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ENTRY MECHANISM, TRAFFICKING AND LOCALISATION OF Macrobrachium rosenbergii (De Man, 1879) NODAVIRUS IN SF9 INSECT CELLS

UMMI FAIRUZ BINTI HANAPI

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UMMI FAIRUZ BINTI HANAPI

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THE ENTRY MECHANISM, TRAFFICKING AND LOCALISATION OF *MACROBRACHIUM ROSENBERGII* NODAVIRUS IN SF9 INSECT CELLS

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

Macrobrachium rosenbergii nodavirus (*Mr*Nv) is a *Gammanodavirus* that was isolated from infected giant freshwater prawn juveniles. *Mr*Nv is the major cause of white tail disease (WTD) in prawn hatcheries and the mortality of the infected post-larvae is 100% in just 3 days. Vertical transmission and widen host range contribute to a worldwide economical crisis. No effective treatments are available to stop the virus infection. This study was aimed to identify the trafficking mechanism involved in *Mr*Nv infection and its localisation in the infected cells by using the virus-like particles (VLPs) of *Mr*Nv. The RNA2 of *Mr*Nv that codes for the viral capsid was previously cloned into pTrcHis2-TOPO expression vector. The recombinant *Mr*Nv capsid (*Mr*Nvc) protein with the size of about 46 kDa produced VLPs in *Escherichia coli* with undistinguishable properties from the native *Mr*Nv. These VLPs were used to study the entry mechanism, trafficking and localisation of the *Mr*Nv in Sf9 insect cells. Live cell observation using the live cell imaging system (LCIS) revealed that the internalisation of *Mr*Nvc VLPs was initiated by VLPs binding to the cell surface. Ammonium chloride inhibition study and LCIS showed that the *Mr*Nvc VLPs entry was mediated by acidic endosomal pathway. The number of the green fluorescent granules in Sf9 cells incubated with *Mr*Nvc VLPs decreased in the presence of 0.1 mM and 1.0 mM NH_4Cl which blocks the endosomal acidification. From LCIS data, green fluorescent 'ring-like shape' was observed as a result of attached VLPs being accumulated around the membrane pits. Green granules of endosomes enclosing VLPs were produced and later, the shape and size of the endosomes become disproportionate. The VLPs escape from the endosomal membrane when the fluorescent green granules faded and disappeared. *Mr*Nvc VLPs localised in the cell cytoplasm and nucleus as spotted from the Z-stack images of the fluorescence microscopy and the Western blotting of the Sf9 sub-cellular fractionation. His-tag located at the C-terminal end of the *Mr*Nvc can still be detected by anti-His antibody suggesting that *Mr*Nvc is still intact upon internalisation and

nuclear translocation. The mutants of the N-terminally truncated capsid proteins [9Δ*Mr*Nvc, 19Δ*Mr*Nvc 29Δ*Mr*Nvc and (20-29)Δ*Mr*Nvc] were used to study the function of the N-terminal residues in nuclear translocation. The 29Δ*Mr*Nvc and (20-29)Δ*Mr*Nvc without the positively-charged RNA-binding region (^{20}KRRKRSRRNR^{29}) showed no effect in VLPs entry into Sf9 cells but these mutants were found much lesser in the cell nucleus. This study revealed that *Mr*Nvc internalised Sf9 cells by receptor-mediated endocytosis and localised in the cell cytoplasm and nucleus. The endosomal escape mechanism of *Mr*Nv is different from that of Flock House virus (FHV), a model for non-enveloped virus entry, which involves gamma (γ) peptide cleavage at the C-terminal end of its capsid protein. It is suggested that ^{20}KRRKRSRRNR^{29} sequence has dual function as RNA-binding sequence and nuclear targeting sequence of *Mr*Nv. This close up examinations on the cellular level of *Mr*Nv infection will contribute to its elimination and control in giant freshwater prawn farming. Understanding the mechanism involved in *Mr*Nvc VLPs internalisation, trafficking and localisation in its host's cell will be useful for other studies such as drug nano-delivery, gene transfer and vaccine development.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

