UNIVERSITI PUTRA MALAYSIA

DISPLAY OF HEPATITIS B VIRUS 'a' DETERMINANT ON THE SURFACE OF Macrobrachium rosenbergii (de Man, 1879) NODAVIRUS-LIKE PARTICLE CAPSID PROTEIN

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By

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

Complications resulting from hepatitis B account for ~1.45 million deaths each year. To date, none of the available treatments is curative and the prophylactic hepatitis B vaccines can only protect ~90% of vaccinated individuals. Other limitations of current vaccination include poor immunogenicity in people with pre-existing conditions and people with unresponsiveness to yeast-derived vaccines. Overall, this necessitates the continuous development of novel hepatitis B vaccines with improved efficacy. This study is aimed at developing a novel hepatitis B vaccine candidate by producing a chimeric virus-like particle (VLP) displaying the hepatitis B virus (HBV) 'a' determinant (aD). The aD is the immuno-dominant region of HBV that induces the production of HBV-neutralising antibodies during infection. Furthermore, aD is conserved among different strains of HBV making it suitable for use in the development of HBV vaccines. In this study, the aD was fused to the C-terminus of the *Macrobrachium rosenbergii* nodavirus (*Mr*NV) capsid protein (Nc) and expressed in *Spodoptera frugiperda* (*Sf*9) cells. SDS-PAGE analysis showed that the expressed protein was ~52 kDa in size. Subsequently, dynamic light scattering (DLS) analysis revealed that the recombinant Nc-aD protein assembled into heterogeneous particles ranging from ~23.4 to ~58.0 nm in diameter. Also, transmission electron microscopy (TEM) confirmed that these particles were spiky spherical virus-like particles (VLPs) with a diameter ranging from ~21 to ~55 nm. Circular dichroism (CD) spectroscopy further revealed that these Nc-aD VLPs consisted of β-sheets (44.8%), random coils (38.7%), α-helices (16.1%) and β-turns (0.3%) with a melting temperature (Tm) of ~56.2 ℃. Furthermore, enzyme-linked immunosorbent assay (ELISA) of these Nc-aD VLPs revealed that the aD was significantly antigenic when probed with the anti-hepatitis B surface antigen (HBsAg) monoclonal antibody. Subcutaneous immunisation of BALB/c mice with three doses of these purified Nc-aD VLPs (100 µL; 0.34 mg/mL) elicited a robust humoral immune response that was sustained for 126 days. The elicited humoral immune response was significantly higher ($p<0.001$)

than those elicited by a commercially available hepatitis B vaccine and those of *Escherichia coli*-produced Nc-aD. In addition, immunophenotyping showed that the *Sf*9-produced Nc-aD VLPs induced an increase of cytotoxic T-lymphocytes (CTL) (0.65 CD8+/CD4+ ratio) and NK1.1 natural killer cells (13.8%). Memory B cell enzyme-linked immunospot (ELISPOT) analysis was performed 126 days after the administration of the second booster injection. The analysis showed the presence of aD-specific antibody-secreting memory B cells in polyclonally activated mice splenocytes (26.67 spots). The significant humoral, CTL, natural killer cell and memory B cell immune responses induced by these *Sf*9-produced Nc-aD VLPs suggest that this recombinant protein presents good prospects for use as a hepatitis B vaccine candidate.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PEMAPARAN PENENTU 'a' HEPATITIS B VIRUS PADA PERMUKAAN PARTIKEL PROTEIN KAPSID MENYERUPAI NODAVIRUS *Macrobrachium rosenbergii* (de Man, 1879)

