DEVELOPMENT AND APPLICATION OF DENGUE VIRUS NS2B/NS3 PROTEASE INHIBITION ASSAY USING ALPHASCREEN® TECHNOLOGY

MUHAMMAD ASYRAF ABDURAMAN

UNIVERSITI SAINS MALAYSIA

2015

DEVELOPMENT AND APPLICATION OF DENGUE VIRUS NS2B/NS3 PROTEASE INHIBITION ASSAY USING ALPHASCREEN® TECHNOLOGY

by

MUHAMMAD ASYRAF ABDURAMAN

Thesis submitted in fulfillment of the requirements for the degree of Master of Science

NOVEMBER 2015

ACKNOWLEDMENT

First and foremost, I would like to express my highest gratitude and my heartiest thanks to my project supervisor, Assoc. Prof. Dr. Tan Mei Lan and Co-supervisor, Prof. Dr. Habibah A. Wahab, for their kindness and patience in providing me valuable guidance, support, advice, understanding and comments throughout the project, especially on *in vitro* and *in silico* techniques. I would like to acknowledge ScienceFund Grant for providing me financial support in completing this research project. Same appreciation goes to the Ministry of Higher Education, which has sponsored my tuition fees through the MyBrain15 program in completing my Master studies.

Apart from this, my warmest thanks and appreciation goes to all members of Lead Optimization, IPharm for their encouragement and useful direct and indirect guidance towards my research when I am facing problems. The token of appreciation also goes to all members of Pharmaceutical Design & Simulation (PhDS) Laboratory, School of Pharmaceutical Sciences, USM for guiding me in computational *in silico* docking simulation.

Last but not least, no words are ever sufficient to express my everlasting gratitude, appreciation and thanks to my beloved, wonderful parents for their constant supports, prayers and inspiration. My heartfelt love and thanks to my family members, who played an important role in encouraging me with moral and financial support throughout this research project.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

2D	two-dimensional
ADE	antibody dependent enhancement
ADMET/PK	absorption distribution metabolism excretion toxicology/pharmacokinetic
AMC	7-aminomethyl-4-coumarin
Arg	arginine
Asn	asparagine
Asp	aspartic acid
ATP	adenosine triphosphate
BPTI	bovine pancreatic trypsin inhibitor
BSA	bovine serum albumin
CADD	computer aided drug design
CAMD	computer aided molecular design
CAMM	computer aided molecular modelling
DENV	dengue virus
DENV-1	dengue virus serotype 1
DENV-2	dengue virus serotype 2
DENV-3	dengue virus serotype 3
DENV-4	dengue virus serotype 4
DF	dengue fever
DHF	dengue hemorrhagic fever
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid

dsRNA	double stranded RNA
DSS	dengue shock syndrome
EDS	expanded dengue syndrome
ER	endoplasmic reticulum
FDA	Food and Drug Administration
Gly	glycine
Gln	glutamine
HCV	hepatitis c virus
His	histidine
HTS	high-throughput screening
IC_{50}	half maximal inhibitory concentration
K_i	inhibition constant
Leu	leucine
Lys	lysine
M	membrane
Met	methionine
mRNA	messenger RNA
N	nitrogen
NaCl	sodium chloride
NaOH	sodium hydroxide
NCI	National Cancer Institute
NGO	non-government organization
NKV	no-known vector
NME	new molecular entities
NS	non-structural protein

O	oxygen
OD	optical density
PDB	Protein Data Bank
Phe	phenylalanine
prM	pre-membrane
Pro	proline
RdRp	RNA dependent RNA polymerase
RFU	relative fluorescence unit
RM	Ringgit Malaysia
RMSD	root mean square deviation
RNA	ribonucleic acid
RTPase	RNA triphosphatase
Ser	serine
Thr	threonine
Trp	tryptophan
Tyr	tyrosine
UD	unusual dengue
UF	undifferentiated febrile illness
WHO	World Health Organization
WNV	West Nile virus
YFV	Yellow fever virus

LIST OF SYMBOLS

α	alpha
~	approximately
β	beta
-	dash
=	equals
>	more than
<	less than
/	or
®	registered trademark
™	trade mark
X	times

LIST OF UNITS

%	percentage
^{o}C	degree Celsius
^{o}N	degree north
^{o}S	degree south
Å	Ångström
Da	Dalton
Kcal/mol	kilocalorie per mole
M	molar
Min	minute
mL	milliliter
mM	millimolar
nm	nanometer
nM	nanomolar
psi	pounds per square inch
U/mL	unit per milliliter
µg	microgram
µl	microliter
µm	micrometer
µM	micromolar

PEMBANGUNAN DAN APLIKASI ASAI PERENCAT PROTEASE NS2B/NS3 VIRUS DENGGI DENGAN TEKNOLOGI ALPHASCREEN®

ABSTRAK

Penyakit denggi yang disebabkan oleh virus denggi merupakan suatu masalah kesihatan di peringkat global. Protease NS2B/NS3 merupakan protein bukan struktur yang memainkan peranan penting dalam replikasi dan kematangan virus denggi. Kompleks protein ini merupakan sasaran utama dalam pembangunan ubat-ubatan anti-denggi. Dalam kajian ini, objektif utama adalah untuk membangunkan asai perencatan protease NS2B/NS3 dengan menggunakan teknologi AlphaScreen® dan seterusnya penyaringan sekumpulan sebatian yang telah disintesis berasaskan penilaian sebatian melalui kajian *in silico*. Interaksi molekul antara sebatian yang aktif dengan tapak aktif protease NS2B/NS3 juga telah ditentukan. Pembangunan asai AlphaScreen® melibatkan penggunaan protease NS2B/NS3-rekombinan dan substrat peptida NS3/NS4A, berserta manik penderma StrepTactin® dan manik penerima kelat nikel. Secara ringkasnya, satu eksperimen titratan silang telah dijalankan dan graf berbentuk loceng telah menunjukkan kepekatan optimum bagi protease NS2B/NS3 dan substrat peptida, iaitu masing-masing pada 100 nM dan 300 nM. Sistem asai ini kemudiannya dioptimumkan dalam format plat 384-perigi dan kandungan larutan asai yang optimum merangkumi 10 mM HEPES, 20 mM NaCl, 0.20 % (v/v) BSA, pada pH 9.0. Untuk tujuan pengesahan asai dan aktiviti perencatan protease NS2B/NS3, aprotinin, panduratin A dan 15 sebatian yang telah dikenal pasti dalam kajian *in silico* telah disaring menggunakan asai AlphaScreen® yang telah dioptimumkan. Aprotinin, yang dikenali sebagai perencat serine protease dan panduratin A, sebatian semulajadi yang menunjukkan aktiviti perencatan