MEKANISMA KEMASUKAN, PENGANGKUTAN DAN LOKASI *MACROBRACHIUM ROSENBERGII* NODAVIRUS DI DALAM SEL SERANGGA SF9

Oleh

UMMI FAIRUZ BINTI HANAPI

November 2015

Pengerusi: Professor Tan Wen Siang, PhD
Fakulti: Bioteknologi dan Sains Biomolekul

Nodavirus *Macrobrachium rosenbergii* (*Mr*Nv) adalah *Gammanodavirus* yang dipencilkan daripada anak udang galah yang terjangkit. *Mr*Nv adalah penyebab utama penyakit ekor putih di kawasan penetasan udang. Kematian pos-larva yang terjangkit adalah 100% dalam masa hanya 3 hari. Pemindahan vertikal dan lingkungan perumah yang meluas menyumbang kepada krisis ekonomi sedunia. Tiada rawatan efektif yang telah dicipta untuk menghalang jangkitan virus ini. Kajian ini dijalankan untuk mengenalpasti mekanisma kemasukan yang terlibat dalam jangkitan *Mr*Nv dan lokasi *Mr*Nv di dalam sel terjangkit dengan menggunakan partikel menyerupai virus (VLPs) dari *Mr*Nv. RNA2 *Mr*Nv yang mengkod kapsid virus pada awalnya telah diklonkan ke dalam vektor pengekspresan pTrcHis-TOPO2. Protein kapsid rekombinan *Mr*Nv (*Mr*Nvc) dengan anggaran saiz 46 kDa menghasilkan VLPs di dalam *Escherichia coli.* VLPs ini menyerupai *Mr*Nv yang asli. VLPs ini digunakan untuk mengkaji mekanisma kemasukan, pengangkutan dan lokasi *Mr*Nv di dalam sel serangga Sf9. Pemerhatian langsung sel dari sistem pengimejan langsung sel (LCIS) menunjukkan bahawa kemasukan *Mr*Nv bermula dengan perlekatan VLPs pada permukaan sel. Kajian perencatan ammonium klorida dan LCIS menunjukkan bahawa kemasukan VLPs *Mr*Nvc adalah melalui laluan asidik endosoma. Bilangan butiran fluoresen hijau di dalam sel Sf9 yang dieram dengan VLPs *Mr*Nvc berkurangan dengan kehadiran 0.1 mM and 1.0 mM NH_4Cl yang menghalang pengasidan endosoma. Data LCIS menunjukkan bahawa fluoresen hijau berbentuk cincin dapat diperhatikan akibat daripada pengumpulan VLPs yang melekat di permukaan sel di sekitar lengkuk membran. Butiran hijau endosoma yang mengandungi VLPs terhasil dan kemudian, saiz dan bentuknya menjadi tidak menentu. VLPs melepasi membran endosoma apabila butiran fluoresen hijau menjadi pudar dan hilang. *Mr*Nvc VLPs terletak di dalam sel sitoplasma dan nukleus seperti yang diperhatikan dalam imej Z-stack mikroskop fluoresen dan analisis pemblotan Western fraksi sub-sel Sf9. His-tag yang terletak di

hujung terminal-C *Mr*Nvc masih dapat dikesan oleh antibody anti-His. Ini mencadangkan bahawa *Mr*Nvc kekal utuh selepas memasuki dan diangkut ke nukleus. Mutasi protein kapsid pada terminal-N *Mr*Nvc [9Δ*Mr*Nvc, 19Δ*Mr*Nvc 29Δ*Mr*Nvc and (20-29)Δ*Mr*Nvc] telah digunakan untuk mengkaji fungsi jujukan asid amino yang disingkirkan pada terminal-N ini dalam translokasi nukleus. Protein 29Δ*Mr*Nvc and (20-29)Δ*Mr*Nvc yang tidak mempunyai kawasan perlekatan-RNA bercaj positif (^{20}KRRKRSRRNR^{29}) tidak menunjukkan sebarang kesan terhadap kemasukan VLPs ke dalam sel Sf9 tetapi bilangannya di dalam nukleus sel didapati sangat sedikit. Kajian ini mendedahkan bahawa *Mr*Nvc memasuki sel Sf9 melalui reseptor-perantara endositosis dan berkumpul di dalam sel sitoplasma dan nukleus. Mekanisma perlepasan endosoma bagi *Mr*Nv adalah berlainan dari virus Flock House (FHV), satu model bagi virus tidak bersalut yang melibatkan pemotongan peptida gama (γ) pada terminal-C protein kapsid. Jujukan ^{20}KRRKRSRRNR^{29} mempunyai dua fungsi; sebagai jujukan prlekatan-RNA dan jujukan sasaran nukleus bagi *Mr*Nv. Pemerhatian terperinci terhadap jangkitan *Mr*Nv pada tahap sel akan menyumbang kepada pemusnahan dan kawalan virus ini dalam industri pertanian udang galah. Pemahaman tentang mekanisma yang terlibat dalam kemasukan, pengangkutan dan lokasi *Mr*Nvc VLPs di dalam sel perumahnya akan berguna untuk kajian lain seperti penghantaran-nano ubatan, terapi gen dan penghasilan vaksin.