Oleh

NINYIO NATHANIEL NYAKAAT

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Pengerusi : Profesor Wen Siang Tan, PhD
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Komplikasi akibat hepatitis B menyumbang kepada ~1.45 juta kematian setiap tahun. Sehingga kini, rawatan yang sedia ada tidak dapat memberikan kesembuhan dan vaksin pencegahan hepatitis B hanya dapat melindungi ~90% individu yang telah divaksinasi. Limitasi lain vaksin semasa termasuklah keimunogenan yang rendah pada individu dengan keadaan sedia ada dan individu yang tidak bertindak balas dengan vaksin yang berasaskan yis. Secara keseluruhannya, vaksin hepatitis B ini perlu dibangunkan secara berterusan dengan keberkesanan yang lebih baik. Kajian ini bertujuan untuk membangunkan vaksin hepatitis B dengan menghasilkan partikel menyerupai virus (VLP) yang terdiri daripada virus hepatitis B (HBV) penentu 'a' (aD). aD adalah kawasan dominan-immuno pada HBV yang akan mendorong penghasilan antibodi peneutralan HBV semasa jangkitan. Tambahan pula, aD ini terpelihara di antara strain HBV yang berbeza yang menjadikannya sesuai digunakan untuk perkembangan vaksin HBV. Di dalam kajian ini aD telah dicantumkan ke terminal-C protein kapsid (Nc) *Macrobrachium rosenbergii* nodavirus (*Mr*NV) dan diekspresskan di dalam sel *Spodoptera frugiperda* (*Sf*9). Analisis SDS-PAGE menunjukkan saiz protein yang diekspresskan adalah ~52 kDa. Selain itu, analisis penyebaran cahaya dinamik (DLS) menunjukkan protein rekombinan Nc-aD berkumpul menjadi partikel heterogenus berdiameter di antara ~23.4 hingga ~58 nm. Mikroskopi elektron transmisi (TEM) menunjukkan bahawa zarah-zarah ini adalah zarah menyerupai virus dengan diameter di antara ~21 hingga ~55 nm. Spektroskopi dikromisme pekeliling (CD) seterusnya mendedahkan bahawa VLP Nc-aD terdiri daripada helaian beta (44.8%), gegelung rawak (38.7%), heliks alpha (16.1%) dan selekoh beta (0.3%) dengan suhu lebur (Tm) ialah ~56.2^{o}C. Seterusnya, pemeriksaan imunosorben berkait enzim (ELISA) dengan VLP Nc-aD ini mendedahkan bahawa aD adalah sangat antigenik apabila diuji dengan antibodi monoklonal terhadap antigen permukaann hepatitis B (HBsAg). Imunisasi subkutan tikus BALB/c dengan tiga dos VLPs Nc-aD yang telah ditulenkan (100 μL; 0.34 mg/mL) menunjukkan tindak balas

imun humoral yang kuat yang dikekalkan selama 126 hari. Tindak balas imun humoral yang ditunjukkan adalah jauh lebih tinggi ($p<0.001$) daripada yang telah ditunjukkan oleh vaksin hepatitis B yang telah ada secara komersial dan vaksin Nc-aD yang dihasilkan oleh *Escherichia coli*. Selain itu, imunofenotiping menunjukkan bahawa VLPs Nc-aD yang dihasilkan oleh *Sf*9 mendorong proliferasi sitotoksik T-limfosit (CTL) (0.65 CD8+/CD4+ ratio) dan sel pembunuh semula jadi NK1.1(13.8%). Analisis memori B sel ujian imunospot berkaitan enzim (ELISPOT) dilaksanakan 126 hari selepas pemberian suntikan penggalak yang kedua. Analisis menunjukkan bahawa terdapat sel B yang dapat menghasilkan antibodi daripada splenosit tikus yang diaktifkan secara poliklon yang dirangsang (26.67 tempat). Kepentingan humoral, CTL, sel pembunuh semula jadi dan tindak balas imun sel memori B yang didorong oleh penghasilan VLPs Nc-aD oleh *Sf*9 menunjukkan bahawa protein rekombinan ini memaparkan prospek yang baik untuk dijadikan sebagai calon vaksin hepatitis B.

