_2SO_4$	ammonium sulphate
NiV	Nipah virus
nm	nano meter
NMR	nuclear magnetic resonance
NNV	nervous necrosis virus
NoV	Nodamura virus
NP	nucleoprotein
NS4B	non-structural protein 4B
nt	nucleotide
OD_{595}	Optical Density at absorbance 595 nm
OD_{600}	Optical Density at absorbance 600 nm
OGNNV	orange-spotted grouper nervous necrosis virus
OR	origin of replication
PaV	Pariacoto virus
PCR	polymerase chain reaction
pg	pico-gram
PL	post-larvae
PMSF	phenylmethylsulfonyl fluoride
Prdx	peroxiredoxin
PVDF	polyvinylidene fluoride
q-RT-PCR	real-time reverse transcription polymerase chain reaction
RBSDV	*rice black-streaked dwarf virus*
RdRp	RNA dependent RNA polymerase

RGNNV	red-spotted grouper nervous necrosis virus
Rh	hydrodynamic radius
RNA	ribonucleic acid
RNAi	RNA interference
RNA/µg	RNA per microgram
ROS	reactive oxygen species
rpm	revolution per minute
RT-LAMP	reverse transcription loop mediated isothermal amplification
RT-PCR	reverse transcription polymerase chain reaction
s	sedimentation coefficient
S	Svedberg unit
SARS	severe acute respiratory syndrome
SB	sodium boric acid base buffer
scFv11	anti-West Nile virus E antibody 11
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC	size exclusion chromatography
sg	sungai
siRNA	short interfering RNA
SJNNV	striped jack nervous necrosis virus
SOC	Super Optimal Broth
SOCS1	cytokine signalling 1
SSN-1	snakehead-fish *cell line*
STNV	satellite tobacco necrosis virus
S-ELISA	Sandwich enzyme-linked immunosorbent assay

s/n/c	substitution per nucleotide per cell infection
T	triangulation number
T	temperature in Kelvin (K)
TA	Thymine Adenine cloning
TAE	tris acetic acid EDTA
TBS	tris base saline
TBST	Tris base saline Tween 20
TBSV	tomato bushy stunt virus
TEM	transmission electron microscope
TEMED	N,N,N',N'-Tetramethylethylenediamine
Tfl	*Thermus flavus*
TGNNV	tiger puffer nervous necrosis virus
TLR4	toll-like receptor 4
TN	Tris-NaCl
TYMV	turnip-yellow-mosaic virus
U	unit
UV	ultraviolet light
V	voltage
VLPs	virus-like particles
Vt	velocity terminus
v/v	volume per volume
WhNV	Wuhan nodavirus
WSSV	white spot syndrome virus
WTD	white tail disease
w/v	weight per volume

XRD	X-ray diffraction
XSV	extra small virus

CHAPTER 1

INTRODUCTION

Giant freshwater prawn, scientifically known as *Macrobrachium rosenbergii* (*Mr*) and locally known as 'Udang Galah' in Malay, is an important aquaculture for human consumption. It is the largest known caridean shrimp, and can grow until 32 cm (male) and 25 cm (female) in length (Hung, 2013). *Mr* farming can be easily found in Malaysia, making the country one of the major players in this industry. In recent years, due to the high demand, the production and price of this prawn have increased dramatically. Based on the report of the Department of Fisheries (DOF) of Malaysia, the price for giant freshwater prawn was between RM 25 to RM 40 per kilogram in 2003. The price for the prawn increased to RM 45 per kilogram in 2011 (Sahidin and Mustapha, 2011, October).

Currently, a few types of diseases are infecting *Mr*, which cause high mortality, even though this species can be considered a moderately disease-resistant species (Ravi *et al.*, 2009). Based on the Food and Agriculture Organisation of The United Nations (FAO) report (FAO, 2015), there are more than 10 types of diseases which can attack the prawn species. These diseases cause immense economic loss in prawn farming due to the high mortality rates.

White tail disease (WTD) is one of the diseases attacking this moderately disease-resistant species. The clinical signs of WTD infection are lethargy, anorexia and opaqueness of abdominal region of the prawn (Sudhakaran *et al.*, 2007). Upon the infection of the WTD, the tail of the prawn will appear milky. After a few days of infection, the milky appearance will spread toward both the posterior and anterior parts, causing the degeneration of the telson region (Hameed *et al.*, 2004a). Discolorations also will start to appear from the telson region toward the cephalothorax. The milky appearance of the muscle is due to the hyaline necrosis of the muscle fibres (Widada *et al.*, 2003). WTD causes high mortality among the prawns, especially in larvae and post-larvae stages. Hameed *et al.* (2004b) demonstrated that this disease can cause 100% mortality in larvae and post-larvae stages after 2 to 3 days post-infection. The mortality was found to be lower in adult prawns due to a better immune system.