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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 Master of Science. The members of the Supervisory Committee were as follows:

Tan Wen Siang, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Noorjahan Banu Mohamed Alitheen, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

_____

BUJANG KIM HUAT, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

Declaration by Members of Supervisory Committee

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) are adhered to.

Signature: _____
Name of Chairman of Supervisory Committee: Prof. Dr. Tan Wen Siang

Signature: _____
Name of Member of Supervisory Committee: Assoc. Prof. Dr. Noorjahan Banu Mohamed Alitheen

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LIST OF ABBREVIATIONS

α	alpha
β	beta
γ	gamma
µ	micro
µl	microliter
µm	micrometer
µM	micro molar
%	percentage
°C	degree Celsius
+ssRNA	positive sense single stranded ribonucleic acid
A_{595}	absorbance at wavelength 595 nm
APS	ammounium persulphate
BCIP	5-bromochloroindolyl-phosphate
BSA	bovine serum albumin
cm	centimeter
C-terminal	carboxy terminal
CBB	Coomassie Brilliant Blue
cm^2	centimetre square
CPE	cytopathic effect
DGNNV	dragon grouper nervous necrosis virus
dH_2O	distilled water
DMSO	dimethylsulfoxide
DNase	deoxyribonuclease
dsDNA	double-stranded deoxyribonucleic acid
dsRNA	double-stranded ribonucleic acid
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetic acid
EGTA	ethylene glycol tetraacetic acid
F-*Mr*Nvc VLPs	fluorescein labelled *Mr*Nv capsid virus-like particles
FBS	fecal bovine serum
FHV	flock house virus
g	gram
×g	g-force
GGNNV	greasy grouper nervous necrosis virus
h	hour
HCl	hydrogen chloride
HBV	hepatitis B virus
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
His-tag	histidine tag
Hz	Hertz
iASUs	icosahedral asymmetric units
IMAC	immobilized metal affinity chromatography
IPTG	isopropyl β-D-1 thiogalactopyranoside
kb	kilobasepair
kDa	kilodalton

L	liter
LB	Luria-Bertani
LCIS	live cell imaging system
M	molar
mA	milliampere
mg	milligram
$MgCl_2$	magnesium chloride
min	minute
ml	milliliter
mm	millimeter
mM	millimolar
mm^3	cubic millimetre
mmol	millimole
*Mr*Nv	*Macrobrachium rosenbergii* nodavirus
*Mr*Nvc	*Macrobrachium rosenbergii* nodavirus capsid protein
NaCl	sodium chloride
NBT	nitroblue tetrazolium
NH4Cl	ammonium chloride
NHS	5/6-carboxyfluorescein succinimidyl ester
NLS	nuclear localisation signals
nm	nanometer
NoV	nodamura virus
N-terminal	amino terminal
PaV	pariacoto virus
PBS	phosphate buffered saline
PL	post-larvae
PMSF	phenylmethylsulfonyl fluoride
*Pv*NV	*Penaeus vannamei* nodavirus
RdRp	RNA-dependent RNA polymerase
RGNNV	red-spotted grouper nervous necrosis virus
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
RT	room temperature
s	second
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyaccrylamide gel electrophoresis
Sf9	S*pedoptera frugiperda* cell line
ssDNA	single-stranded deoxyribonucleic acid
SSN-1	striped snakehead fry cell line
T=3	triangulation number 3
TEM	transmission electron microscope
TEMED	tetramethylethylenediamine
Tris	2-amino-2-nydroxymethyl-propane-1,3-diol
UL	unclarified lysates
UV	ultra violet

