THE EFFECT OF *Hydrocotyle sibthorpoides* Lam.

EXTRACT ON DENGUE VIRUS 2 INHIBITION *In Vitro*

FITRIEN BINTI HUSIN

UNIVERSITI SAINS MALAYSIA

2015
THE EFFECT OF *Hydrocotyle sibthorpioides* Lam.

EXTRACT ON DENGUE VIRUS 2 INHIBITION *In Vitro*

by

FITRIEN BINTI HUSIN

Thesis submitted in fulfilment of the requirements for the Degree of

Master of Science

(Microbiology)

September 2015
ACKNOWLEDGEMENT

In the name of Allah, the most Merciful and Compassionate, had it not been due to His will and favour, the completion of this study would not been possible.

First and foremost, I would like to express my deepest gratitude and greatest appreciation to my supervisor, Dr. Rafidah Hanim Shueb, whom I cannot thank enough for her continuous assistance and advice during the whole study, co researchers, Prof. Siti Amrah Sulaiman, Prof. Gan Siew Hwa, Associate Prof. Dr. Chan Yean Yean, and Dr. Nabilah Ismail for their time and continuous supports.

To my beloved parent, family and husband (Mohd Salihin Selamat) whose irreplaceable, no words can describe on how much I adore their love, never ending concerns and supports throughout my life.

My sincere and special thanks to staff of Department of Medical Microbiology & Parasitology and Department of Pharmacology, especially Mr. Chan and Mr. Owi who helps me in offering the facilities for the cell culture work.

Lastly, for my colleagues, Nurul Izzati, Tg. Ahmad Akram, Om Prakash, Mohd Lukman, Nik Zuraina and Adila, Amalina, Izzah and other students, bunch thanks for their immeasurable assistance, cooperation and encouragement. May Allah bless them all. Ameen.

I hope this thesis will be a useful reference in the future in assisting other study or planning further work.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>ACKNOWLEDGEMENT</th>
<th>II</th>
</tr>
</thead>
<tbody>
<tr>
<td>TABLE OF CONTENTS</td>
<td>III</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>XI</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>XII</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>XV</td>
</tr>
<tr>
<td>ABSTRAK</td>
<td>XVII</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>XX</td>
</tr>
<tr>
<td>CHAPTER 1: LITERATURE REVIEW</td>
<td></td>
</tr>
<tr>
<td>1.1 Overview of dengue</td>
<td>1</td>
</tr>
<tr>
<td>1.1.1 Disease burden</td>
<td>1</td>
</tr>
<tr>
<td>1.1.2 Epidemiology</td>
<td>4</td>
</tr>
<tr>
<td>1.1.3 Dengue virus</td>
<td>6</td>
</tr>
<tr>
<td>1.1.4 Genome structure and viral protein</td>
<td>6</td>
</tr>
</tbody>
</table>
1.1.4.1 NS-1 protein