terhadap protease NS2B/NS3 telah digunakan sebagai sebatian kawalan positif. Aprotinin didapati secara aktif merencat aktiviti protease NS2B/NS3 dengan 74.00 % perencatan pada kepekatan maksimum 5 µM. Sementara itu, panduratin A menunjukkan 53.40 % perencatan pada kepekatan maksimum 100 µM. Kesemua 15 sebatian tidak mempamerkan aktiviti perencatan yang baik terhadap protease NS2B/NS3, kecuali sebatian MH005 yang merencat protease NS2B/NS3 secara lemah dengan 50.20 % perencatan pada kepekatan 1 mM. Purata faktor Z' untuk asai ini adalah 0.5 manakala nisbah isyarat latar belakang (S/B) bagi isyarat maksimum secara keseluruhannya adalah 196:1. Bagi nilai koefisien variasi (CV), nilai purata bagi isyarat maksimum pada hari 1, 2, 3 masing-masing adalah 3.00 %, 1.90 % dan 2.10 %. Nilai purata bagi kumpulan isyarat minimum pada hari yang sama masing-masing adalah 4.20 %, 5.10 % dan 7.20 %, yang memaparkan kesesuaian asai AlphaScreen® ini sebagai asai penabiran celusan tinggi (HTS). Kajian *in silico* secara terperinci bagi panduratin A dan MH005 telah mengenal pasti ikatan hidrogen dan interaksi hidrofobik dengan sisa asid amino yang dilaporkan penting untuk pengikatan dan perencatan protease NS2B/NS3. Pengubahsuaian struktur sebatian berdasarkan sebatian MH005 kemungkinan boleh menghasilkan perencatan yang lebih kuat terhadap protease NS2B/NS3. Sebagai kesimpulan, asai penabiran celusan tinggi (HTS) baru yang berasaskan teknologi AlphaScreen® bagi perencatan protease NS2B/NS3 virus denggi telah berjaya dibangunkan dan telah digunakan dalam penyaringan sebatian. Sebatian dasar yang mungkin berpotensi sungguhpun dengan aktiviti perencatan yang lemah terhadap protease NS2B/NS3 juga telah dikenal pasti di dalam kajian ini.

DEVELOPMENT AND APPLICATION OF DENGUE VIRUS NS2B/NS3 PROTEASE INHIBITION ASSAY USING ALPHASCREEN® TECHNOLOGY

ABSTRACT

Dengue is an infectious disease caused by the dengue virus and is a global health problem. NS2B/NS3 protease is a non-structural protein which plays a pivotal role in viral replication and maturation of the dengue virus. This complex is a primary target for the development of anti-dengue drugs. In this study, the main objectives were to develop a specific NS2B/NS3 protease inhibition assay using AlphaScreen® technology and subsequently to screen a group of synthesized compounds which were potentially active as determined using *in silico* studies. Molecular interactions of the potentially active compound within the active site of the NS2B/NS3 protease were also further determined. The development of specific NS2B/NS3 protease inhibition assay involved utilizing a recombinant NS2B/NS3 protease and NS3/NS4A peptide substrate with proprietary StrepTactin® donor beads and nickel chelate acceptor beads. Briefly, a cross-titration experiment was carried out and a typical bell-shaped curve revealed the optimum concentrations of NS2B/NS3 protease and the peptide substrate at 100 nM and 300 nM, respectively. The assay system was subsequently optimized in a 384-well plate format and the optimal assay solution consisted of 10 mM HEPES, 20 mM NaCl, 0.20 % (v/v) BSA, and at pH 9.0. To validate the assay and NS2B/NS3 protease inhibition activities, aprotinin, panduratin A and 15 synthesized compounds were screened using this optimized AlphaScreen® assay. Aprotinin, a known serine protease inhibitor and panduratin A, a natural compound which exhibited inhibitory activity against NS2B/NS3 protease were used as positive controls. Aprotinin was found to actively

inhibit NS2B/NS3 protease with 74.00 % of inhibition at the maximum concentration of 5 µM. Meanwhile, panduratin A showed 53.40 % of inhibition at the maximum concentration of 100 µM. Of all 15 compounds, none exhibited good inhibitory activity against NS2B/NS3 protease, except for MH005 which weakly inhibit NS2B/NS3 protease with 50.20 % of inhibition at 1 mM. The average Z' factor for this assay was 0.5 and the overall signal to background (S/B) ratio of the maximum signal was 196:1. As for the coefficient of variation (CV), the mean values for the maximum signal groups on the day 1, 2, 3 were 3.00 %, 1.90 % and 2.10 %, respectively. The mean values for the minimum signal groups on the same day were 4.20 %, 5.10 % and 7.20 %, respectively, which indicated the reliability of this AlphaScreen® assay for high-throughput screening purpose. A detailed *in silico* studies of panduratin A and MH005 had identified both hydrogen bonds and hydrophobic interactions with residues reported to be essential for the binding and inhibition of the NS2B/NS3 protease. Further modifications of the compound structure based on MH005 may yield a stronger inhibitor of the NS2B/NS3 protease. As a conclusion, a new high-throughput screening assay for dengue NS2B/NS3 protease inhibition based on AlphaScreen® technology has been successfully developed and utilized in screening of compounds. A possible lead but with weak NS2B/NS3 protease inhibition activity was also identified in this study.

CHAPTER 1

INTRODUCTION

1.1 Flavivirus

Flavivirus genus belongs to the family of *Flaviviridae*. Flaviviruses comprise tick-borne, no-known-vector (NKV) and mosquito-borne viruses and this genus include the dengue virus (DENV), yellow fever virus (YFV), West Nile virus (WNV), tick-borne encephalitis virus, and other viruses (Zuo et al., 2009; Grard et al., 2010). The members of these flaviviruses are important human pathogens and they exhibit the ability to cause significant morbidity and mortality (Yang et al., 2011). Flaviviruses are associated with emerging human diseases, such as the Japanese encephalitis disease, Kyasanur Forest hemorrhagic disease (Pattnaik, 2006), dengue hemorrhagic fever (DHF), WNV encephalitis disease (Lanciotti et al., 1999) and Rocio virus disease (Medeiros et al., 2007). About 70 recognized flaviviruses belong to this genus have a widespread geographical dispersion and are antigenically related (Gaunt et al., 2001). Flaviviruses have positive-sense RNA genome and they replicate in the cytoplasm of host cells. The genome of flaviviruses mimics the cellular mRNA molecule in all aspects except for the absence of poly-adenylated (poly-A) tail (Guo et al., 2000). The members of the flavivirus genus possess similarity in intracellular life cycles. The infection of arthropod-borne flaviviruses begins when the vector takes a blood meal and the virus is introduced into the host (Clyde et al., 2006).

1.2 Dengue virus

1.2.1 Epidemiology of the dengue virus

Dengue is an endemic infectious disease found in most parts of the world, particularly in tropical and subtropical regions. The dengue infectious disease has

been reported since the 18th century. During that time, major dengue epidemics occurred at intervals of 10 to 40 years in North America, Africa and Asia (Carrington et al., 2005; Dash et al., 2006; Moi et al., 2010). In recent decades, the incidence of dengue infection has grown dramatically around the world. According to World Health Organization (WHO), nearly 50–100 million dengue infections occurred worldwide every year (WHO, 2012). An estimated 500, 000 people with severe dengue require hospitalization each year, a large proportion of whom are children and the mortality rate of those affected by dengue is about 2.50 %. The WHO estimates that 2.5 billion people live in over 100 endemic countries and areas where DENV can be transmitted (WHO, 2014a). Dengue infection is not only a health burden to developing countries, it poses an emerging problem worldwide (Melino and Paci, 2007). Brady and co-workers estimated that up to 3.97 billion people are at risk of dengue infection in 128 countries (Brady et al., 2012). In 2012, dengue was ranked as the fastest spreading vector-borne viral disease with an epidemic potential in the world, registering a 30-fold increase in disease incidence over the past 50 years (Thomas and Moloo, 2013).

In Malaysia, an increasing number of dengue infections have been reported since 1980. For example, a huge number of dengue cases were reported, registering a 7-fold increase from year 2000 to 2010 (Mudin, 2013; Md Shahin et al., 2013; Mohd-Zaki et al., 2014). As for 2015, an estimated number of 53, 823 cases were reported until June which was about 25.00 % increase as compared with the same period in 2014 (Ministry of Health Malaysia, 2015). In addition, the number of fatality cases increased about 51.00 % as compared with the same period in 2014 (Ministry of Health Malaysia, 2015).