V	voltage
v/v	volume per volume
VLPs	virus-like particles
w/v	weight per volume
WTD	white tail disease
XSV	extra small virus

CHAPTER 1

INTRODUCTION

Macrobrachium rosenbergii nodavirus (*Mr*Nv) causes muscle necrosis with opaque appearances and white lesions on infected giant freshwater prawns or scientifically known as *Macrobrachium rosenbergii* (Tang *et al.*, 2007). The larvae of the giant freshwater prawns can live for 2 to 3 days once it was infected by *Mr*Nv. However, the adults can survive from this virus. *Mr*Nv contains two single stranded positive-sense RNA molecules; RNA1 codes for the RNA-dependent RNA polymerase and RNA2 codes for the viral capsid (Li *et al.*, 2007; Bonami & Widada, 2011). The capsid sequence was previously cloned into a pTrcHis2-TOPO vector and introduced into *Escherichia coli* [BL21 (DE3)] cells. The capsid protein of *Mr*Nv (*Mr*Nvc) produced in the *E. coli* cells are easily purified by using the immobilised metal ion affinity chromatography (IMAC) which binds to the His-tag that at the C-terminal end of the *Mr*Nvc. The *Mr*Nvc formed virus-like particles (VLPs) with a diameter of about 30 nm. The shape and size of the particles are indistinguishable with the native virus but the particles encapsidated *E. coli* RNA rather than *Mr*Nv genome. Therefore, *Mr*Nvc VLPs are not infectious (Goh *et al.*, 2011).

From the previous study (Goh *et al.*, 2011), it was proven that *Mr*Nvc VLPs have the same characteristics with the native virus, but are these VLPs able to infect insect cells like the native virus? Therefore, the purified VLPs were used to infect Sf9 cells, insect cells derived from *Spedoptera frugiperda*. To date, the mechanisms of *Mr*Nv entry into its host's cells are still lacking, although some researchers have proposed an entry model from Flock House virus (FHV), an *Alphanodavirus* of the same *Nodaviridae* family. FHV has shown to utilize acidic endosomal pathway or receptor-mediated pathway and produced lytic peptide (gamma peptide) for endosomal escape (Odegard *et al.*, 2010). Studies on *Betanodavirus* entry mechanism have observed another entry pathway which was through the formation of macropinosome, a large endosome (Liu *et al.*, 2005). There is a question on how *Mr*Nv, a *Gammanodavirus* enter its host cells, either endosome, macropinosome or both. Once viruses enter cells, they will co-localise in specific cellular organelles for viral replication and assembly. Some viruses co-localise in the cell cytoplasm, mitochondria and nucleus and others just injecting their viral cargo into the cells. To understand the downstream process of *Mr*Nv infection, the translocation of the virus particles has to be identified.

It was hypothesized that *Mr*Nvc VLPs enter cells through acidic endosomal pathway. The most studied nodaviruses, FHV, red-spotted grouper nervous necrosis virus (RGNNV) and dragon grouper nervous necrosis virus (DGNNV) utilised endosomal pathway to enter their host cells (Liu *et al.*, 2005; Adachi *et al.*, 2007; Odegard *et al.*, 2009). However, there are several endosomal pathways that can be utilised by virus such as clathrin-mediated endocytosis, caveolar-mediated endocytosis, clatrin/caveolin-independent pathway and macropinocytosis. Clathrin-mediated and macropinocitosis