1.1.5 Virus replication

1.2 Dengue transmission

1.2.1 The vector

1.3 Clinical features of dengue

1.4 Dengue case classification

1.4.1 The 1997 dengue case classification

1.4.2 The 2009 dengue case classification

1.5 Host immune response

1.5.1 Primary and secondary dengue infections

1.6 Laboratory diagnosis of dengue

1.7 Management and prevention of dengue

1.8 Antiviral study

1.8.1 Model of infection

1.8.1.1 Animal model
1.8.1.2 Cell culture 29

1.9 Medicinal plants for treatment of dengue 31

1.9.1 Phytochemicals with anti-dengue activity 37

1.9.2 Hydrocotyle sibiriphioides Lam. 40

1.10 Rationale and objectives of the study 41

CHAPTER 2: MATERIALS AND METHODS

2.1 Chemicals and reagents 43

2.2 Preparation of plant extracts 44

2.2.1 Plant collection 44

2.2.2 Plant extraction 44

2.3 Preparation of media 46

2.3.1 Growth media 46

2.3.2 Foetal bovine serum (FBS) 46

2.3.3 Tryptose phosphate broth (TPB) 47

2.3.4 Trypsin EDTA 47
2.3.5 Antibiotic

2.3.6 Phosphate buffer saline (PBS)

2.3.7 L15 complete growth media

2.3.8 DMEM complete growth media

2.3.9 Overlay medium

2.3.10 Cryopreservation medium

2.4 Cell lines preparation

2.4.1 C6/36 cells

2.4.2 Vero cells

2.4.3 HepG2 cells

2.4.4 PscloneD cells

2.5 Cryopreservation of cells

2.6 Retrieval of cell lines from liquid nitrogen

2.7 Cell counting

2.8 Dengue virus
2.8.1 Virus propagation 53

2.8.2 Multiplicity of infection (m.o.i) 54

2.8.3 Viral growth characteristics 54

2.9 Antiviral assay 55

2.9.1 Determination of maximum non-toxic dose (MNTD) 56

2.9.2 Pre-treatment of cells prior to DENV-2 infection 57

2.9.3 Post treatment of cells after DENV-2 infection 57

2.9.4 Virucidal treatment 58

2.9.5 Concurrent treatment 58

2.10 Microscopy observation 58

2.11 Plaque assay 59

2.11.1 Preparation of methylene blue staining 60

2.12 Immunofluorescence assay 60

2.12.1 Preparation of antibody 60

2.13 NS-1 ELISA 61
2.13.1 Standard curve for NS-1 protein concentration 61

2.14 RNA extraction 62

2.14.1 Isolation of viral RNA from cell culture 62

2.14.2 Isolation of viral RNA from cell culture supernatants 62

2.15 Quantitative RT-PCR 63

2.16 Primers 64

CHAPTER 3: RESULTS

3.1 Morphology of healthy cell lines 65

3.2 Presentation of CPE in various cell lines 67

3.3 Kinetics of DENV replication in C6/36 and Vero cells 70

3.4 Determination of MNTD of *H. sibthorpioides* water and methanol extracts on cell lines 77

3.5 Antiviral activity of *H. sibthorpioides* extracts on Vero and C6/36 cells 82

3.5.1 Pre-treatment prophylactic effect of *H. sibthorpioides* plant extracts on Vero and C6/36 cells 82

3.5.1.1 Morphological changes of Vero and C6/36 cells 83
3.5.1.2 IFA analysis

3.5.1.3 Dengue viral titres

3.5.2 Post treatment therapeutic effect of *H.sibthorpioides*

plant extracts on Vero and C6/36 cells

3.5.2.1 Morphological changes of Vero and C6/36 cells

3.5.2.2 IFA analysis

3.5.2.3 Dengue viral titres

3.6 Antiviral of *H.sibthorpioides* plant extracts on HepG2 cells

3.6.1 NS-1 protein expression

3.6.2 Morphological changes of HepG2 cells

3.6.3 Dengue viral titres

3.6.3.1 Virucidal effect of *H.sibthorpioides* plant extracts on HepG2 cells

3.6.3.2 Concurrent treatment effect of *H.sibthorpioides*

plant extracts on HepG2 cells

3.6.3.3 Post treatment therapeutic effect of

*H.sibthorpioides* plant extracts on HepG2 cells
CHAPTER 4: DISCUSSION  124

CHAPTER 5: CONCLUSION  135

REFERENCES  136

APPENDICES  152
LIST OF TABLES

Table 1.1  Estimated burden of dengue in 2010, by continent  3

Table 1.2  Traditional plants that had been identified to have anti-dengue activity  33

Table 1.3  Overview of some phytochemicals having anti-dengue properties along with their sources  34

Table 1.4  Plant classification of Hydrocotyle sibthorpioides Lam.  43

Table 2.1  Chemicals and reagents used in the study  43

Table 3.1  Morphological changes in DENV-2 infected Vero cells pre-treated with H.sibthorpioides water and methanol extracts  84

Table 3.2  Morphological changes in DENV-2 infected C6/36 cells pre-treated with H.sibthorpioides water and methanol extracts  85

Table 3.3  Morphological changes in DENV-2 infected Vero cells post treated with H.sibthorpioides water and methanol extracts  98

Table 3.4  Morphological changes in DENV-2 infected C6/36 cells post treated with H.sibthorpioides water and methanol extracts  99