1.2.2 Dengue virus serotypes

DENV possess four closely related/distinct serotypes; DENV-1, DENV-2, DENV-3 and DENV-4, and these viruses have been reported as the cause of dengue infection (Fatima et al., 2011). These four viruses are called serotypes because each serotype has different interactions with the antibodies in human blood serum (Weasley and Barrett, 2008). The four DENV's are similar in their viral genomes (65.00 % similarity) (Guzman et al., 2010). According to Guzman and co-workers, all four serotypes of dengue circulate together, particularly in tropical and subtropical regions around the world as depicted in Figure 1.1 (Guzman et al., 2010). The DENV serotypes are further classified into multiple subtypes or genotypes based on their genomic diversity (Weaver and Vasilakis, 2009). The genotypes of DENV serotypes are genetically classified by T1 RNase fingerprinting (Santos et al., 2003). Extensive viral exchanges of Asian genotypes occur in East Asian countries, while in other localities, such as Africa and the Arabian Peninsula, strains are apparently introduced.

1.2.2.1 Dengue virus serotype 1

DENV-1 is one of the four serotypes of arthropod-borne viruses that cause dengue (Theiler and Downs, 1973; Karabatsos, 1985). The distribution of DENV-1 genotypes of the strain is geographically-dependent whereby genotype 1 was found in Asia, genotype 2 mainly found in Thailand, genotype 3 was found in Asia, including the sylvatic strain collected in Malaysia and genotype 4 was found in Asia and Pacific with an invasion into the Indiana Ocean, Australia, as well as the Americas (Villabona-Arenas and Zanotto, 2013). On the other hand, genotype 5 mostly found in American and The Caribbean, is widely distributed and suggest an

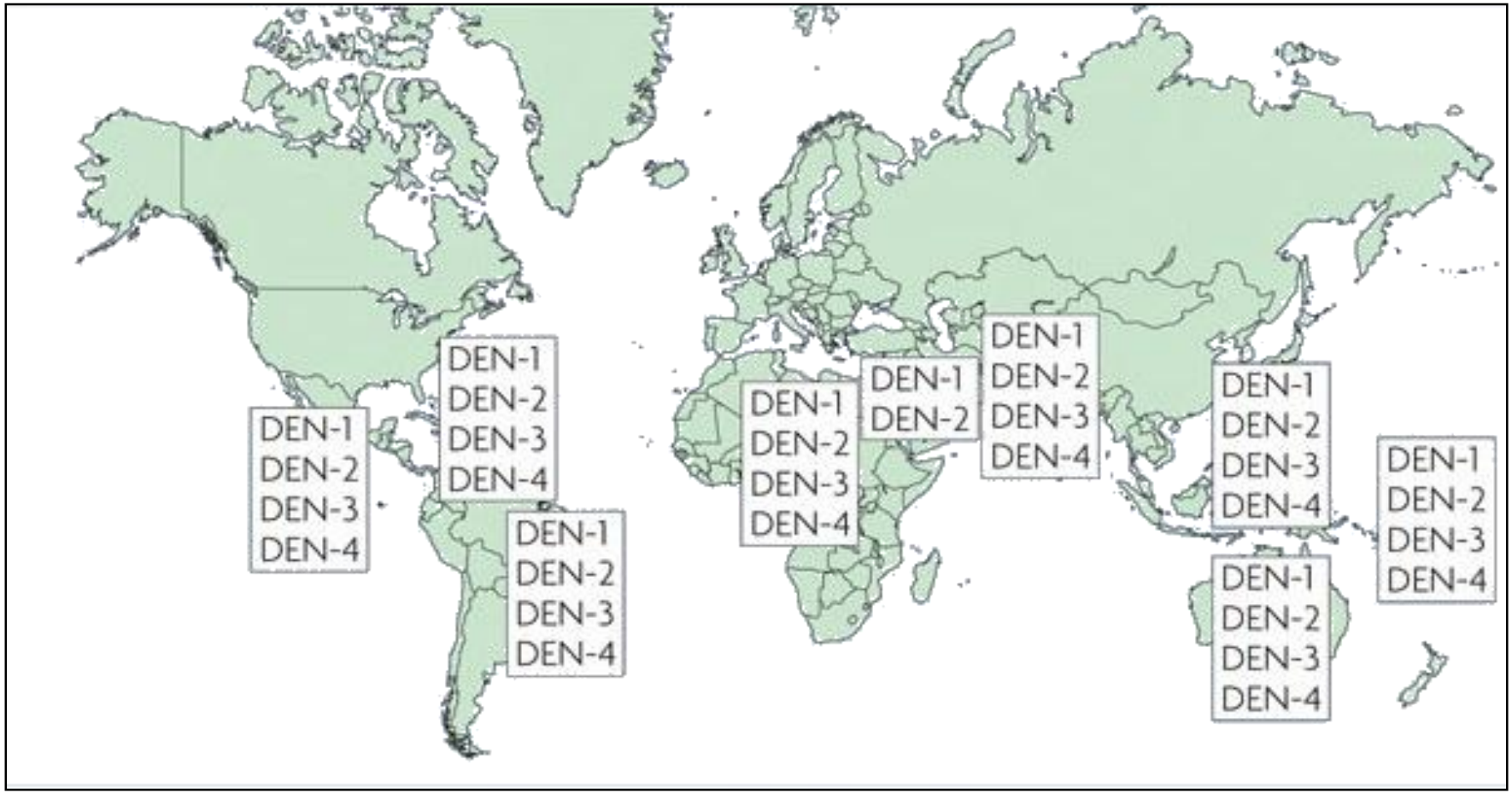

Figure 1.1 The geographical areas where DENV serotypes are distributed and circulated (Guzman et al., 2010).

in situ evolution among the susceptible human population after its introduction from Asia (A-Nuegoonpipat et al., 2004).

The biogeographical patterns of dispersal for DENV-1 have been revealed using 1812 strains-based and taxa associations (Chen and Vasilakis, 2011). The role of Southeast Asian countries was highlighted as the source of dengue epidemics and the overall topologies within each DENV-1 genotype are characterized by the basal location of the oldest strains followed by newer isolates, which revealed a pattern of evolution radiating around spatially-defined geographic clades (Chen and Vasilakis, 2011). In addition, the collection of isolated DENV strains from different countries showed diversity in genotype and having sequence divergence in determining DENV serotypes (Villabona-Arenas and Zanotto, 2013). The wide spread of DENV-1 across the world was demonstrated with the first outbreak in Brazil, Latin America in 1981 and following the outbreak in 1986 (in Brazil), dengue infection has become a nationwide public health issue (Schatzmayr et al., 1986).

A study conducted by Carvalho and co-workers revealed a strong evidence of relatively pervasive recombination based on the full analysis of genome sequence sampled from the geographically structured, but low-diversity Latin American DENV-1 population (Carvalho et al., 2010). The three clear recombination events that have been characterized occurred between relatively divergent virus genotypes and most likely arose during mixed DENV-1 infections. Thus, this suggested the existence of various country-specific DENV-1 effects, which may have adopted geographical structuring in the Americas (Carvalho et al., 2010).