WTD was first reported in French West Indies in 1995 (Arcier *et al.*, 1999). Within 10 years, the disease has spread to different countries all over the world including India (Hameed *et al.*, 2004a), China (Qian *et al.*, 2003), Thailand (Yoganandhan *et al.*, 2006), Australia (Owens *et al.*, 2009), Taiwan (Wang *et al.*, 2008a) and most recently Malaysia (Saedi *et al.*, 2012). An immersion challenge by using the virus isolated from the WTD infected prawn successfully caused similar appearances on healthy prawns suggesting that the virus might be the main causative agent. This was later identified as a nodavirus-like particle and was named as *Macrobrachium rosenbergii* nodavirus (*Mr*Nv). There is another type of virus known as extra small virus (XSV) which is associated with the *Mr*Nv (Qian *et al.*, 2003). Both *Mr*Nv and XSV are icosahedral in structure. *Mr*Nv is about 26 nm in diameter while XSV is about 15 nm in diameter (Ravi *et al.*, 2009). Both viruses are non-enveloped (Tripathy *et al.*, 2006) and located at the cytoplasm of the infected prawn cells. *Mr*Nv and XSV have positive-sense, single-stranded RNA molecules. *Mr*Nv genome is composed of two linear positive-

sense RNAs which are encapsidated by the capsid assembled from CP-43 (Bonami *et al.,* 2005). The genome of *Mr*Nv only codes for 3 proteins. RNA1, which codes for RNA dependent RNA polymerase (RdRp), is only 2.9 kb in length. RNA2, which codes for the capsid protein, has a genome of 1.3 kb in length. As for RNA3, which codes for protein B-2, researchers have yet to understand its function. For XSV, the genome consists of one linear positive sense, single-stranded RNA which has 796 nucleotides that codes for one capsid protein; CP-17 (Widada and Bonami, 2004). Yoganandhan *et al.* (2006) showed that not every post-larvae that are infected by *Mr*Nv contain XSV. Therefore, the relationship between *Mr*Nv and XSV is still unclear. This also suggests that WTD can be due to the infection of a prawn by *Mr*Nv alone.

This newly emerged *Mr*Nv poses many challenges to virologists. The genotypes of this virus share little similarity among the members in *Nodaviridae* family. Hence, this virus was grouped into a new sub-genus known as *Gammanodavirus* (NaveenKumar *et al.,* 2013). The genome of *Mr*Nv isolated from different countries showed differences in nucleotide sequence as analysed with the Molecular Evolutionary Genetics Analysis software (MEGA) (Saedi *et al.,* 2012). *Mr*Nv has been detected in Malaysian prawn farms since 2012, but the problems are Malaysian *Mr*Nv capsid sequence has yet to be identified and the relationship between Malaysian isolate with the other available isolates is still unclear. Thus far, the analyses done on this newly emerged member of *Nodaviridae* family were not in depth. The important domain and characteristics for the virus are still not available and have thus delayed the progress to develop a vaccine to encounter the virus outbreak. Therefore, the objectives for the first study will be the nucleotide sequence of *Mr*Nv capsid isolated from Malaysia prawns will be analysed and the sequence of the *Mr*Nv capsid, Malaysian isolate will be compared with other isolates to identify the nucleotide differences and the relationship of the Malaysian isolate with others.

*Mr*Nv can be transmitted horizontally or vertically (Ravi *et al.,* 2009) either by sharing virus-polluted water sources from native host to non-native host (Sudhakaran *et al.,* 2006) or from parental prawns to offsprings (Hameed *et al.,* 2004b). Transmission of this virus from a marine shrimp (*Penaeus* sp.) to a freshwater prawn (Sudhakaran *et al.,* 2006) further validates the horizontal transmission theory. A high mutation rate in RNA viruses due to the lack of a proof-reading RNA polymerase (Elena and Sanjuán, 2005) preliminarily poses a threat to the aquatic invertebrate where the *Mr*Nv was found to cause mortality in post-larvae in *Penaeus vannamei* (Senapin *et al.,* 2012), suggesting that the virus mutation might transmit to other prawn species and pose a threat to them in future. *Mr*Nv outbreaks in many countries since the last decade suggest that the virus can be transmitted easily and the mutations occurring in the virus RNA genome may start to cross infect *Penaeus vannamei*, endangering other aquatic crustaceans. Currently there is still no vaccine to prevent nor a drug to treat the virus infection. Early screening and better water management schemes are the only solutions to prevent the virus outbreak.