pathway were proposed for nodavirus entry as previous studies observed the attachment of nodaviruses on the cell receptors and the formation of invaginations or membrane ruffles on infected cells (Liu *et al.*, 2005; Odegard *et al.*, 2010). For *Betanodavirus* such as greasy grouper nervous necrosis virus (GGNNV), the protein α was translocated into the cell cytoplasm and the cell nucleus (Guo *et al,*. 2003), while Wuhan nodavirus (WhNV) localised its protein A into mitochondria (Qiu *et al.*, 2013) once it passed through the cell membrane. It is hypothesized that *Mr*Nvc VLPs will co-localise in the cell cytoplasm and nucleus of the infected cells. It is also believed that the RNA binding site located at the N-terminal region of the *Mr*Nvc is involved in the viral translocation into the cell nucleus. The amino acid sequence of *Mr*Nv's RNA binding site is ^{20}KRRKRSRRNR^{29} which is highly rich in positively-charged basic residues (Goh *et al.*, 2014). This site is similar to the nucleolus localisation signal of GGNNV (^{23}RRRANNRRR^{31}). These residues are believed to have high affinity to the acidic regions in nucleolus such as negatively charged rRNA, B23 or ribosomal proteins (Guo *et al.*, 2003).

This research was conducted to study the mechanism of *Mr*Nv entry into Sf9 cells. Ammonium chloride pre-treatment on Sf9 cells that raises the endosomal pH was employed in order to observe the involvement of acidic endosomal pathway in VLPs entry. The VLPs internalisation was also observed in a closer range on infected cell by live cell imaging system (LCIS). To identify the location of VLPs inside the infected Sf9 cells at post-internalisation, Sf9 sub-cellular fractionation was done. RNA binding region on *Mr*Nvc protein is positively-charged and has high affinity to negatively-charged RNA and some viruses exploit this region as nuclear localisation signals (NLS), therefore, the N-terminal deletion mutants of *Mr*Nvc were utilised in this study to investigate the interaction of this regions with the nuclear translocation of *Mr*Nvc VLPs.

The importance of knowing the entry mechanism and co-localisation of *Mr*Nvc VLPs in Sf9 organelles is to find the idea on how to stop this virus from progressing at the very early stage at the cellular level. Currently, the diagnostic tools available are sophisticated but not fast enough. Once diagnosed, there is limited time left to stop this virus because *Mr*Nv only takes 2 to 3 days to kill a whole pond of giant freshwater prawn juveniles. Furthermore, there are no effective treatments against this virus. As found in this study that *Mr*Nvc VLPs can target to its host's nucleus, this particle can be utilised and engineered for clinical use to target the specific cell nucleus for drug delivery or gene therapy. These particles exhibit additional advantage as they are readily and rapidly produced in *E. coli* cells. More importantly these particles are not infectious and can be disassembled or re-assembled to be used as a nano-carrier. Findings from this research will contribute to filling the gaps in diagnostic, treatments and knowledge that the world is still lacking now.

The objectives of this study were:

1) To investigate the ability of *Mr*Nvc VLPs to internalise Sf9 cells.
2) To identify the localisation of *Mr*Nvc VLPs upon internalisation into Sf9 cells.
3) To identify the entry mechanism involves in *Mr*Nvc VLPs internalisation into Sf9 insect cells.
4) To recognise the nuclear targeting sequence on the *Mr*Nvc.

The hypotheses of the study were that:

1) *Mr*Nvc VLPs co-localise in the cytoplasm and nucleus of Sf9 cells upon internalisation.
2) *Mr*Nvc VLPs enter Sf9 cells by receptor-mediated acidic endosomal pathway.
3) The highly positively-charged residues located at the amino acids 20-29 of the *Mr*Nvc involve in the translocation of *Mr*Nvc VLPs into the Sf9 nucleus.

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PUBLICATIONS

This thesis dissertation resulted in the following publications:

Publications

Hanapi, U.F., Yong, C.Y., Goh, Z.H., Alitheen, N.B., Yeap, S.K. and Tan, W.S. (2017). Tracking the virus-like particles of *Macrobrachium rosenbergii* nodavirus in insect cells. *PeerJ*5:e2947. doi: 10.7717/peerj.2947

Proceedings/ Conferences

Hanapi, U.F., Alitheen, N.B.M and Tan, W.S. Entry mechanism of the virus-like particle of *Macrobrachium rosenbergii* nodavirus in insect cells (No. 62; poster presentation). 39th Annual Conference of the Malaysian Society for Biochemistry and Molecular Biology (MSBMB), 25th -26th August 2014, Sama-Sama Hotel, Selangor.

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