In Asia, the first dengue outbreak caused by DENV-1 genotype 1 occurred in Vietnam and Myanmar in 2001 (Chen and Vasilakis, 2011). Meanwhile, in Japan, a dengue epidemic outbreak caused by DENV-1 occurred in Yap state that began in

the last week of May 2004 (Nukui et al., 2006). The epidemic outbreak caused dengue fever (DF), however, no fatality or DHF or dengue shock syndrome (DSS) cases were reported within the same year (Martin, 2005). Following the epidemic outbreak, DENV-1 infection was serologically confirmed in patients by an immunoglobulin M (IgM) capture enzyme-linked immunosorbent assay (ELISA) and an IgG ELISA at the National Institute of Infectious Disease in Tokyo, Japan (Nukui et al., 2006). Meanwhile, studies in symptomatic travelers have revealed that 90.00 % of cases frequently introduced into Japan and Korea are DENV-1 strains from Southeast Asia (Ito et al., 2007). Since then, dengue infection caused by DENV-1 has been an important health problem due to the frequency of international travel in developing countries (Chen and Vasilakis, 2011; Villabona-Arenas and Zanotto, 2013).

1.2.2.2 Dengue virus serotype 2

Five distinct genotypes of DENV-2 were recognized, including the West African sylvatic strains (Rico-Hesse, 1990). Current phylogenies of DENV-2 based on E protein gene sequences confirmed 5 major genotypes. The first genotype (genotype 1) originated from Asian countries, namely, the Asian genotype or Asian sub-genotype 1A, representing strains from Malaysia and Thailand, and Asian sub-genotype 1B representing strains from Vietnam, China, Taiwan, Sri Lanka and the Philippines, respectively (Rico-Hesse, 1990; Rico-Hesse et al., 1997). Meanwhile, genotype 2 was denoted the cosmopolitan genotype, representing the widely geographic distributed strains, including Australia, East and West Africa, the Pacific and Indian ocean islands, the Indian subcontinent and the Middle East. Genotype 3 or the American genotype represents strains from Latin America and older strains

collected from the Caribbean, the Indian subcontinent and Pacific Islands in the 1950s and 1960s. Genotype 4 or the Southeast Asian/American genotype, represents strains from Thailand and Vietnam and strains collected in the Americas over the last 20 years while genotype 5 or the sylvatic genotype, represents strains collected from humans, forest mosquitoes or sentinel monkeys in West Africa and Southeast Asia (Rico-Hesse, 1990; Rico-Hesse et al., 1997; Wang et al., 2000).

DENV was first hypothesized to originate from sylvatic habitats in West Africa, which was demonstrated from phylogenetic studies of DENV-2 strains and suggested that the strains were distinct from all others (Rico-Hesse, 1990). Malaysia is considered an area where all DENV serotypes evolved independently from a sylvatic ancestral lineage (Wang et al., 2000). The sylvatic lineage of DENV is mostly spread among animals such as monkey before the human was infected (Rico-Hesse, 1990; Wang et al., 2000). The evolutionary origins of DENV have been shown to reflect the geographic origin of DENV strains from four continents (Rico-Hesse, 1990), and this has allowed the identification of Southeast Asia as the source of DENV-2 (Rico-Hesse et al., 1997).

In the 1970s, both DENV-1 and DENV-2 were found in Central America and Africa, and all four serotypes were present in Southeast Asia (Guzman et al., 2010). DHF was first reported in Cuba in 1981 following the introduction of DENV-2 genotype mainly from Southeast Asian countries (Rico-Hesse et al., 1997; Rico-Hesse, 1990). Since then, other countries in the Americas have reported DHF associated with DENV-2 genotype, but not with the American genotype (Rico-Hesse, 1990). In Brazil, DENV-2 was first identified in the State of Rio de Janeiro in 1990, where the first cases of DHF and DSS were documented (Nogueira et al., 1993). It was followed by a fast spread of DENV-2 across the country becoming

endemic in some areas and causing severe clinical forms (Siqueira et al., 2005; Jean-Yves Zimmer et al., 2010).

DENV-2 has been isolated in Malaysia for more than three decades. Dengue has since become endemic in Malaysia with an average incidence of about 400 to 7000 cases of DF annually (Chee and AbuBakar, 2003). Though DENV-2 emerged as a major serotype in the late 1960s until the early 1970s, it has since persisted in the country and has been responsible for the major outbreaks that occurred from late 1980s to early 2000s (Chee and AbuBakar, 2003). Malaysia has reported a switch of dengue cases from DENV-3 to DENV-2 throughout the year of 2013 and most of the evidence was found in Johor and Malacca of which the fatality rate is remarkably high (Zulkefli and Kheong, 2014).

DENV-2 was revealed by Santos and co-workers to exhibit a higher viral load in the patient compared to DENV-1, which was in agreement with immunological assay results (Santos et al., 2003). Another study conducted by Vaughn and co-workers revealed that the highest secondary antibody responses are observed in patients infected with DENV-2 and they are more likely to have DHF compared to other serotypes. A higher viremia titer of DENV-2 is associated with more severe disease and this is consistent with viral virulence or the replicative ability of DENV-2 (Vaughn et al., 2000). Thus, DENV-2 is said to be more prevalent for dengue diseases compared with other serotypes (Frimayanti et al., 2011).

1.2.2.3 Dengue virus serotypes 3 and 4

Earlier studies have described intra-serotypic antigenic variation in DENV-3 as strains from Puerto Rico and Tahiti were shown to be antigenically and biologically different from Asian DENV-3 (Russel and McCown, 1972). Among

them, genotype 3 of DENV-3 has been frequently associated with severe dengue outbreaks in Asia, Africa, and Latin America (Rico-Hesse, 1990; Gubler, 2002). The first outbreak of DENV-3 in Latin America, particularly Venezuela was reported in 1964 and re-appeared in 2003 after 32 years of disappearance (Uzcategui et al., 2003). Phylogenetic studies revealed that DENV-3 circulating during the 1960s Latin American outbreak was a genotype 5 virus and this genotype belongs to the sylvatic strains found in Malaysia (Lanciotti et al., 1994; Weaver and Vasilakis, 2009).

Geographically independent evolution of DENV-3 was identified by Lanciotti and co-workers using parsimony analysis of the prM/M and E gene sequences of 23 DENV-3 strains (Lanciotti et al., 1994). Genotype 1 was identified in Indonesia, Malaysia, Philippines and South Pacific Islands while genotype 2 was found in Thailand. Virus strain from Sri Lanka, India, Africa and Samoa was categorized as genotype 3 while genotype 4 was found in Puerto Rico and Tahiti and genotype 5 belongs to the sylvatic strain found in Malaysia (Lanciotti et al., 1994).

Relatively, few comprehensive studies of DENV-4 genetic diversity have been undertaken, particularly in Southeast Asia. DENV-4 is of particular interest because it was the first dengue serotype to diverge in phylogenetic analysis of the genus flavivirus (Gaunt et al., 2001). It is generally found in low frequency in Southeast Asia. To date, three major genotypes of the DENV-4 have been described (Lanciotti et al., 1997); one found in Southeast Asia (genotype 1), a second described in Southeast Asia and the Americas (genotype 2), and the third genotype found exclusively as sylvatic strains in Malaysia (genotype 3) (Lanciotti et al., 1997). The emergence of DENV-4 in Malaysia was reported in 1967, and it was the predominant serotype (DENV-4) that previously responsible for about 40.00 to 64.00 % of total dengue cases in 1967. However, from total cases of dengue infections in a decade,

only 5.00 % of total dengue fever (DF) are caused by DENV-4 (AbuBakar et al., 2002). It has been reported that the invading strain of DENV-4 in Malaysia is genotype IIA (AbuBakar et al., 2002). In America, DENV-4 was first reported in 1981, where it caused epidemics of DF throughout the region (Carrington et al., 2005). The invading strain was reported from genotype IIA, a strain originated from the Asian countries (Lanciotti et al., 1997). It dispersed rapidly throughout the region, causing DF but only sporadic cases of DHF and DSS (Gubler, 1998).