Table 3.5  Morphological changes in DENV-2 infected HepG2 cells treated with H.sibthorpioides water and methanol extracts  115
### LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Countries/ areas at risk of DENV transmission (dark-grey shading)</td>
<td>5</td>
</tr>
<tr>
<td>1.2</td>
<td>Schematic representation of the organisation of the genes for Flavivirus</td>
<td>7</td>
</tr>
<tr>
<td>1.3</td>
<td>Dengue virus replication</td>
<td>11</td>
</tr>
<tr>
<td>1.4</td>
<td>Life cycle of <em>Aedes aegypti</em> from egg to adult mosquito</td>
<td>14</td>
</tr>
<tr>
<td>1.5</td>
<td>Manifestations of dengue virus infection</td>
<td>18</td>
</tr>
<tr>
<td>1.6</td>
<td>The 2009 revised dengue case classification</td>
<td>20</td>
</tr>
<tr>
<td>1.7</td>
<td>An immune response to dengue infection</td>
<td>23</td>
</tr>
<tr>
<td>1.8</td>
<td>Images of densely populated <em>Hydrocotyle sibthorpioides</em> showing long creeping stems and scalloped leaf edges</td>
<td>39</td>
</tr>
<tr>
<td>2.1</td>
<td>Schematic view of Soxhlet apparatus</td>
<td>45</td>
</tr>
<tr>
<td>3.1</td>
<td>Morphology of confluent healthy (A) C6/36 cells (B) Vero cells (C) HepG2 cell and (D) PscloneD cells monolayer grown in T25cm² tissue culture flask viewed under inverted microscope (220 x magnification)</td>
<td>66</td>
</tr>
<tr>
<td>3.2</td>
<td>Changes in C6/36 cell morphology following DENV-2 Infection</td>
<td>68</td>
</tr>
<tr>
<td>3.3</td>
<td>Replication of different DENV serotypes in C6/36 cells</td>
<td>73</td>
</tr>
<tr>
<td>3.4</td>
<td>Replication of different DENV serotypes in Vero cells</td>
<td>75</td>
</tr>
</tbody>
</table>
Figure 3.5  (A) Morphology of Vero cells from incubation within MNTD concentrations range and (B) the toxicity effect of *H. sibthorpioides* extract on Vero cells above the MNTD range of concentrations

Figure 3.6  Determination of maximum non toxicity dose of *H. sibthorpioides* water and methanolic extracts in (A) Vero cells (B) C6/36 cells and (C) HepG2 cells as determined by MTS assay

Figure 3.7  Effect of *H. sibthorpioides* extracts on DENV-2 replication in pre-treatment assay

Figure 3.8  Effect of *H. sibthorpioides* extracts on DENV-2 replication in pre-treatment assay

Figure 3.9  DENV-2 infectivity in Vero cells pre-treated with (A) water extract and (B) methanolic extract of *H. sibthorpioides*

Figure 3.10  DENV-2 infectivity in C6/36 cells pre-treated with (A) water extract and (B) methanolic extract of *H. sibthorpioides*

Figure 3.11  Effect of *H. sibthorpioides* extract on DENV-2 replication in post treatment assay

Figure 3.12  Effect of *H. sibthorpioides* extract on DENV-2 replication in post treatment assay

Figure 3.13  DENV-2 infectivity in Vero cells post treated with (A) water extract and (B) methanolic extract of *H. sibthorpioides*

Figure 3.14  DENV-2 infectivity in C6/36 cells post treated with (A) water extract and (B) methanolic extract of *H. sibthorpioides*

Figure 3.15  Standard curve for NS-1 protein level

Figure 3.16  NS-1 protein expression in post treated HepG2 cells with *H. sibthorpioides* (A) water extract and (B) methanolic extract
Figure 3.17  Virucidal activity of *H. sibthorpioides* extracts on DENV-2 infectivity  118

Figure 3.18  Concurrent treatment of *H. sibthorpioides* extracts on DENV-2 infectivity  120

Figure 3.19  Post treatment effect of *H. sibthorpioides* extracts on DENV-2 infectivity  123
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMC</td>
<td>carboxymethyl cellulose</td>
</tr>
<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>CPE</td>
<td>cytopathic effect</td>
</tr>
<tr>
<td>DENV</td>
<td>dengue virus</td>
</tr>
<tr>
<td>DF</td>
<td>dengue fever</td>
</tr>
<tr>
<td>DHF</td>
<td>dengue haemorrhagic fever</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DSS</td>
<td>dengue shock syndrome</td>
</tr>
<tr>
<td>EC₅₀</td>
<td>effective concentration</td>
</tr>
<tr>
<td>FBS</td>
<td>foetal bovine serum</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>HBV</td>
<td>hepatitis B virus</td>
</tr>
<tr>
<td>HSV</td>
<td>herpes simplex virus</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>inhibitory concentration</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>L15</td>
<td>leibovitz media</td>
</tr>
<tr>
<td>m.o.i</td>
<td>multiplicity of infection</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffer saline</td>
</tr>
<tr>
<td>PES</td>
<td>polyethersulfone</td>
</tr>
</tbody>
</table>
pfu  particle forming unit
RNA  ribonucleic acid
TNF  tumor necrosis factor
TPB  tryptose phosphate broth
WHO  World Health Organisation

dengan pengesanan virus antigen menggunakan IFA dan seterusnya penilaian titer virus menggunakan asai plak dan qRT-PCR.


