THE STUDY OF SUSCEPTIBILITY AND CYTOKINE PRODUCTION IN DENGUE VIRUS TYPE 2 (DENV2)-INFECTED MONOCYTES OF INDIVIDUALS WITH GLUCOSE-6-PHOSPHATE DEHYDROGENASE (G6PD) DEFICIENCY

ABDULLAH ABDULL JABBAR AHMED AL-ALIMI

UNIVERSITI SAINS MALAYSIA

2013

THE STUDY OF SUSCEPTIBILITY AND CYTOKINE PRODUCTION IN DENGUE VIRUS TYPE 2 (DENV2)-INFECTED MONOCYTES OF INDIVIDUALS WITH GLUCOSE-6-PHOSPHATE DEHYDROGENASE (G6PD) DEFICIENCY

By

ABDULLAH ABDULL JABBAR AHMED AL-ALIMI

Thesis Submitted in Fulfilment of the Requirement for the Degree of Doctor of Philosophy

July, 2013

ACKNOWLEDGEMENT

Firstly, I am thankful to ALLAH for the blessings and kindness given me. I would also like to express my deepest gratitude to my supervisor Prof. Dr. Narazah Mohd Yusoff for her patience, support, guidance and advice that was instrumental in the completion of this work. I also would like to thank my co-supervisor Assoc. Prof. Fauziah Mohd Idris for her selfless support throughout this study.

I would also like to thank Dr. Faisal Muti Al-hassan and give a million 'thank you' to Dr. Syed Ali Atif, for the very insightful suggestions and their guidance that helped me in my work. Dr Hasnah Hashim, Dr. Abdullah Aldhabily, Dr. Ryidh Al-Alimi Mahmmoud Al-Areefi, Dr. Abdul Raheem Al-alimi, Dr. Khalil Al-alimi and Mr. Nazuan also deserve my deepest gratitude for supporting me in the statistical analysis.

My special thanks go to Prof. Shamalah Devi, Department of Virology, University Malaya, as well as to Dr. Jamilah Barhom, Dr. Soo and the nurses in Blood Bank Hospital Pulau Pinang (HPP) for their assistance and co-operation in blood specimen collection from donors.

Finally, my special thanks go to my friends, Suleiman Fati as well as Salem Bashanfer, Ashwaq Hamid, Ahmed Al-Rifaee, and Dr. Mohammed Alhoot. I would like to express my appreciation to all staffs AMDI for their help and support.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

Ab	Antibody
ADE	Antibody dependent enhancement
AIDS	Acquired immunodeficiency syndrome
APC	Antigen presenting cells
ATCC	American type culture collection
Bcl-2	B-cell lymphoma-2
CAT	Catalase
CD	Cluster of differentiation
CO_2	Carbon dioxide
CGD	Chronic granulomatous disease
C	Capsid protein
CCL	Chemokine (C-C Motif) ligand
Cl_2	Chloride
CNSHA	Chronic non-spherocytic haemolytic anaemia
CPE	Cytopathic Effect
CHO	Chinese hamster ovary
DC	Dendritic Cells
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-3-Grabbing non- integrin
DAPI	Diamidino-2-phenylindole
DENV	Dengue virus

DENV-1	Dengue virus type-1
DENV-2	Dengue virus type-2
DENV-3	Dengue virus type-3
DENV-4	Dengue virus type-4
DF	Dengue fever
DHF	Dengue haemorrhagic fever
DMEM	Dulbecco's modified eagle's medium
DNA	Deoxyribonucleic acid
DPBS	Dulbecco’s phosphate buffer saline
DSS	Dengue shock syndrome
E	Envelope protein
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme-linked immune sorbent assay
MEM	Modified eagle’s medium
ER	Endoplasmic reticulum
G6PD	Glucose-6-phosphate dehydrogenase deficiency
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte macrophage colony stimulating factor
GSSG	Glutathione disulfide (oxidized)
GSH	Glutathione (reduced)
GRx	Glutathione reductase
GPx	Glutathione peroxidase
FcR	Fc-Receptor

FACS	Fluorescence activated cell sorting
FITC	Fluorescein isothiocyanate
FBS	Fetal bovine serum
Hb	Haemoglobin
HRP	Horse radish peroxidase
HCV	Hepatitis C virus
HBV	Hepatitis B virus
HepG2	Human Liver carcinoma cell line
HIV	Human immunodeficiency virus
H_2O_2	Hydrogen peroxide
HCF	Human cytotoxic factor
HMEC-1	Human dermal microvascular endothelial cells-1
HOCL	Hypochlorous acid
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IFN	Interferon
IFN-α	Interferon-alpha
IFN-β	Interferon-beta
IFN-γ	Interferon-gamma
IL	Interleukin
IL-1β	Interleukin (1β)
IL-2	Interleukin (2)
IL-4	Interleukin (4)

IL-5	Interleukin (5)
IL-6	Interleukin (6)
IL-7	Interleukin (7)
IL-8	Interleukin (8)
IL-10	Interleukin (10)
IL-12	Interleukin (12)
IL-13	Interleukin (13)
IL-15	Interleukin (15)
IL-16	Interleukin (16)
IL-18	Interleukin (18)
L-15	Leibovitz-15 media
LPS	Lipopolysaccharide
MAb	Monoclonal Aantibody
MACS	Magnetic activated cell sorting
MBL2	Mannan-binding lectin 2
MCP-1	Monocyte chemo attractant protein-1
MDM	Monocyte derived macrophages
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
MPO	Myeloperoxidase
MOH	Ministry of health
MOI	Multiplicity of infection
NAC	N-acetyl cysteine

NADPH	Nicotinamide adenine dinucleotide phosphate
NF-kB	Nuclear factor kappa-enhancer of activated B cells
NGC	New guinea clone
NK	Natural killer
NKT	Natural killer T
NO	Nitric oxide
NaN3	Sodium azide
NS	Non-structural protein
OD	Optical density
O_2•-	Superoxide anion
ONOO	peroxynitrite
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
PE	Phycoerythrin
PKC	Protein kinase C
PFU	Plaque forming units
prM	Premembrane protein
Prx	Peroxiredoxins
PPP	Pentose phosphate pathway
RBCs	Red Blood Cells
RANTES	Regulated upon activation normal t-cell expressed and secreted

RNS	Reactive nitrogen species
RNA	Ribonucleic acid
RPMI	Roswell park memorial institute
ROS	Reactive oxygen species
RT	Room temperature
SOD	Superoxide dismutase
STAT	Signal transducers and activators of transcription
TGF-β	Transforming growth factor-beta
TGN	Trans golgi network
Th1	T helper type 1
Th2	T helper type 2
TNF-α	Tumor necrosis factor-alpha
TMB	Tetramethylbenzidine
UV	Ultraviolet
WHO	World health organization

KAJIAN KERENTANAN DAN PENGHASILAN SITOKIN DALAM VIRUS DENGGI JENIS 2 (DENV2) – MONOSIT TERKESAN DARIPADA INDIVIDU YANG KEKURANGAN GLUKOSA-6-FOSFAT DEHIDROGENASE (G6PD).