1.2.3 Infection by the dengue virus

Aedes aegypti mosquitoes, the main vector of DENV, are found worldwide between latitudes 35 ºN and 35 ºS and the principle areas affected include South East Asia, Indian sub-continent, Australia, the Pacific Islands, Hawaii, Mexico, Caribbean, South and Central America, and Africa (WHO, 2012). During the usual five-day viremia period, the mosquitoes become infected when they feed on human blood (Guzman et al., 2010). The transmission of the virus begins from the mosquito intestinal tract to the salivary glands, after an extrinsic incubation period, about 10 days and is most rapid at high ambient temperature (Watts et al., 1987). After the extrinsic incubation period, mosquito bites result in an infection that is promoted by mosquito salivary proteins (Schneider et al., 2004). The mosquito salivary proteins comprise D7 proteins (Calvo et al., 2006) with diverse functions to facilitate blood-feeding (Chisenhall et al., 2014). These proteins are known to be anti-hemostatics, have platelet aggregation inhibition, and anti-vasoconstrictive activities, and consist of allergens and immune-modulatory compounds (Ribeiro et al., 1984; Sim et al., 2012; Surasombatpattana et al., 2012). Subsequently, DENV infection occurs in the immature dendritic cells in the skin. This infection process is aided by the receptor of

dendritic cell known as specific ICAM3-grabbing non-integrin (DC-SIGN) (Kwan et al., 2005; Libraty et al., 2001; Wu et al., 2000). The matured infected dendritic cells will migrate to local or regional lymph nodes, presenting the viral antigens to T cells, and thus initiating the cellular and humoral immune responses (Jessie et al., 2004). Infection by DENV is depicted in Figure 1.2.

The co-circulation of 4 dengue serotypes infections in a given population might be enhanced by the antibody-dependent enhancement (ADE) phenomenon mediated by both macrophages and monocytes (Halstead, 1979), and will occur when the non-neutralizing antiviral proteins such as IFITM3, facilitate the virus entry. ADE is postulated to contribute to the infection by increasing the number of ADE infection pathway to suppress antiviral molecules, hence increases the viral production (Chareonsirisuthigul et al., 2007). ADE significantly enhanced virus titer when the virus-antibody complexes enter into monocytic cells via Fc receptors (Shresta et al., 2004).

DENV infection produces several syndromes that are conditioned by age and immunological status (Guzman et al., 2010). During initial DENV infections, most children experience a subclinical infection or mild undifferentiated febrile syndromes. Meanwhile, secondary infections produce dramatic changes of the disease pathophysiology, particularly in sequential infections, such as when infection with DENV-1 is followed by dengue virus type 2 (DENV-2) or DENV-3, or infection with DENV-3 is followed by infection with DENV-2 (Alvarez et al., 2006; Guzmán et al., 2000). Such secondary infections can result in an acute vascular permeability syndrome known as dengue shock syndrome (DSS) (Guzman et al., 2010).

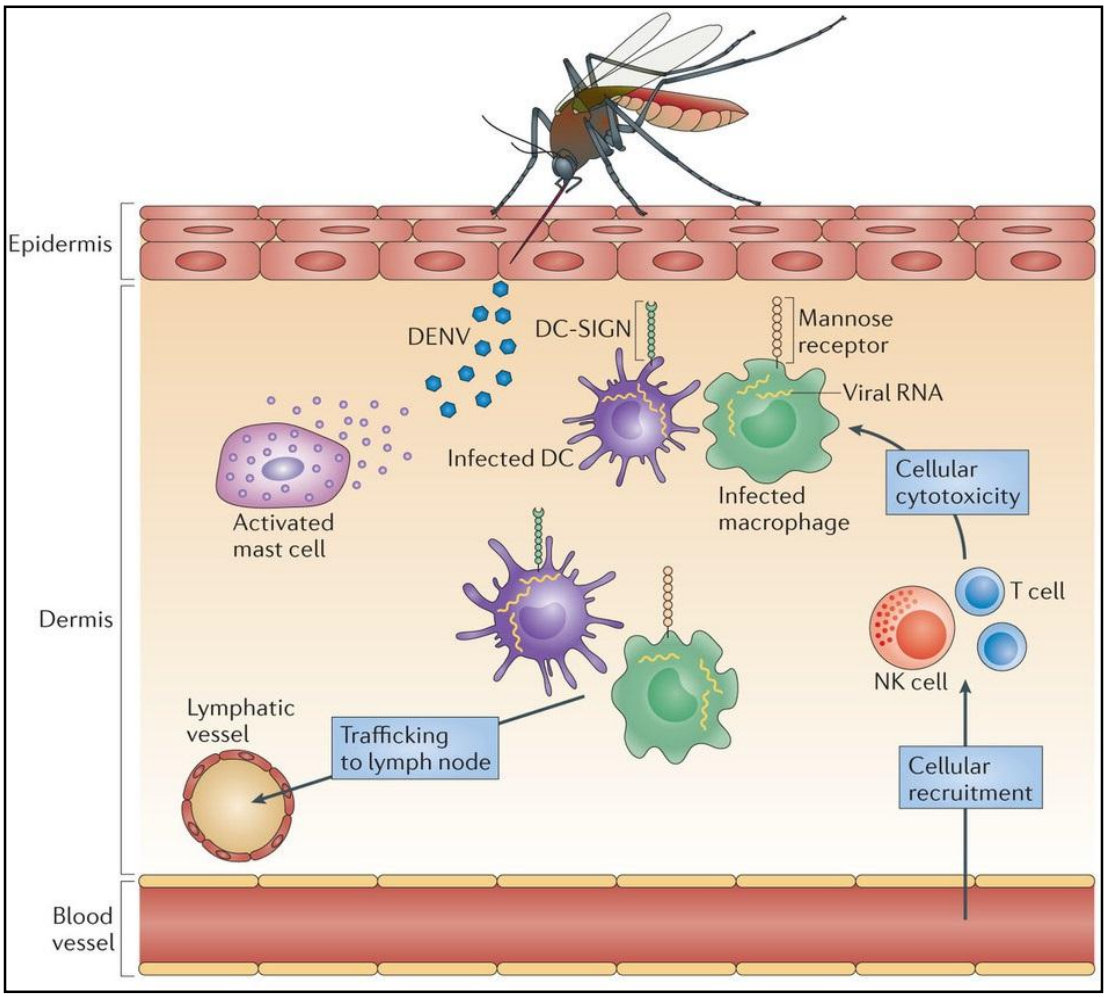

Figure 1.2 An overview DENV infection occurring after subcutaneous injection of the virus into the skin by the vector. The released of viral particles may infect nearby cells (predominantly monocytes or DCs) or activate resident immune cells such as mast cells (St John et al., 2013).

DENV infection will lead to either DF, life-threatening DHF, or DSS (Carrington et al., 2005; Chua et al., 2004; Moi et al., 2010). Primary infections with 4 DENV serotypes in adult, particularly with DENV-1 and DENV-3, often results in DF (Guzman et al., 2010). Some outbreaks of primary DENV-2 infections have been predominantly subclinical (Guzmán et al., 2000). Individuals with asthma, diabetes and other chronic diseases can result in life-threatening situations when infected with the virus (Halstead et al., 1970; Bravo et al., 1987; Kouri et al., 1987). The classical DSS or severe disease complicated by hemorrhages is usually a result of secondary dengue infections in adults (Guzman et al., 2010).