THE EFFECT OF Hydrocotyle sibthorpioides Lam. EXTRACT ON DENGUE VIRUS 2 INHIBITION In Vitro

ABSTRACT

For thousands of years the practice of traditional medicine, using plant and herbs, has been effective in the treatment of various infections. Despite decades of efforts, there is no proven effective antiviral for DENV infection and attempts at vaccine development have been hampered by several major obstacles e.g. different serotypes. The aim of this study therefore was to investigate the potential antiviral activity of Hydrocotyle sibthorpioides Lam. (*H. sibthorpioides*) towards DENV-2 infection *in-vitro* in three cell lines, Vero cells, C6/36 cells and HepG-2 cells.

The *H. sibthorpioides* was extracted using two different solvents, water and methanol. Toxicity tests were initially performed to evaluate and determine the highest tolerable dose of *H. sibthorpioides* extracts in the tested cell lines. The *H. sibthorpioides* methanolic extract was found to be more toxic than the water extract. Vero cells had a wider tolerance range to *H. sibthorpioides* extracts followed by C6/36 and HepG-2 cells. Antiviral assays were performed in two different stages, pre- and post-treatment to evaluate the *H. sibthorpioides* prophylactic and therapeutic effects on DENV-2 replication. The activities were scored by observing the morphological changes and CPE appearances, followed by detection of viral antigen by IFA and quantitation of viral titres by either plaque assay or qRT-PCR.
The results demonstrated that *H. sibthorpioides* extracts possess mild prophylactic activity against DENV-2 replication in Vero cells but not in C6/36 cells. Pre-treatment of Vero cells showed that *H. sibthorpioides* pretreatment had 2% - 44% protective effect against DENV-2 in Vero cells using water extract, while 18% - 30% protective effect were seen when *H. sibthorpioides* methanolic extract was used. On the contrary, in general, *H. sibthorpioides* pre-treatment on C6/36 cells had low to no cellular protective effect against DENV-2 infection. Only few concentrations of *H. sibthorpioides* caused 5% - 13% inconsequential inhibition on C6/36 cells.

The post treatment of DENV-2 infected-Vero cells with *H. sibthorpioides* methanolic extract presented higher therapeutic effect when compared with the *H. sibthorpioides* water extract. The DENV-2 viral replication was inhibited by 6% - 31% when various concentrations of *H. sibthorpioides* water extract were used, while 2% – 42% of DENV-2 titre reduction was seen in Vero cells post treated with *H. sibthorpioides* methanolic extract. Surprisingly, post treatment of C6/36 cells resulted in an enhancement of DENV-2 replication. An enhancement effect of DENV-2 by 7% - 59% was seen in infected-C6/36 cells post treated with *H. sibthorpioides* water and methanolic extracts.

The antiviral assay was further extended in HepG-2 cells and the results demonstrated that the methanolic extract of *H. sibthorpioides* was better than the water extract in reducing DENV-2 replication. Post treatment of *H. sibthorpioides* extracts against DENV-2 showed more potent antiviral activity followed by virucidal treatment and concurrent treatment in HepG2 cells. As a conclusion, *H. sibthorpioides* had variable effects on DENV-2 replication, depending on treatment types, solvent types and cell lines.
used; providing important novel insight on the phytomedicinal properties of the plant on DENV. Further studies are needed to verify which compounds could be responsible and how they exert their antiviral effects.
CHAPTER ONE
LITERATURE REVIEW

1.1 Overview of dengue

Dengue fever (DF), the most prevalent arthropod-borne viral illness in humans, is caused by dengue virus (DENV). Dengue is a one disease entity with different clinical presentations and often with unpredictable clinical evolution and outcomes (WHO, 2009). Originally, dengue virus infections occurred mainly as epidemics in tropical and subtropical countries, but over time, with increasing globalisation and the geographic spread of inhabitants of *Aedes aegypti* and *Aedes albopictus* mosquitos, dengue virus infection has steadily penetrated every corner of the world (Noisakran *et al.*, 2010).