ABSTRAK

Virus denggi adalah endemik di Semenanjung Malaysia. Manifestasi klinikal berubah-ubah bergantung ke tempoh inkubasi virus serta tahap keimunan pesakit. Kekurangan Glukosa-6-fosfat dehidrogenase (G6PD) adalah prevalen di Malaysia, yang keberlakuannya adalah 5.2%. Dicatatkan bahawa individu kekurangan G6PD menderita lebih teruk jika dijangkiti infeksi denggi. Kajian ini bertujuan mengkaji kerentanan / suseptibiliti monosit terhadap infeksi DENV2, respons oksidatif, dan penghasilan sitokin dalam monosit individu yang mengalami kekurangan G6PD. Sampel darah dikumpulkan daripada penderma setelah mendapat kelulusan daripada Jawatankuasa Etika Penyelidikan USM dan Kementerian Kesihatan. Ujian G6PD dijalankan dengan keadah titik pendarflour (florescent spot method) diikuti dengan asai kuantitatif. Monosit daripada individu kekurangan G6PD dan individu yang sihat (G6PD normal) diasingkan dan diberikan DENV2. Kepelbagaian infeksi (multiplicity of infection, MOI) 0.1 dan kadar infeksi intrasel diukuir dengan sitometri aliran dan ekstrasel menggunakan asai plak. Tahap spesies oksidatif, anion superoksida (O_2•-), nitrik oksida (NO), stres oksidatif dan sitokin ditentukan dan dibandingkan dengan kawalan. Keputusan menunjukkan bahawa kerentanan yang tinggi daripada monosit kekurangan G6PD terhadap DENV2 dan tahap NO dan O_2•-, secara signifikannya rendah dalam monosit kekurangan G6PD dibandingkan dengan kawalan-sihat. Pada keseluruhan, stres oksidatif pada individu kekurangan G6PD secara signifikannya amat tinggi dibandingkan dengan kawalan-sihat. Kajian korelasi di antara replikasi viral dan

keadaan oksidatif monosit mengesahkan dapatan ini. Di samping itu, pro-inflamatori sitokin IL-6, IL-8, IL-12, TNF-α dan MCP-1 secara signifikannya meningkat ($P<0.05$) dan mencapai puncak pada 48 jam dalam monosit kekurangan G6PD dibandingkan dengan individu sihat. Secara kontras, anti-inflamatori sitokin IL-10 secara signifikan lebih tinggi ($P<0.05$) dengan puncak maksimum pada 48 jam dan seterusnya menjadi semakin berkurangan. IFN-α secara signifikan berkurangan dalam monosit kekurangan G6PD dibandingkan dengan G6PD-normal ($p<0.05$). Sebagai tambahan, kajian korelasi di antara replikasi viral dan penghasilan sitokin menyokong hipotesis bahawa kekurangan G6PD, beban DENV2 yang lebih tinggi, dan stres oksidatif dalam sel boleh menyumbang terhadap peningkatan penghasilan sitokin inflamatori. Keputusan kajian menunjukkan bahawa individu yang kekurangan G6PD didapati lebih rentan terhadap infeksi DENV2 dibandingkan dengan individu sihat. Dengan kata lain, penghasilan spesies oksigen reaktif (reactive oxygen species, ROS) berkurangan dalam individu kekurangan G6PD. Ini menjelaskan bahawa infeksi denggi tinggi di kawasan yang mana kekurangan G6PD adalah prevalen. Beban viral yang tinggi, stres oksidatif yang semakin meningkat, dan penghasilan sitokin yang diaruh adalah patogenesis DHF yang amat pemting.

THE STUDY OF SUSCEPTIBILITY AND CYTOKINE PRODUCTION IN DENGUE VIRUS TYPE 2 (DENV2)-INFECTED MONOCYTES OF INDIVIDUALS WITH GLUCOSE-6-PHOSPHATE DEHYDROGENASE (G6PD) DEFICIENCY

ABSTRACT

Dengue virus is endemic in peninsular Malaysia. The clinical manifestations vary depending on the incubation period of the virus as well as the immunity of the patients. Glucose-6-phosphate dehydrogenase (G6PD) deficiency is prevalent in Malaysia, where the incidence is 5.2%. It has been noted that G6PD-deficient individuals suffer from more severe clinical presentation of dengue infection. The aim of this study is to investigate the susceptibility of monocytes to DENV2 infection, the oxidative responses, and cytokine production in monocytes from G6PD-deficient individuals. Blood samples were collected from donors after being approved by the Research Ethical Committees of USM and Ministry of Health. Screening for G6PD was performed using the florescent spot method followed by the quantitative assay. Monocytes from G6PD-deficient and healthy individuals (G6PD-normal) were isolated and infected with DENV2, whereby multiplicity of infection (MOI) 0.1 and intracellular infection rate were measured by flow cytometry and extracellular by plaque assay. Levels of oxidative species, superoxide anions (O_2•-), nitric oxide (NO), oxidative stress and cytokines were determined and compared with normal controls. The findings indicate that early and high susceptibility of monocytes with G6PD-deficiency to DENV2 and levels of NO and O_2•-, were significantly lower in the monocytes of

individuals with G6PD-deficiency compared to the healthy controls. Furthermore, the overall oxidative stress in individuals with G6PD-deficiency was significantly higher when compared to the healthy controls. Correlation studies between viral replication and monocyte oxidative state further confirmed these findings. Moreover, pro-inflammatory cytokines IL-6, IL-8, IL-12, TNF-α and MCP-1 were significantly increased ($P<0.05$) and peaked on 48 hours in infected G6PD-deficient monocytes compared to those obtained from healthy individuals. Anti-inflammatory cytokine IL-10 was significantly higher ($P<0.05$) with maximum peak at 48 hours and decreased thereafter. IFN-α were significantly reduced in infected monocytes with G6PD-deficiency compared to G6PD-normal ($p<0.05$). In addition, correlation studies between viral replication and cytokine production confirmed and thus supported the hypothesis that in G6PD-deficiency, the higher DENV2 load, and oxidative stress in the cell may contribute to enhance production of inflammatory cytokines. The results of the study demonstrated that individuals with G6PD-deficiency are more susceptible to DENV2 infection compared to healthy individuals. The likely explanation for this is reduced production of reactive oxygen species (ROS) in individuals with G6PD deficiency. This may explain the reason for high prevalence of dengue infection in areas where G6PD deficiency is prevalent. High viral load, elevated oxidative stress and induce cytokines production are the most important of pathogenesis of DHF.

CHAPTER ONE

1.0 Introduction

Dengue infection disease is a leading cause of morbidity and mortality in the tropics and subtropics, and can lead to extensive outbreaks in urban areas (Dammert *et al.*, 2009). The disease is a global problem, given that as many as 100 million people are infected, of whom 25,000 die annually (Gubler & Meltzer, 1999). Dengue virus (DENV) is primarily transmitted to humans through the bite of infected *Aedes* mosquitoes, particularly *Aedes aegypti* (Green & Rothman, 2006). Dengue infection is caused by DENV, a positive-strand RNA virus of the family *Flaviviridae*. Distinct variations or serotypes of the virus include DENV1, DENV2, DENV3, and DENV4. All four serotypes are capable of causing a full spectrum of the disease symptoms, with several degrees of severity (McBride & Bielefeldt-Ohmann, 2000).