Early febrile stage of dengue infection includes symptoms, such as rash, body pains, malaise, fever, and headache. During this stage, clinicians cannot predict which patient will progress to severe disease (Guzman et al., 2010; WHO, 2014a). Symptoms such as bleeding, thrombocytopenia of < 100 000 platelets mm^{-3}, ascites, pleural effusion, hematocrit > 20.00 % and clinical warning signs, such as severe and continuous abdominal pain, restlessness, and/or somnolence, persistent vomiting and a sudden reduction in temperature associated with profuse perspiration, adynamia and sometimes fainting during defervescence may be indicative of plasma extravasation and the imminence of shock (Guzman et al., 2010).

1.2.4 Dengue virus morphology and life cycle

The virus particle (virion) is about 50 nm in diameter and is surrounded by structural proteins; envelope (E) protein, membrane (M) protein on the surface, and nucleocapsid; consists of capsid (C) protein and genomic RNA, in the inner part (Lindenbach et al., 2007). The cell surface receptor-mediated endocytosis are used by DENV, as well as other flaviviruses for cell entry (Rodenhuis-Zybert et al., 2010).

This process is followed by membrane fusion of the viral envelope with the host cell membrane, catalyzed by acidic pH of the environment, to un-coat the nucleocapsid and release the viral genome into the host cell cytosol. Subsequently, the RNA genome is translated as a single polyprotein by the host ribosomes, which is then translocated across the endoplasmic reticulum membrane (ER) (Lindenbach et al., 2007; Rodenhuis-Zybert et al., 2010). The polyprotein is then processed co- and post-translationally by the cellular (host) and virus-derived proteases into three structural proteins and seven nonstructural proteins (Lindenbach et al., 2007; Rodenhuis-Zybert et al., 2010). The NS3 protease of the virus possesses autocatalytic characteristic and plays an important role in this process (Chambers et al., 1990; Preugschat et al., 1990). Subsequently, the nonstructural proteins initiate the replication of viral RNA genome right after the protein translation and facilitate folding into individual proteins (Clyde et al., 2006).

The replication of RNA is catalyzed by virus replicase, which associates with virus membranes through interactions involving nonstructural proteins, viral RNA and probably some host factor (Lindenbach et al., 2007). Subsequently, the newly synthesized RNA is packaged by the C protein to form a nucleocapsid, meanwhile the prM and E proteins form heterodimers that are oriented into the lumen ER. The immature virion budding subsequently takes place through encapsulation of nucleocapsid by the prM/E heterodimers. However, the engulfment mechanism of the nucleocapsid by prM/E protein is still unclear (Rodenhuis-Zybert et al., 2010). The immature virions formed in the ER will be transported and released via the host secretory pathway by travelling to the Golgi compartment and maturing in the secretion (Clyde et al., 2006; Lindenbach et al., 2007; Rodenhuis-Zybert et al., 2010). Mature virions formed are able to infect new cells when prM protein is

cleaved into soluble pr-peptide and M protein by host protease, furin (Stadler et al., 1997; Clyde et al., 2006; Lindenbach et al., 2007; Rodenhuis-Zybert et al., 2010). The life cycle of DENV is depicted in Figure 1.3.

1.3 Management of dengue infection

1.3.1 Clinical presentation and therapeutic management of dengue infection

Clinical manifestations of dengue virus infections vary from asymptomatic to symptomatic with severe life-threatening illness cases such as undifferentiated febrile illness (UF), DF, DHF, DSS, and unusual dengue (UD) or expanded dengue syndrome (EDS). DHF is classified into four severity grades, with grades III and IV being defined as DSS (Kabra et al., 1999; WHO, 2008; Kalayanarooj, 2011). Most dengue infections in young children are mild and indistinguishable from other common causes of febrile illnesses and the majority of cases are UF and DF (Kalayanarooj, 2011).

DF is a flu-like illness with a variety of nonspecific signs and symptoms and should be suspected when a high fever (40 ^{o}C) concurrently appearing with two of the following symptoms: pain behind the eyes, nausea, severe headache, vomiting, swollen glands or rashes, muscle and joint pains (Lindenbach et al., 2007; Guzman et al., 2010; Rodenhuis-Zybert et al., 2010; WHO, 2012). These symptoms usually occur following an incubation period of 3 to 14 days after being infected by the virus (WHO, 2012). On the other hand, DHF is a potentially deadly complication that is characterized by high fever and can cause dengue hemorrhagic manifestations, which may lead to DSS (Gubler, 1998; WHO, 2012). The warning signs such as severe abdominal pain, rapid breathing, bleeding gums, restlessness fatigue, persistent vomiting and/or blood in vomit appear 3-7 days after the first symptoms