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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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LIST OF ABBREVIATIONS

aD	'a' determinant
*Ac*MNPV	*Autographa carlifornica* multiple nuclear polyhedrosis virus
AIDS	Acquired immunodeficiency syndrome
APC	Allophycocyanin
ASC	Antibody secreting cell
cccDNA	Covalently closed circular DNA
CD	Circular dichroism
CHO	Chinese hamster ovary
Cryo-EM	Cryo-electron microscopy
CTL	Cytotoxic T-lymphocyte
DLS	Dynamic light scattering
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunospot
ER	Endoplasmic reticulum
FDA	Food and Drug Administration
FITC	Fluorescein isothiocyanate
HBV	Hepatitis B virus
HBcAg	Hepatitis B core antigen
HBeAg	Hepatitis B e-antigen
HBsAg	Hepatitis B surface antigen
HIV	Human immunodeficiency virus
IFN	Interferon
IRF	Interferon regulatory factor
MHC	Major histocompatibility complex
MHR	Major hydrophilic region

*Mr*NV	*Macrobrachium rosenbergii* nodavirus
Nc-aD	Nodavirus capsid-'a' determinant
Nc	Nodavirus capsid
NK cells	Natural killer cells
NKG2D	Natural killer group 2D
ORF	Open reading frame
PE	Phycoerythrin
pgRNA	Pregenomic RNA
rcDNA	Relaxed circular DNA
RdRp	RNA-dependent RNA polymerase
*Sf*9	*Spodoptera frugiperda*
SSE	Secondary structure estimation
TEM	Transmission electron microscopy
Tm	melting temperature
VLP	Virus-like particle
WHO	World Health Organisation

CHAPTER 1

INTRODUCTION

Hepatitis B and complications associated with it have since been classified by the World Health Organisation (WHO) to be a major health concern of the 21st century. About 250 million people, globally, are living carriers of the hepatitis B virus (HBV) (Tsai et al., 2018) and yearly recorded deaths resulting from HBV infection and related complications have been estimated to be 1.45 million (Anikhindi et al., 2018), higher than yearly deaths that result from malaria and HIV/AIDS with a yearly estimation of 0.44 million and 1.06 million deaths, respectively. Unlike the aforementioned communicable diseases, cases of HBV infections and related deaths have been on the increase, especially in developing nations (Seto et al., 2018; Tan & Ho, 2014). Therefore, more effective curative and preventive measures are being explored.

Since the USA's Food and Drug Administration (FDA) department's approval of the first hepatitis B vaccine in 1981, very significant milestones in the development of prophylactic and therapeutic measures targeted at hepatitis B, have been achieved (Seto et al., 2018). Based on common practice, injection with three separate doses of the HBV vaccine is still the most effective control measure, especially when the first dose is administered within the first 24 hours of birth (Hou et al., 2018).

However, several concerns about the prophylactic efficacies of the currently available hepatitis B vaccines have been raised. These concerns include the capability of these vaccines to elicit sustained protective immunity in some individuals with obesity, advanced age and pre-existing health conditions (Coates et al., 2001; Gerlich, 2017), the ineffectiveness of these vaccines in producing protective immunity in chronically infected subjects (Bengsch & Chang, 2016), induction of a poor immune response in about 10% of adult vaccinated subjects (Lerous-Roels et al., 2001) and their inability to confer protective immunity in subjects that are unresponsive to yeast-derived vaccines (Shouval, 2003). Furthermore, the spread of vaccine HBV escape mutants has made it necessary to develop newer HBV vaccine candidates with improved efficacy against HBV (Carman et al., 1990; Gerlich, 2017; Gerlich, 2015).

In an effort by WHO to eradicate Hepatitis B by 2030, a couple of treatments have been licensed for treatments of HBV infection (Mitra et al., 2018). These treatment regimens include interferons and nucleotide/nucleoside analogues which are capable of inhibiting HBV polymerases involved in viral replication. These treatments are reportedly effective in patients suffering from hepatitis B, however, none of these treatments is curative (Childs et al., 2018; Whitsett et al., 2019). The absence of a cure for hepatitis B further substantiates the need for developing more effective preventive vaccines while a cure is being sought.