A significant percentage (~80%) of individuals infected with DENV show only mild symptoms, such as dengue fever (DF), whilst some develop a more severe dengue illness called dengue haemorrhage fever (DHF)/dengue shock syndrome (DSS) (Whitehorn & Farrar, 2010). DF is a self-limiting illness characterized by fever, headache, myalgia, arthralgia, nausea, and fatigue (Whitehorn & Farrar, 2010). The high viral load in DHF/DSS patients can be a life-threatening form of dengue infection characterized by a high fever, haemorrhage, vascular permeability, thrombocytopenia and shock (Rigau-Pérez *et al.*, 1998). DHF/DSS is one of the leading causes of

paediatric hospitalization in Southeast Asia and has become endemic in all Pacific Countries.

DENV2 has been involved in most Dengue outbreaks in the last 20 years and is associated with the severity of the disease‘s outcome (Méndez *et al.*, 2012). Moreover, several epidemic reports from different studies indicated that a severity of dengue infection was associated with a higher titer DENV2 viremia than the other DENV serotypes (Balmaseda *et al.*, 2006; Clyde *et al.*, 2006). In addition, DENV2 has been identified as a cause of DHF/DSS, rather than other serotypes of DENVs (Hesse, 2007). In Malaysia, DENV2 emerged as the major serotype responsible for recent outbreaks of dengue infection (Chee & AbuBakar, 2003).

Monocytes have been considered the major target cells of DENV replication by a number of authors (O'Sullivan & Killen, 1994; Chao *et al.*, 2008). They act with the aim to regulate the mechanism of the immune system during infection, resulting in production of several cytokines/chemokines and chemical mediators. These cytokines include tumour necrosis factor alpha (TNF-α) (Hober *et al.,* 1996), interferon alpha (IFN-α) (Kurane & Ennis, 1988), interleukin-6 (IL-6) (Chaturvedi *et al.*, 1999), interleukin-8 (IL-8) (Chaturvedi *et al.*, 2000; Bosch *et al.*, 2002), interleukin-10 (IL-10), interleukin-12 (IL-12) (Green *et al.*, 1999b), and monocyte chamoattarctic protein-1 (MCP-1) (Yang *et al.*, 1995), which play a key role in both innate and adaptive immune responses. Therefore, these cytokines play an important role in enhanced activation of other immune cells that may contribute to the DHF pathogenesis, and they have been implicated widely in conditions associated with vascular leakage, as well as hemorrhagic disorders in DHF/DSS patients (Martina *et al.*, 2009). Therefore, inflammatory and anti-

inflammatory cytokines play an important role in the pathogenesis of dengue virus infection and serum levels of certain cytokines are elevated during dengue infection (Martina *et al.*, 2009).

Dengue haemorrhagic fever is one of the serious causes of morbidity and mortality in children, in contrast to other infections in Southeast Asian countries (Dejnirattisai, 2004). DHF is now a leading cause of hospitalization and death among children (Dejnirattisai, 2004). However, it is not entirely clear why some individuals are more at risk of severe forms DHF/DSS of dengue infection while others are not.

The pathogenesis of DENV remains unclear, due to the complex interplay of viral and host factors. Several conducted studies identified the risk factors associated with the severity of disease, including the specific serotype of DENV, the host immune status, age, and the genetic background of the patients (Clyde *et al.*, 2006; Noisakran & Perng, 2008). The data from genetic epidemiology studies has demonstrated that a certain host susceptible genes promote the development of severe DENV infection (Chaturvedi *et al.*, 2006), particularly alleles of human leukocyte antigen (HLA) class I and II (Lan *et al.*, 2008). Additionally, polymorphism in gene‘s coding for TNF-α (Fernández-Mestre *et al.*, 2004), transforming growth factor β (TGFβ) (Chen *et al.*, 2009), and Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN) (Sakuntabhai *et al.*, 2005) have been linked with an increased risk of severe dengue complications. Moreover, another genetic abnormality that is reported to have a link with DHF/DSS is the deficiency of glucose-6-phosphate dehydrogenase (G6PD)—an ubiquitous X-linked enzyme.

G6PD deficiency is primarily found in populations originating from tropical and subtropical areas of the world, and its geographic distribution is similar to that of malaria. G6PD deficiency, a common enzymopathy in human cells, enhances the viral replication (Ho *et al.*, 2008; Wu *et al.*, 2008) and cytokine production (Wilmanski *et al.*, 2007). Although the relationship between G6PD deficiency and cytokine modulation is very important, it has not been investigated thus far. Intracellular redox status changes in G6PD-deficient cells may have an impact on a modulator of cytokine production, thereby potentially increasing the severity of microbial infection complications (Wilmanski *et al.*, 2007). Moreover, modulator of cytokine production was found to influence the antiviral mechanism of G6PD-deficient cells (Wu *et al.*, 2008).

1.1 Rationale of the Study

Globally, dengue infection continues to affect more than 2.5 billion people living in 120 countries in endemic areas at risk of dengue infection (Chaturvedi *et al.*, 2006; Chaturvedi & Nagar, 2008). Approximately 40% of the world‘s population live in areas where the disease can be acquired from mosquitoes (Morens & Fauci, 2008). Recent epidemic reports from Western Pacific, Southeast Asia, Eastern Mediterranean, Africa and some parts of South America have demonstrated that DENV infection is a serious cause of morbidity and mortality, with much higher prevalence compared to other infections (Halstead, 2007). The number of DF cases has increased 30-fold in the last 50 years, and the associated complications cause an estimated 100 million infections, 500,000 hospitalizations by DHF, and 25,000 deaths annually (Phillips, 2008).

In Malaysia, the first case of DF was reported in 1902 (Skae, 1902). Penang was the first to be affected with DF (Rudnick *et al.*, 1965). The primary DHF outbreak was also recorded in Penang in 1962 (Rudnick *et al.*, 1965; George, 1992). Currently, DF and DHF have become the major public health problems in Malaysian Peninsula (Wallace *et al.*, 1980; George, 1992), all states, including East Malaysia, similarly affected. More recently, a large outbreak of DENV infection occurred in 2008, with the total of 49355 cases and 122 deaths, according to the Ministry of Health reports. Annually, epidemiology of the disease and the number of cases increased in all Malaysian states. Clearly, there is an urgent need to determine whether genetic factors, rather than environmental factors, are associated with increased prevalence of dengue fever.

G6PD deficiency and dengue infection are still major health problems in countries where these diseases are common, such as Malaysia. At present, our understanding of the pathogenesis of dengue virus is incomplete, especially in affected individuals who suffer from genetic diseases such as G6PD-deficiency, thalassemia, and sickle cell anaemia. G6PD deficiency is the most common enzymopathy in human cells, which affects approximately more than 500 million individuals throughout the world (Sirdah *et al.*, 2012). The majority of the affected individuals reside mainly in Africa, Mediterranean countries, Southeast Asian countries and Northern Europe (Sirdah *et al.*, 2012).

Deficient in G6PD enzyme affects production of reactive nitrogen (RNS) and oxygen species (ROS), such as nitric oxide (NO), superoxide (O_2•-), and hydrogen peroxide (H_2O_2), resulting in alterations of normal redox state of immune cells, which

produce cytokines and RNS/ROS in order to clear invading pathogens (Wu *et al.*, 2008). Alteration of the redox state may render immune cells ineffective against invading organisms, resulting in an increased severity of the infection (Wu *et al.*, 2008). Recent studies have indicated that the G6PD deficiency enhances viral replication and hence the virulence of a virus (Ho *et al.*, 2008; Wu *et al.*, 2008). Moreover, recurrence of microbial infections in G6PD-deficient individuals has been previously reported (Abu-Osba *et al.*, 1989; Costa *et al.*, 2002).