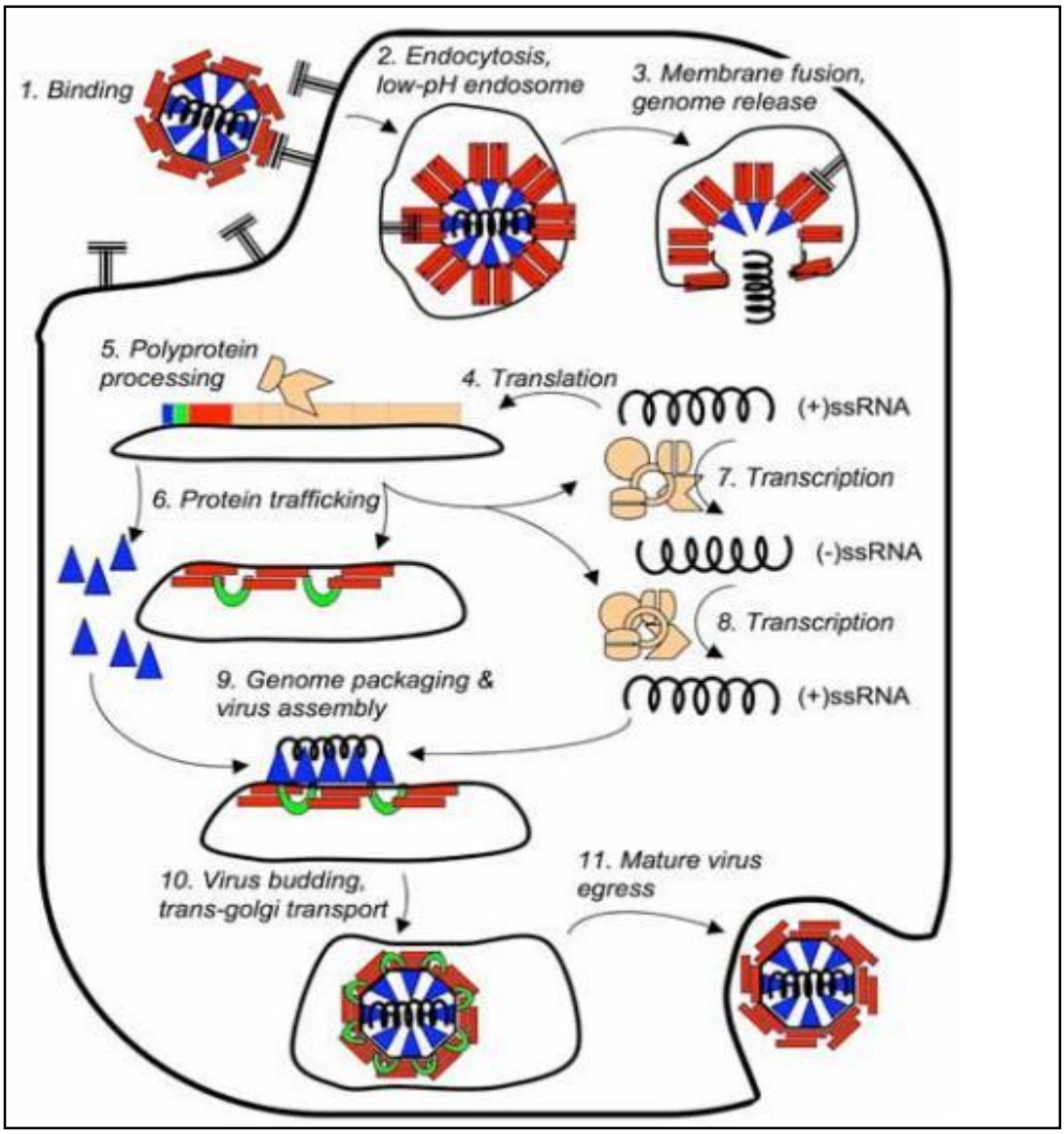

Figure 1.3 An overview of the DENV life cycle (Tomlinson et al., 2009).

along with a decrease in temperature below than 38 ^{o}C (WHO, 2012a). Patients in shock may die within 8 to 24 hours, but usually recover following anti-shock therapy (Gubler, 1998). As a result, various studies have been carried out to develop therapeutics against dengue, including anti-dengue drugs (Leung et al., 2001; Whitby et al., 2005; Tan et al., 2006a; Tan et al., 2006b; Frimayanti et al., 2011) and vaccines (Durbin et al., 2001; WhiteHead et al., 2003; Putnak et al., 2005; Edelman, 2007).

Early diagnosis of dengue infection using tourniquet test, involves raising the blood pressure to midway between systolic and diastolic pressure is a simple helpful tool in diagnosis (Singhi et al., 2007; Kalayanarooj, 2011). Based on the tourniquet test, more than 10 petechiae per 2.5 cm^2 indicates the probability of having dengue infection. In an epidemic situation, the test is positive in 50.00 % of infected patients on the first day, and in 80.00 % of infected patients by the end of the febrile phase (Singhi et al., 2007). The management of febrile phase involves reduction of high fever by taking paracetamol and oral rehydration solution and promote oral feeding (Kalayanarooj, 2011). In the early detection of DHF, the infected patients require regular monitoring by the physician (Singhi et al., 2007). The most important element of treatment in a critically ill patient or in a patient with DSS is providing intensive care with close monitoring of blood pressure, hematocrit levels, platelet count, urinary output, hemorrhagic manifestations and level of consciousness (Singhi et al., 2007). However, at present, there is no effective vaccine or antiviral drugs available in the market to manage the spectrum of dengue infections (Singhi et al., 2007; Ismail, 2014a; Ismail, 2014b; WHO, 2014a).

1.3.2 Dengue vaccine

In the absence of effective antiviral drugs, vaccination offers a good option for decreasing the incidence of dengue diseases (Qi et al., 2008). The most effective way to control dengue diseases in future will include the use of a safe and effective vaccine (Thisyakorn and Thisyakorn, 2014). The first dengue vaccine was evaluated in 1929, with an attempt to prevent virus transmission using infectious human plasma treated with ox bile or virus grown in live mosquitoes and inactivated with formalin (Thomas, 2011). Development of effective and safe dengue vaccine faces many hurdles to succeed, including lack of full understanding of the pathogenesis of severe dengue disease and an inadequate animal disease model for all four DENV serotypes (Vaughn et al., 2008). Very few dengue vaccine candidates are currently being developed (Guirakhoo et al., 2002; Sun et al., 2003; Zhang et al., 2004; Putnak et al., 2005; Simmons et al., 2006; Villar et al., 2015). Some of the dengue vaccines include recombinant subunit protein vaccines, virus-vectored DENV vaccines, biologically-derived live-attenuated dengue vaccines, inactivated dengue vaccines, and DNA vaccines (Durbin and Whitehead, 2010). The live-attenuated vaccine entails the induction of both humoral and cellular immune responses, mimicking the natural infection of DENV and it appears to have a higher success rate in vaccine development (Whitehead et al., 2007). Live attenuated DENV vaccines have been the most extensively evaluated in clinical trials and are furthest along the development pipeline (Durbin and Whitehead, 2010).

The developing of dengue vaccine has proven equally complex due to the uniqueness of dengue virus that possesses 4 different serotypes. Two tetravalent vaccine candidates comprise live attenuated viruses have been evaluated in Phase 2 clinical trials in North America and Southeast Asia countries (Vaughn et al., 2008;

Durbin and Whitehead, 2010). Butrapet and co-workers developed recombinant vaccine consists of DENV-2 16681 strain and PDK-53 strain. Phenotype PDK-53 caused no mortality, however, the replication efficacy is low in mice. Unlike PDK-53, vaccine 16681 strain causes 50.00 % mortality in animals (Butrapet et al., 2002). In another study, Blaney and co-workers developed 4 different vaccine candidates for DENV-1 by replacing the structural genes of DENV-1 with recombinant DENV-4 structural genes and these vaccines were found to replicate efficiently in rhesus monkeys. Of 4 vaccines candidates, only rDEN4Δ30(ME) appeared to induce 66.00 % seroconversion and protection in the rhesus monkey. However, the protection was only against DENV-1 challenge and not the other serotypes, indicating the difficulties in developing a dengue vaccine (Blaney et al., 2007). At present, no licensed or approved dengue vaccine is available in preventing dengue diseases (Durbin and Whitehead, 2010; Thisyakorn and Thisyakorn, 2014).

1.3.3 Preventive management

Dengue infection is a worldwide health problem, particularly in Malaysia, and at present, no specific treatment and vaccine can protect human against dengue (WHO, 2014c; WHO, 2014b). Preventive management of dengue diseases involves environmental management, personal protection, biological control, and chemical control. WHO has proposed various strategies to prevent or control the transmission of DENV including preventing mosquitoes from accessing egg-laying habitats by environmental management and modification such as disposing of solid waste, covering, emptying and cleaning domestic water storage containers on a weekly basis (WHO, 2014a). WHO responds to dengue cases by supporting the countries involved through its collaborating network of laboratories and provides the technical

support and guidance for the effective management of dengue outbreaks. In an attempt to reduce dengue outbreaks, several programs were carried out by the Ministry of Health Malaysia such as "Program Jom Hapus Tempat Pembiakan Aedes" (Ministry of Health Malaysia, 2014b). Several guidelines to protect against dengue infections were also provided by the Ministry of Health Malaysia and that includes wearing long sleeve shirt, bright shirts, and the use of household insecticides.

Currently, the use of genetically modified vectors to prevent dengue outbreak was undertaken. The genetically modified mosquito targets the wild mosquito as a potential mate, with a high species specificity and minimal off-target effects (Alphey and Alphey, 2014). For example, Blandine and co-workers have developed OX513A, a transgenic strain of engineered *Aedes aegypti*. This transgenic strain was able to compete with wild males in the field in seeking for potential mates, hence, the strain can suppress a population of *Aedes aegypti* (Blandine et al., 2013). Meanwhile, Gu and co-workers developed an efficient recombinant mosquito-densovirus that mediates the RNA interference system (Gu et al., 2011). The recombinant mosquito-densovirus will infect the wild mosquito and causes RNA interference to the wild mosquito's genome. It was postulated that this infected mosquito will then transmit the densovirus to other mosquitoes. However, the infected mosquito with defective genome was unable to execute a secondary transmission of densovirus to suppress the mosquito's population (Gu et al., 2011). Overall, despite having much preventive management to reduce dengue disease cases, the number of cases still increases tremendously (Ministry of Health Malaysia, 2014a). The failure of dengue prevention and control strategies has contributed to the increase in dengue infections and it remained as a worldwide major health problem (Ismail, 2014b; WHO, 2014b).