Recombinant virus-like particles (VLPs) which are exploited as new hepatitis B vaccine candidates have shown promising prospects in inducing protective immunity in mice (Hyakumura et al., 2015; Kingston et al., 2019; Netter et al., 2001; Netter et al., 2003). Chimeric VLPs, consisting of two or more fused proteins from different viruses, are even more immunogenic and specific than VLPs consisting of a single viral protein (Ryu et al., 1997). Of interest to this study are VLPs of *Macrobrachium-rosenbergii*-nodavirus-(*Mr*NV) capsid protein (Nc) and their application as nanocarriers to display foreign epitopes. The immunogenicity of *Escherichia coli* (*E. coli*)-expressed chimeric VLPs, which consist of the-Nc displaying foreign viral epitopes, has been studied in mice (Ong et al., 2019; Yong et al., 2015a).

The *Mr*NV, from which the recombinant Nc is derived, is implicated in the aetiology of the whitetail disease in *Macrobrachium rosenbergii* (commonly known as the giant freshwater prawns). Outbreaks of *Mr*NV infection often result in 100% mortality in the infected prawn population thereby, causing severe financial losses to the aquaculture industry (Murwantoko et al., 2016). The *Mr*NV genome is a positive sense single-stranded bipartite RNA molecules, known as RNA 1 (3.1 kb) and RNA 2 (1.2 kb). The former codes for the viral RNA-dependent RNA polymerase (RdRp) while the latter codes for the viral capsid protein (Nc) (Goh et al., 2011; Hanapi et al., 2017).

Previously, *E. coli* and *Spodoptera frugiperda* (*Sf*9) were used to express Nc VLPs (Goh et al., 2011; Kueh et al., 2016) and the VLPs were shown to display foreign protein epitopes at the Nc C-terminal region. Chimeric Nc VLPs displaying foreign viral epitopes have beenβproducedβinβ*E. coli*β(Yong et al., 2015a; Yong et al., 2015b), suggesting that the Nc is an effective carrier protein to display of foreign viral epitopes in *E. coli* as it has been shown to prevent carrier protein-induced suppression of the displayed epitope (Ong et al., 2019). However, the performance of the Nc as an epitope-displaying carrier protein in *Sf*9 expression systems is yet to be explored. Using the baculoviral expression system for insect cell lines, previous studies have shown that *Sf*9 cells are better than the *E. coli* expression system with regards to the production of higher yields of stable and distinctly assembled VLPs (Ho et al., 2017; Kueh et al., 2016; López-Vidal et al., 2015; Rendic et al., 2008).

In this study, chimeric virus-like particles consisting of the HBV 'a' determinant (aD) fused to the C-terminus of the-*Mr*NV-capsid-protein-(Nc) were-produced-in *Sf*9 insect cells via the baculovirus expression system. The aD of HBV is located in the *S*-domain of the HBV surface antigen (HBsAg) and it is conserved among the different strains of HBV (Bengsch & Chang, 2016; Hassemer et al., 2017). Furthermore, during infection, the aD is the component of the virus that induces the production of HBV-neutralising antibodies within the infected hosts and as such, this makes it an important epitope to be exploited in the development of new hepatitis B vaccines (Howard & Allison, 1995).

This study hypothesises that *Sf*9 cells are an effective host for the production of chimeric *Mr*NV Nc fused with HBV aD, in which Nc functions as a carrier protein. Also, this study hypothesises that *Sf*9 cell-expressed Nc-aD will assemble into VLPs,

which will, in turn, elicit an aD-induced humoural, cellular and memory immune responses in BALB/c mice. The general objective of this study was to determine the prospect of the *Sf*9-produced Nc-aD as a new hepatitis B vaccine by measuring its immunogenicity in mice. Specifically, the objectives of this study include:

1. To construct recombinant bacmid DNA harbouring the coding regions of the HBV 'a' determinant fused to the C-terminus of the *Mr*NV capsid protein.
2. To produce virus-like particles of Nc-aD in *Sf*9 cells.
3. To characterise the chimeric Nc-aD virus-like particles.
4. To determine its immunogenicity in BALB/c mice.

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