According to the findings of a study conducted in Thailand, G6PD-deficient individuals were significantly (19.1 %) prone to developing DHF/DSS compared to non-G6PD-deficient individuals (Tanphaichitr *et al.*, 2002). More recent study have shown that monocytes from G6PD-deficient individuals were more susceptible to DENV2 infection with higher replication ability than those from healthy controls (Chao *et al.*, 2008). Although there appears to be a connection between G6PD-deficiency and increased severity of DENV2 infection, no studies have been carried out to elucidate the mechanism behind this relationship and the immune status.

In Malaysia, so far no research has been conducted to investigate the association of G6PD deficiency as a genetic defect and dengue infection. Moreover, until now, there have been no studies on G6PD deficiency and its effect on cytokine production from DENV-infected monocytes of individuals with G6PD deficiency.

1.2 Hypothesis

Many genetic alterations have contributed to the development of dengue disease.

One of the most recently identified genetic factors believed to be implicated in

pathogenesis dengue infection is G6PD-deficiency. It was hypothesized that monocytes from G6PD-deficient individuals will:

- be more susceptible to DENV2 infection
- produce lower levels of nitric and oxygen species following DENV2 infection.
- accumulate higher oxidative stress following DENV2 infection
- produce higher levels of cytokines following DENV2 infection

1.3 General of the Study

To investigate the association between G6PD deficiency and DENV2 infection

1.3 .1 Specific Objectives

1. To find out whether monocytes from G6PD-deficient individuals were more susceptible to DENV2 infection
2. To investigate the levels of nitrogen and oxygen species production following DENV2 infection in monocytes from G6PD-deficient individuals
3. To investigate levels of oxidative stress accumulation following DENV2 infection in monocytes from G6PD-deficient individuals
4. To investigate the levels of cytokines production following DENV2 infection in monocytes from G6PD-deficient individuals

CHAPTER TWO

LITERATURE REVIEW

2.1 Dengue Virus (DENV)

The DENV belongs to the genus flavivirus of the family *Flaviviridae* (Kurane, 2007). There are four serotypes that are closely antigenically related and designated as DENV1, DENV2, DENV3 and DENV4 (Lindenbach & Rice, 2003). When humans are infected by one serotype, this stimulates long-life protection immunity against reinfection by the same serotype, but it does not protect the affected individual from infection with other serotypes (Gujarati & Ambika, 2012).

2.1.1 Viral Composition

DENV is a small spherical particle covered by a lipid-enveloped RNA virus. DENV is comprised of three structural proteins, which include capsid (Core C) protein, a membrane (M) protein, an envelope (E) protein and seven non-structural (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5) proteins as shown in Figure 2.1 (Lambeth, 2007). The E protein is the major surface protein that plays a key role in DENV entry and fusion into target cells (Rey, 2003). M protein is a small proteolytic fragment of prM protein, which is essential for growth and maturation of the virus into an infectious form that can attack new cells (Netsawang, 2010; Smit *et al.*, 2011).

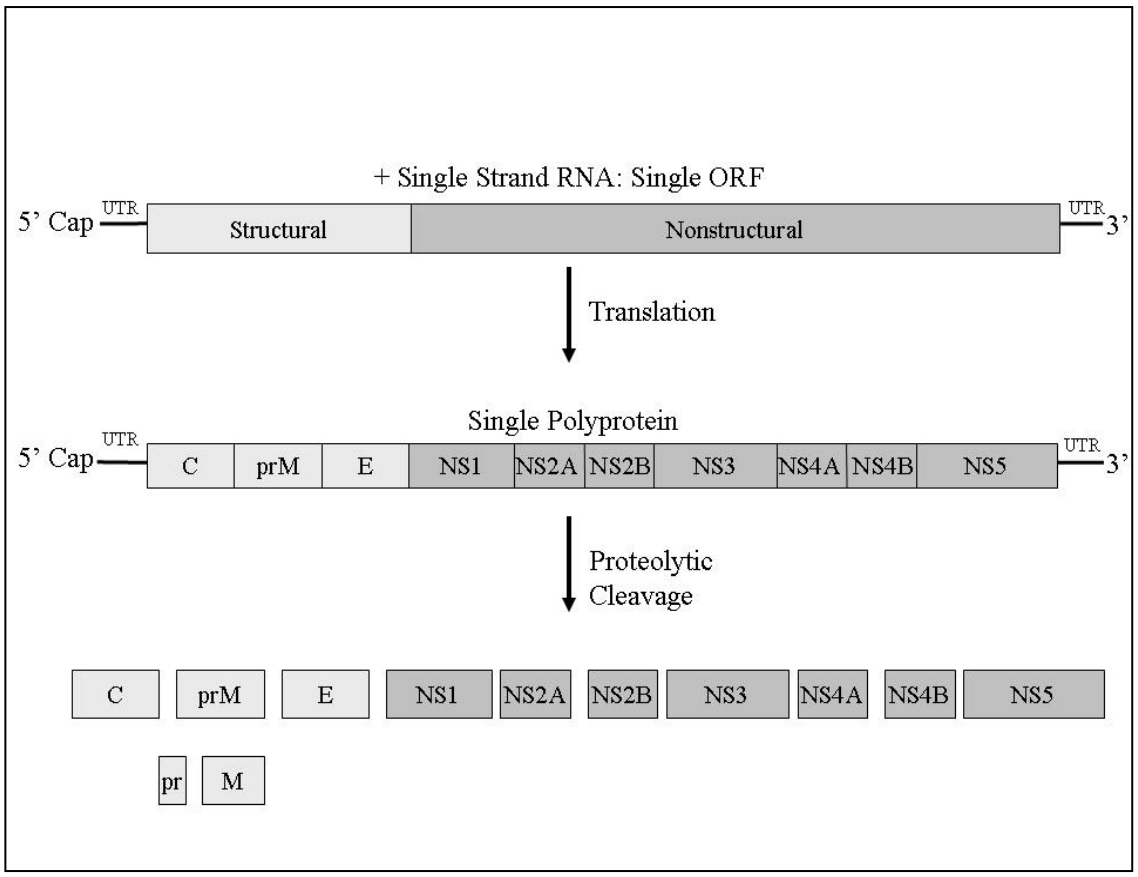

Figure 2.1: Dengue virus genome (Lambeth, 2007). The positive single stranded RNA is translated to capsid (C), pre-membrane (M), and envelope (E). The seven non-structural proteins as divided into NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5.

2.1.2 Dengue Virus Life Cycle and Replication

DENV is a lipid-enveloped RNA *flavivirus* replicate in the cytoplasm of susceptible cells (Perera *et al.*, 2008). Figure 2.2 summarized the initial stages of the viral life cycle start with bonding of the virus to the susceptible cells by receptor-mediated endocytosis (Van Der Schaar *et al.*, 2007; Perera *et al.*, 2008). The entry process is initiated by the interaction of E protein with glycosaminoglycans and heparan sulphate on monocyte/macrophage target cells (Jain, 2005). After taking up the virus particles, the viruses are carried into endocytic compartment to form endosome in which low pH triggers a conformational change in the viral E and allows it to fuse with the endosomal membrane releasing the capsid into the cytoplasm. This mechanism has been reported with mosquito cells and human peripheral blood monocytes (Van Der Schaar *et al.*, 2007; Perera *et al.*, 2008; Umareddy, 2009).