1.4 Dengue virus polyprotein

1.4.1 Structural protein

Flavivirus genus has 3 structural regions, namely; capsid (C), membrane (M) and envelope (E) (Yusof et al., 2000; Nall et al., 2004; Shiryaev et al., 2007a; Shiryaev et al., 2007b). These 3 structural proteins are involved in the replication of the virus particles. The C protein is a highly basic protein of about 11 kDa that serves to encapsulate the viral RNA genome for protecting and disseminating the viral RNA to suitable hosts (Lucas, 2001). C protein has been detected in the nucleus and nucleoli, as well as the cytoplasm, of DENV-infected cells (Weasley and Barrett, 2008). The interaction of C protein hydrophobic region with RNA or DNA can induce isolated C protein dimers to assemble into nucleocapsid-like particles, however, the function of C protein dimers and the mechanisms of this interaction is currently unclear (Kunkel et al., 2001; Lindenbach et al., 2007). The pre-membrane (prM) protein is about 18 kDa and it is the glycoprotein precursor of M protein with a major function of preventing E protein from undergoing acid-catalyzed rearrangement to the T3 fusogenic form during transport through the host secretory pathway (Zybert et al., 2008; Rodenhuis-Zybert et al., 2010). The M protein, about 8 kDa, is a small proteolytic fragment of the precursor prM protein, produced after being cleaved by host protease, furin during maturation of nascent virus particles within the host secretory pathway (Melino and Paci, 2007). Meanwhile, the function of E protein (50 kDa) is to mediate the binding and fusion during the virus entry, and it is the main antigenic determinant on the virus particle as the target for the immune system (Seema and Jain, 2005).

During the translation of the polyprotein, the structural proteins are translocated and anchored in the endoplasmic reticulum by various signal sequences

(Lindenbach et al., 2007). The capsid protein contains a hydrophobic signal sequence that aids in the translocation of prM into the lumen of the endoplasmic reticulum. The prM protein has two transmembrane spanning domains, which contains stop transfer sequence and a signal sequence (Rodenhuis-Zybert et al., 2010). E protein is translocated into the lumen, aided by prM. Subsequently, after the appropriate proteolytic cleavages, the capsid protein, and viral RNA are localized in the cytoplasm and the capsid remains associated with the endoplasmic reticulum membrane. The prM and E proteins will form a stable heterodimer on the luminal side of the endoplasmic reticulum after the translation process begins (Mukhopadhyay et al., 2005).

1.4.2 Nonstructural protein (NS)

The precursor polyproteins comprised seven non-structural proteins arranged in the order of NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5 (Chambers et al., 1990; Falgout et al., 1991; Preugschat et al., 1990; Preugschat and Strauss, 1991; Mukhopadhyay et al., 2005). NS1 glycoprotein is about 46 kDa in size (Lindenbach et al., 2007). It is an important antigen along with E protein used in immunization of mice for human vaccine development (Zhang et al., 1988). However, in recent years, it had been used for the early detection of DENV infection (Hua Xu et al., 2006; Hang et al., 2009; Lima et al., 2011). One example of a commercially available dengue NS1 detection kit is Platelia Dengue NS1 Ag (Bio-Rad, USA).

The NS2A protein is a hydrophilic protein about 22 kDa in size (Lindenbach et al., 2007). It plays an important role in virus assembly (Leung et al., 2008). On the other hand, NS2B protein, which is about 14 kDa, is a membrane-associated protein.

It forms a stable complex with NS3 and also acts as a cofactor for the activation of NS3 serine protease (Lindenbach et al., 2007).

NS3 protein is about 70 kDa, is a large multifunctional protein, containing several functions required for polyprotein processing and RNA replication (Lindenbach et al., 2007). It consists of a trypsin-like serine protease domain within the N-terminal 180 residues and a domain with NTPase/helicase activity at the C - terminal (Li et al., 1999). The active site of the NS3 serine protease carries the catalytic triad, comprising of three amino acid residues, namely His51, Asp75, and Ser135, with NS2B acting as a cofactor of NS3 serine protease for optimal catalytic activity (Preugschat et al., 1990). The catalytic triad residues are involved in the peptide bond hydrolysis of substrate. Briefly, an exposed loop of a large hydrophobic residue on the substrate allows the serine protease enzyme to bind it. Subsequently, the aspartate (Asp75) will form a hydrogen bond with histidine (His51), allowing histidine to deprotonate serine. Serine (Ser135) will serve as a nucleophile, of which the carbonyl carbon of the substrate will be attacked, leading to the formation of a tetrahedral intermediate by accepting an electron from serine. This intermediate will then collapse, causing histidine to donate its proton to nitrogen in the carbon atom, and thus, the nitrogen and the attached C-terminal of substrate is fragmented and leave as a product (2 separated fragment) (Dodson and Wlodawer, 1998).

NS4A and NS4B are both hydrophobic proteins of about 16 kDa and 27 kDa in sizes (Lindenbach et al., 2007). These two proteins are membrane-associated and are reported to play an important role in RNA replication by localization with replication complexes, involving NS3 (Preugschat and Strauss, 1991). There is a signal sequence for the translocation of the adjacent NS4B into the ER lumen (Miller et al., 2007).

NS5 is a multifunctional protein, about 103 kDa in size, with methyltransferase (MTase) and RNA-dependent RNA polymerase (RdRp) activities (Lindenbach et al., 2007). The NS5 MTase is responsible for methylating the viral RNA cap structure to cap-1 structure, important for polyprotein translation (Zhuo et al., 2007). On the other hand, the NS5 RdRp catalyzed the viral replication by synthesizing a transient double-stranded replicative RNA intermediate which consist of viral plus- and minus-strand RNAs (Bartholomeuzs & Thompson, 1999, Yap et al., 2007). The newly synthesized minus strand is subsequently used as a template for synthesizing additional plus-strand RNAs (Bartholomeuzs & Thompson, 1999, Yap et al., 2007). The overall functions of each protein in dengue polyprotein are depicted in Figure 1.4.

1.4.3 NS2B/NS3 protease of dengue virus

NS2B is a 14 kDa non-structural protein and it comprises hydrophobic and hydrophilic regions in a complex structure (Champreda et al., 2000; Lindenbach et al., 2007). It is located immediately upstream of NS3 protease in the polyprotein precursor (Champreda et al., 2000). The sequence of NS2B includes three to four transmembrane helicases that anchor the NS2B/NS3 heterodimer to the endoplasmic reticulum (Shiryaev et al., 2007b). NS2B protein plays a pivotal role in the protease cleavage activity. As demonstrated by *in vitro* studies with synthetic peptide substrates and natural polyprotein precursors, the activated NS2B cofactor is a necessity for catalytic activity of the NS3 protease (Falgout et al., 1991; Zuo et al., 2009). Comparison of the kinetic properties of NS3 and NS2B/NS3 protease suggests that NS2B generates additional specific interactions with the substrate residues to form sub-pocket 2 (interactions with Asp75, Asn152 of NS3) and sub-pocket 3 (Chambers et al., 1990).