To counter the virus outbreak and contain its spreading, knowledge on the virus life cycle and virus morphologies is needed. Introduction of virus genome into an expression system ensures the production of virus protein throughout the virus analysis. The use of bacterial expression host in virus study can be observed since decades ago such as the expression of coat protein of potyvirus, Johnsongrass mosaic

virus (JGMV) (Jagadish *et al.*, 1991), hepatitis C virus core antigen (Lorenzo *et al.*, 2001) and VP2 protein of canine parvovirus (CPV) (Xu *et al.*, 2014). These recombinant proteins expressed by bacteria hosts retain the native virus properties and were shown to form virus-like particles (VLPs). These recombinant VLPs were successfully used to understand the virus assembly, genome encapsidation (Jagadish *et al.*, 1991), virus aggregation (Lorenzo *et al.*, 2001) and vaccine production (Xu *et al.*, 2014). Extra small virus (XSV) which associates with the infection of MrNv has been produced in Escherichia coli and studied. The XSV capsid gene has been successfully cloned into an expression vector and introduced into a bacterial host (Wang et. al., 2008). This study showed that the recombinant capsid protein of XSV can be expressed in a bacterial cell and was able to retain the capsid forming properties when analysed under a transmission electron microscope (TEM). The bacteria expression system has been widely used in many virus studies and the recombinant virus capsid genes produced by bacteria hosts were able to form into VLPs that resemble the native virus. MrNv has a low incidence rate in Malaysia and this problem has hindered the process to gain information regarding this virus using the native virus. Thus, the objectives for this part of study are to introduce the MrNv capsid gene into a bacteria expression system to ensure large production of virus protein to advance the study for better understanding of MrNv in morphology, assembly and genome encapsidation. Following that, the Malaysian MrNv capsid gene will be cloned into an expression vector and introduced into bacteria cells to ensure the production of the MrNv capsid. This recombinant MrNv capsid protein will then be studied and used as a model to understand the characteristics of the native virus.

Studies on the assembly of Brome mosaic virus (BMV) and Flock House virus (FHV), both of which are positive sense RNA viruses grouped under the families of *Bromoviridae* and *Nodaviridae* respectively, indicating that there are at least two important interactions for the capsid formation: a weaker protein-protein interaction and a stronger RNA-protein interaction (Marshall and Schneemann, 2001; Kaper, 1975). The modelling of RNA-protein interaction of Pariacoto virus (PaV) has been discussed by Harvey *et al.* (2009) and the process of capsid formation has also been predicted by Devkota *et al.* (2009). These studies suggest that these non-enveloped, icosahedral RNA viruses tend to form particles after a certain concentration of RNA has been neutralised by the positively-charged amino acids. This reaction favours the condensation of RNA-protein complex and pulls the capsid protein subunits together. Interactions between protein-protein subunits take part after the condensation of the complexes and further strengthen the capsid formation (Devkota *et al.*, 2009). This suggests that disruption of the RNA-protein interaction might alter or halt the viral assembly. Marshall and Scheemann (2001) showed that RNA-protein interaction is important for recognising and packaging of viral genome in FHV. Nevertheless, the RNA-protein interaction site for *Mr*Nv, a newly emerged member of *Nodaviridae* family, has not yet been fully understood and this give rise to the research problem for the last part of this study. *Mr*Nv, which significantly shows differences in terms of nucleotide sequence with other nodaviruses, suggesting that it is of interest to locate the RNA binding site of *Mr*Nv capsid protein. Removal of the RNA interaction sites might affect the virus morphology and hence halt the virus assembly.

Amino acid sequence analysis of the first 50 amino acid residues of *Mr*Nv capsid available in the Genbank revealed that 11 residues of these 50 amino acids are positively-charged and 8 out of 10 amino acids located at positions 20^{th} to 29^{th} of the

N-terminal end are rich in positively-charged amino acids. The positively-charged amino acids are found to accumulate at this position but their functions are yet to be understood. This suggests that this rich positively-charged amino acid region of the *Mr*Nv capsid might be the RNA interaction site. Thus, the objective for the last part of this study is to determine the RNA binding site for this virus. Deletion or point mutation of the positively-charged amino acids located at residue 20^{th} to 29^{th} might result in the absence of RNA molecules in the mature virus cavity. Therefore, a study will be carried out to identify the RNA binding site of this virus via deletion and amino acid substitution mutageneses.

From the hypotheses and research questions mentioned above, the objectives of this study were:

1. To determine the nucleotide sequence of the *capsid* gene of *Macrobrachium rosenbergii* nodavirus (*Mr*Nv) isolated from Malaysia

2. To compare the *capsid* gene of Malaysian *Mr*Nv with other isolates

3. To produce the *Mr*Nv capsid protein in bacteria system via recombinant DNA technology

4. To characterise the recombinant *Mr*Nv capsid protein

5. To identify the RNA binding site of *Mr*Nv capsid protein by deletion and point mutageneses

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