Once the viral RNA is in the cytoplasm, initiation of translation begins; the viral polyprotein is processed co- and post-transnationally into three functional viral proteins (C, prM, and E) and seven non-structural proteins (Perera *et al.*, 2008). This processing is carried out by cellular and viral proteases. RNA replication is processed in the membrane-associated cytoplasmic compartment. Following virus proliferation, newly synthesized viral genome is encapsidated into the capsid proteins and directly buds into the endoplasmic reticulum where the immature virus (prM and E proteins) is surrounded by a lipid envelope containing viral proteins and budded off into the endoplasmic reticulum as undeveloped particles (Van Der Schaar *et al.*, 2007; Netsawang, 2010). Some of these immature particles are transported to Golgi apparatus in which prM is cleaved to generate mature infectious particles in the low pH environment (Van Der

Schaar *et al.*, 2007; Netsawang, 2010). Immature non-infectious and mature infectious particles are released into the extracellular space by exocytosis (Van Der Schaar *et al.*, 2007)

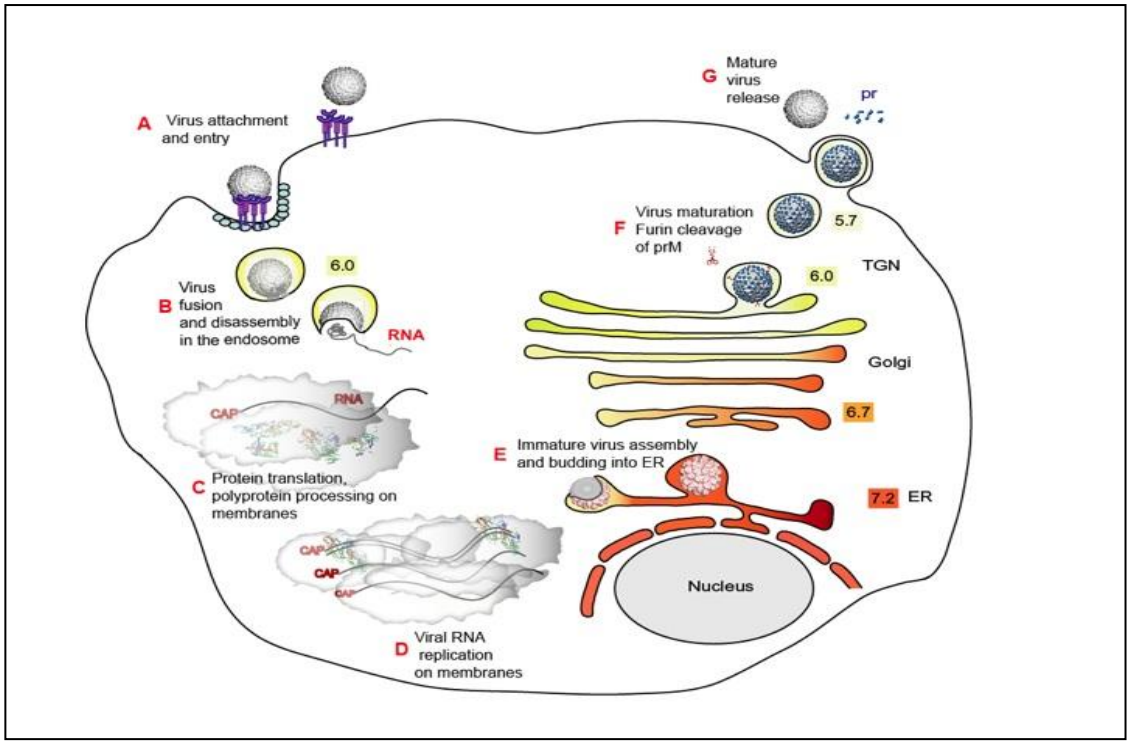

Figure 2.2: The flavivirus life cycle . **A.** Virions bind to cell-surface attachment molecules and receptors and are internalized through endocytosis. **B.** In the low pH of the endosome, viral glycoproteins mediate fusion of viral and cellular membranes, allowing disassembly of the virion and release of RNA into the cytoplasm. **C.** Viral RNA is translated into a polyprotein that is processed by viral and cellular proteases. **D.** Viral non-structural proteins replicate the genome RNA. **E.** Virus assembly occurs at the ER membrane, where capsid protein and viral RNA are enveloped by the ER membrane and glycoproteins to form immature virus particles. **F.** Immature virus particles are transported through the secretory pathway. In the low pH of the trans-Golgi, network (TGN) furin-mediated cleavage of prM drives maturation of the virus. **G.** Mature virus is released into the cytoplasm. Numbers shown in colored boxes refer to the pH of the respective compartments (Perera *et al*., 2008).

2.1.3 Epidemiology

2.1.3.1 Global Epidemic of Dengue Infection

In the 19th century, dengue infection was seen as a periodic disease, causing widespread infections at long intervals (Malavige *et al.*, 2004; Netsawang, 2010). However, variation in this pattern has occurred and recently dengue surpassed other infections to rank the most serious mosquito-borne viral disease in the world. In the last 50 years, its occurrence has increased 30-fold with considerable outbreaks in five of six World Health Organization (WHO) regions (Pinheiro & Corber, 1997; Malavige *et al.*, 2004).

Globally, dengue infection is endemic in more than 120 countries worldwide and about 3 billion people are estimated to be at risk of acquiring dengue infection in tropical and subtropical regions as shown in figure 2.3 (Pinheiro & Corber, 1997; Malavige *et al.*, 2004). Annually, approximately 100 million individuals suffer from self-limited an acute mild DF, while at least 500,000 incidences of DHF have been reported with 0.5% - 3.5% fatalities in Asian countries (Gubler & Clark, 1995; Malavige *et al.*, 2004). Moreover, 90% of those suffering from DHF are children less than 15 years of age (Gubler & Clark, 1995; Malavige *et al.*, 2004).

In past centuries, in tropical regions of the world, outbreaks of DF occurred every 10 to 40 years (Luplertlop, 2005). This pattern has shifted dramatically in the last century and now DF and DHF occur every 3-5 years in many countries in Southeast Asia and are currently a major public health problem in seven of them (Malavige *et al.*, 2004). Epidemics of DF have been reported in the early 1900s, primarily in South Africa

(Gubler & Kuno, 1997), as well as in Yemen in the 1870s (Carey, 1971), and in Mediterranean. In the Southeast Asian countries, the number of outbreaks increased during and after the II World War (Gubler, 1998). However, since then, the situation had deteriorated, as there was a dramatic rise in frequency and in geographic extension of DF into Latin America and Brazil (Monath, 1994; Bozza *et al.*, 2008). In the America, only few cases had been reported until the early 1980s, when a large outbreak in Cuba marked the start of epidemic spread to the Pacific and the American tropics (Monath, 1994).

The first outbreak of DHF in Asia was recorded in Manila, Philippines, in 1953-1955 (Chaturvedi & Nagar, 2008), followed by an outbreak in Thailand in 1958. DHF epidemics in Singapore, Malaysia, and Vietnam were also reported in 1960s (Teo *et al.*, 2009). The incidence of DHF has increased dramatically in recent years with approximately five times more cases reported since 1980 than in the previous 30 years (Gubler & Meltzer, 1999).

DENV infection now causes more illnesses and deaths than any other arboviral illness and has become a significant cause of morbidity and mortality in some parts of the world. Both DF and DHF affect people of all ages, with some studies showing highest incidence rates among infants and elderly (Rigau-Pérez *et al.*, 1998; García-Rivera & Rigau-Pérez, 2003). DHF mostly affects children under 15 years of age and women are more susceptible to the infection compared to men (Lye *et al.*, 2010). The death rate decreases with increasing age, in particular above age of 50 (Guzmán *et al.*, 2002). The risk that a child will die during a secondary infection is nearly 15- fold higher compared to adults (Lye *et al.*, 2010).

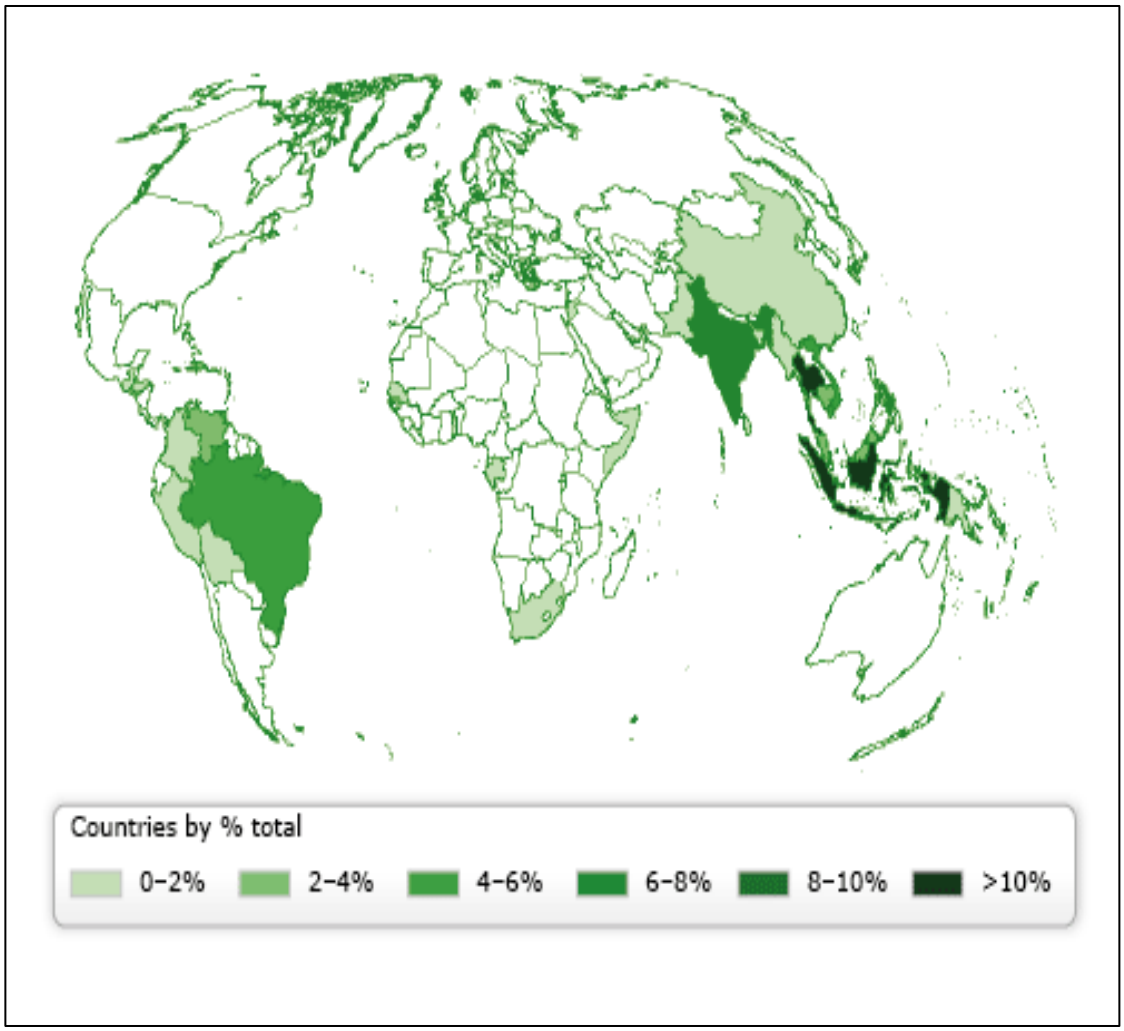

Figure 2.3: Countries and areas at risk of dengue transmission (Jelinek, 2009)

2.1.3.2 Dengue Infection in Malaysia

In Malaysia, a century after its first reported occurrence in 1902 in Penang, dengue continues to be a serious health threat (Skae, 1902). It has become one of the major public health problems in both urban and suburban areas of Malaysia, especially after the emergence of DHF in 1962 (Rudnick *et al.*, 1965). In 1982, the country underwent vast spread of the disease, with 3005 cases, of which 28.4% were cases of DHF with 35 deaths (Fang *et al.*, 1984). Last available data suggested that the major DF and DHF outbreaks in Malaysia follow a periodic pattern, alternating every eight years (Bakar & Shafee, 2002). However, the frequency of these outbreaks had now changed to occur yearly and most cases are associated with severe complications (Senior, 2007).

According to the data from Ministry of Health (MOH), all the states in Peninsular of Malaysia were evenly and similarly affected by the outbreaks. During the past 10 years, there has been a dramatic increase in DENV infections cases and the number of deaths (Figures 2.4 and 2.5.) Thus, the disease greatly affected the health, social and even economic life throughout the country.

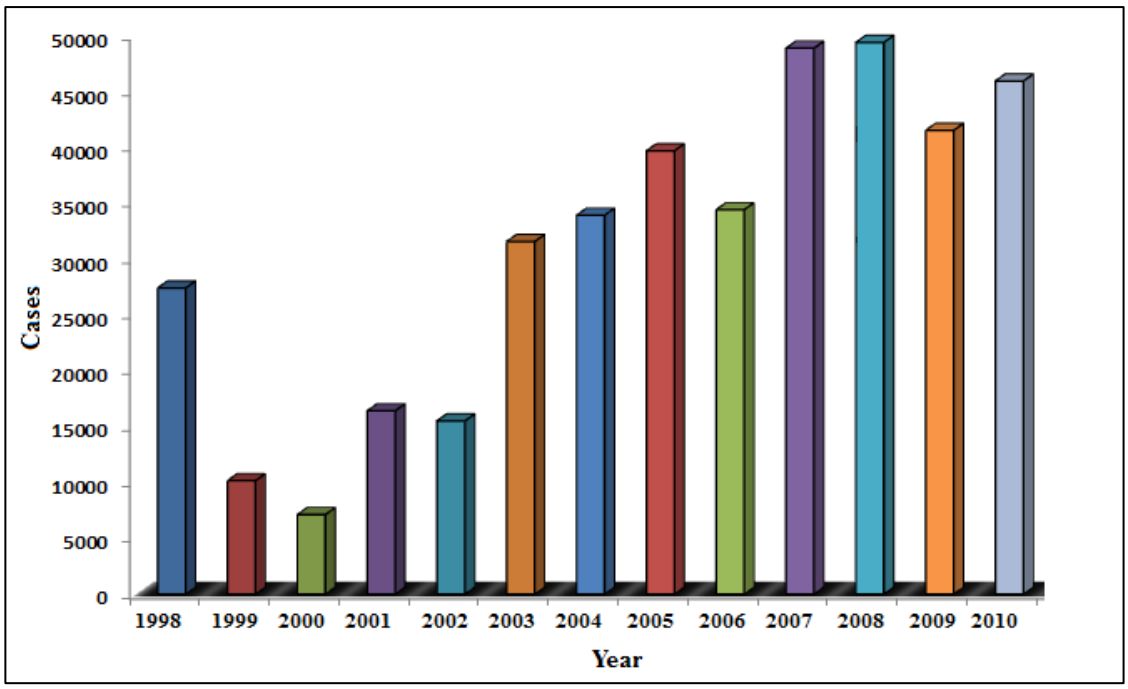

Figure 2.4: Dengue cases within the last decade recorded among Malaysian population (MOH, 2010)

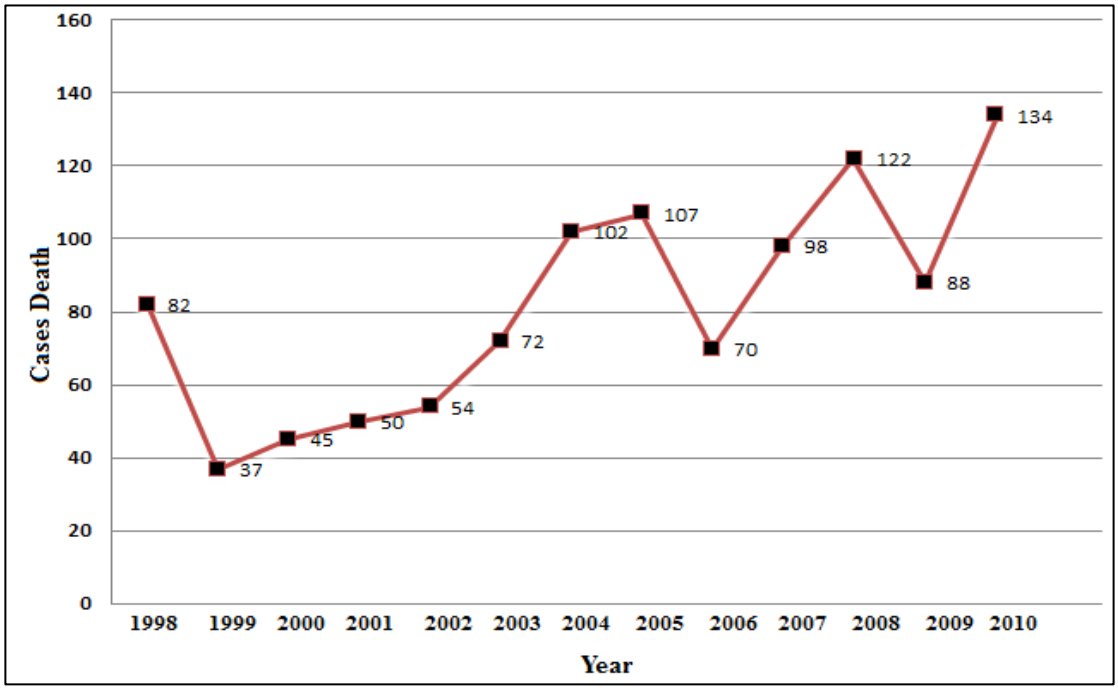

Figure 2.5: Dengue death cases during the last decade among Malaysian population (MOH, 2010)

2.1.4 Clinical Manifestations of Dengue Virus Infection

2.1.4.1 Dengue Fever (DF)

DF is an acute febrile illness characterized by the abrupt onset with a high fever between 39 and 40 ^{o}C that tends to last for 3-7 days. The fever is usually accompanied by severe malaise, headache, retro-orbital pain, myalgia, nausea, vomiting, epigastric pain, lymphadenopathy, weakness, and diarrhoea (Platt *et al.*, 1997). In children, sore throat and abdominal pain are prevalent; thereafter, defervescence occurs between days 3 and 8, and is usually followed by minor haemorrhagic phenomena (petechiae, purpura, epistaxis, gum bleeding, and menorrhagia) and the occurrence of a maculopapular (Gubler, 1998; Rigau-Pérez *et al.*, 1998).

In most cases, DF is self-limiting and the patient usually convalesces from the symptoms without complications 10 days after the onset of disease. Severe symptoms, such as haemorrhagic manifestations, are rare in DF patients, ranging from mild to severe in some cases, and are not restricted to only dengue haemorrhage fever (Gubler, 1998; Rigau-Pérez *et al.*, 1998). Laboratory findings include leukopenia and thrombocytopenia and a positive result of the tourniquet test (Gubler, 1998; Rigau-Pérez *et al.*, 1998).

2.1.4.2 Dengue Haemorrhagic Fever (DHF)/ Dengue Shock Syndrome (DSS)

DHF is a more severe form of dengue fever and mostly affects children under 15 years, although it may also occur in adults (Lye *et al.*, 2010). It is manifested as high fever, haemorrhage, increased vascular permeability, hepatomegaly and marked

thrombocytopenia (Gubler, 1998; Rigau-Pérez *et al.*, 1998). Patients may also present with Vessele's leakage, which leads to plasma discharge, haemoconcentration, low pulse pressure, hypotension, heart failure, and shock, resulting in DSS (Gubler, 1998; Rigau-Pérez *et al.*, 1998). High serum levels of viral progeny, pro-inflammatory and ant-inflammatory cytokines had been associated with DHF(Avirutnan *et al.*, 2006).

World Health Organization (WHO) grouped DHF into four grades. DHF grades 1 and 2 are distinct from classical DF due to the affected individuals developing thrombocytopenia, hepatomegaly, and haemoconcentration(Gubler, 1998). DHF grades 3 and 4 are classified as DSS, which is a more life-threatening dengue disease characterized by failure circulation due to a rapid and weak pulse, low pressure or hypotension with cold clammy skin and restlessness (Gubler, 1998).

The dangerous phase of DSS is characterized by circulation failure that may occur at 24 hours before to 24 hours after the temperature falls to or below normal level (Dejnirattisai, 2004). The crisis usually lasts for 24 to 36 hours and the patients recover rapidly once convalescence starts (Dejnirattisai, 2004). The prognosis in DSS depends on prevention, early diagnosis and treatment of shock (Umareddy, 2009). Once a shock has set in, the fatality rate may be as high as 12 to 44% (Umareddy, 2009). The fatality among DSS patients is usually 25-50% or higher if not properly treated, and less than 5% of such poor cases succumb to the disease, though recovery is rapid and without sequelae (Nimmannitya *et al.*, 1987).

2.1.5 Dengue Pathogenesis

DENV is introduced into a human host through the bite of an infected mosquito, whereby the virus establishes infection by replication in Langerhans cells and dentritic cells (DC) (Wu *et al.*, 2000). It is subsequently disseminated and replicates via monocytes/macrophages (Wu *et al.*, 2000). Pathogenesis of dengue disease depends upon a number of factors, such as viral virulence, antibody-dependent enhancement (ADE) (Halstead, 2007) and a number of host-specific factors that include age, race/ethnicity, genetic status, cytokines, and cellular immune response (Noisakran & Perng, 2008). The factors identified as responsible for the development of serious dengue diseases are viral virulence and abnormal host immune responses to infection (Sakuntabhai *et al.*, 2005).

2.1.5.1 Viral Virulence

A number of researchers have studied the possible relationship between particular DENV serotypes and the severity of disease outcome, some reports indicating that DENV2 and DENV3 serotypes may cause more serious disease than the other serotypes, while DENV4 is responsible for a milder illness (Clyde *et al.*, 2006; Mathew & Rothman, 2008). It is possible that certain genotypes within specific serotypes have also been associated with more severe disease of DHF. Generally, all Asian serotypes appear to be more virulent compared to those found in the Americas and the South Pacific (Clyde *et al.*, 2006). According to phylogenetic analyses, DF is caused by the Native American DENV2 genotype, whereas the Asian DENV2 genotypes are associated with DHF (Cologna & Rico-Hesse, 2003).

Moreover, it was shown that the replication of Asian DENV2 genotype resulted in higher titers in human monocyte/macrophages and dentritic cells (DCs) compared to the American genotype (Rodenhuis-Zybert *et al.*, 2010). Furthermore, when the ability of American and Asiatic lineage‘s genotypes to infect several population of *Aide's aegypti* was analyzed, it was demonstrated that the overall infection rates were higher for the Asiatic DENV2 genotypes, so that the latter may be more transmittable (Armstrong & Rico-Hesse, 2003; Rodenhuis-Zybert *et al.*, 2010).

Maturation of DENV appears to be unproductive as dengue-infected mosquito and mammalian cells have been shown to secrete large numbers (up to 30%) of prM-containing particles that play a role in dengue pathogenesis (Yu *et al.*, 2008; Zybert *et al.*, 2008; Rodenhuis-Zybert *et al.*, 2010). Numerous studies have demonstrated that not fully mature particles lack the ability to infect cells and therefore, these particles are generally believed to be of minor importance in DENV pathogenesis (Rodenhuis-Zybert *et al.*, 2010).

2.1.5.2 Antibody-Dependent Enhancement (ADE) Infection

Antibodies against dengue viruses play a significant role in the development of infection. Recently, several studies have shown a positive relationship between peak viremia titer and disease severity in humans, supporting the idea of the potential importance of ADE in enhancing dengue pathogenesis (Libraty *et al.*, 2002).

Following a primary DENV infection individuals typically develop cross-reactive immune responses of two to three months in duration (Luplertlop, 2005). On the other hand, throughout a secondary infection with a different DENV serotype, a

preexisting, non-neutralizing, cross-reactive heterologous antibody recognizes the heterological infecting virus and forms an antigen-antibody complex (Luplertlop, 2005). The complex is bound to and internalized by immunoglobulin-FcR on the cell membrane of infected monocytes/macrophages (Luplertlop, 2005; Wahala & de Silva, 2011). As the antibody is heterologous, the heterologous virus is not neutralized and is free to replicate inside a monocytes. This mechanism, known as ADE, enhances the infection and replication of dengue virus in monocytes, resulting in the increasing number of dengue-infected cells and levels of viremia, leading to DHF/DSS (Luplertlop, 2005; Martina *et al.*, 2009; Wahala & de Silva, 2011).

The occurrence of DHF during primary dengue virus infection in the first year of life in children who have acquired antibody against dengue viruses transplacentally from dengue-immune mothers also supports the idea of an *in vivo* role for ADE (De Rivera *et al.*, 2008).

2.1.5.3 Cellular Immune Response in Dengue Virus Infection

At present, the focus of the researchers has shifted towards studying aspects of cell-mediated immune responses in the pathogenesis of DHF (Malavige *et al.*, 2004). The DENV can infect CD4+ and CD8+ T-cell lines *in vitro* (Malavige *et al.*, 2004; Nielsen, 2009), even though there is no evidence of CD4+ and CD8+ T-cells serving as a target for dengue virus *in vivo* (Theofilopoulos *et al.*, 1976). There is strong evidence that a high level of T-cell activation is associated with DHF (Green *et al.*, 1999a). Moreover, primary infection induces activation of both serotype-specific and serotype-cross-reactive T-cells against DENV infection. On secondary infection, CD4+ and

CD8+ T-cells were shown to enhance severity of infection by producing various cytokines (Kurane & Ennis, 1994). Thus, enhanced cytokine production indicates severe pathogenesis of DHF/DSS (Chaturvedi *et al.*, 2005).

2.1.5.4 Cytokine Response in Dengue Infection

The immune system maintains the physiological integrity of the body mainly by eradicating foreign material and infectious pathogens (Chaturvedi *et al.*, 2004). During the process of virus establishing an infection, the host responds by initiating a complex defence system by means of phagocytic cells, in order to eradicate and clear the virus from the host (Matsukawa *et al.*, 2000).

The most severe forms of dengue disease are associated with high viral titers and cytokines. Both host genetic determinants and virus characteristics contribute to viral replication; however, rapid replication during the short viraemic period of acute infection can be achieved only if innate immunity, which represents the first line of host defence against pathogens, is delayed or inhibited (Mazzon, 2010). The more efficient the evasion of innate immunity, the higher will be the viral titer and cytokine production, and the more severe the disease outcome (Mazzon, 2010).

Phagocyte cells that serve as the first line of defence in innate immunity play important role in neutralizing and eliminating pathogens (Matsukawa *et al.*, 2000). In contrast, the adaptive immune response is responsible for permanent protection, taking days to develop antigen-specific T-cell receptor (cell-mediated immunity) and immunoglobulin (Esche *et al.*, 2005).

During an infection, both innate and adaptive immune responses play a key role in cytokine and chemokine release (Esche *et al.*, 2005). Inflammatory and anti-inflammatory cytokines might be released either directly from infected monocytes or after interactions with immune cells (Esche *et al.*, 2005; Luplertlop, 2005). Consequently, a number of cytokines are produced, and the complex network of induction further increases the cytokine levels, resulting in the increased vascular permeability, plasma leakage, shock, and the coagulation system dysfunction, which may lead to DHF/DSS (Luplertlop, 2005; Nielsen, 2009).

2.1.5.4.1 Role of Cytokines in Pathogenesis of DF and DHF

Cytokines are groups of soluble proteins with low molecular weight that are produced by different immune cells—autocrine and paracrine regulators—that affect and regulate the activity of target cells (Berczi & Szentivanyi, 2003). In DENV infection, cytokines play an important role in the disease severity, homeostasis regulation (Bozza *et al.*, 2008) and immunopathogenesis of the DENV infection (Chen & Wang, 2002).

In fact, all immune-system cells seem to be activated during dengue infection and produce cytokines, such as T-cells producing helper Th1 and Th2 cytokines during DENV infection (Rabablert, 2005). Th1 cells produce IFN-γ, IL-2 and TNF-α, which are responsible for cell-mediated inflammatory reactions and tissue injury infection (Rabablert, 2005). Th2 cells produce IL-4, IL-5, IL-6, IL-10 and IL-13, which help B-cells to secrete antibodies (Chaturvedi *et al.*, 2000; Rabablert, 2005). The cytokine profiles produced in patients with DF include IFN-γ and IL-2, as well as slightly increased levels of IL-4, IL-6 and IL-10, which is a typical Th1-type response