1.1.1 Disease burden

Dengue is a worsening global health problem. In 19th century, dengue was considered a sporadic disease that caused epidemics at long intervals, a reflection of the slow pace of transport and limited travel at that time and today in contrast, dengue ranks as the most important mosquito-borne viral disease in the world (WHO, 2012). According to the World Health Organization report, there are 50 million dengue infections and 500 000 cases of dengue haemorrhagic fever (DHF) leading to hospitalisation each year.

In Malaysia, dengue cases have risen alarmingly across the country this year, with data showing a 269% rise in the disease from January till May 2014 as compared to the same period last year (KKM, 2014). The number of cases reported annually to WHO ranged from 0.4 to 1.3 million in the decade 1996 – 2005. Underreporting and misdiagnoses are major obstacles to understanding the full burden of dengue and as an
infectious disease, the number of cases varies substantially from year to year (WHO, 2009).

Bhatt et al. (2013) estimated that there were 96 million apparent dengue infections in 2010 (Table 1.1) with Asia bore 70% of this burden, and is characterised by large swathes of densely populated regions coinciding with very high suitability for disease transmission. Recent studies have estimated annual economic burden of dengue in specific countries of Southeast Asia using the average reported cases between 2001 – 2005, in Cambodia, Malaysia and Thailand were at least US$ 3.1 (± 0.2), US$ 42.4 (± 4.3), and US$ 53.1 (± 11.4) million respectively (Shepard et al., 2013). Dengue inflicts all levels of society but the burden may be higher among the poorest who grow up in communities with inadequate water supply and solid waste infrastructure (WHO, 2009).
Table 1.1: Estimated burden of dengue in 2010, by continent (adapted from Bhatt et al. (2013))

<table>
<thead>
<tr>
<th>Continent</th>
<th>Apparent Millions (credible interval)</th>
<th>Inapparent Millions (credible interval)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Africa</td>
<td>15.7 (10.5 – 22.5)</td>
<td>48.4 (34.3 – 65.2)</td>
</tr>
<tr>
<td>Asia</td>
<td>66.8 (47.0 – 94.4)</td>
<td>204.4 (151.8 – 273.0)</td>
</tr>
<tr>
<td>Americas</td>
<td>13.3 (9.5 – 18.5)</td>
<td>40.5 (30.5 – 53.3)</td>
</tr>
<tr>
<td>Oceania</td>
<td>0.18 (0.11 – 0.28)</td>
<td>0.55 (0.35 – 0.82)</td>
</tr>
<tr>
<td>Global</td>
<td>96 (67.1 – 135.6)</td>
<td>293.9 (217.0 – 392.3)</td>
</tr>
</tbody>
</table>
1.1.2 Epidemiology

More than 100 countries are endemic, primarily affecting 2.5 billion inhabitants in the tropical and subtropical region (Figure 1.1) (WHO, 2009). It is estimated that about 2.5 billion individuals, a staggering 40% of the world population, inhabit areas where there is a risk of transmission of DF and that disease burden has increased at least fourfold in the last three decades (Guzman and Istúriz, 2010).

Dengue virus sustains in Asia and Africa probably through vertical transmission in mosquitoes and with regular amplification in non-human primates. The vector has adapted itself to survive in close vicinity to human settlement and is found predominantly between latitudes 35° N and 35° S throughout the globe (Kumar et al., 2010).

Knowledge of the geographical distribution and burden of dengue is essential for understanding its contribution to global morbidity and mortality burdens, in determining how to allocate optimally the limited resources available for dengue control, and in evaluating the impact of such activities internationally (Bhatt et al., 2013).

The factors responsible for the increasing importance of dengue as a global public health problem in the last decades are associated with demographic and social changes including unprecedented global population growth and the associated unplanned and uncontrolled urbanization, especially in tropical developing countries. Substandard housing, crowding, and deterioration in water, sewer, and waste management systems, intimately associated with unplanned urbanization have created ideal conditions for increased transmission of mosquito-borne diseases in tropical urban centres (De Paula and Fonseca, 2004).
Figure 1.1: Countries/areas at risk of DENV transmission (dark-grey shading). The contour lines indicate the potential geographical limits of the northern and southern hemispheres for year-round survival of *Aedes aegypti*, the principal mosquito vector of DENVs (adapted from WHO (2009)).
1.1.3 Dengue virus

DENV, the causative agent of dengue is a member of the family Flaviviridae with four distinct serotypes, dengue serotype 1, 2, 3 and 4 (DENV 1-4). It is a small, enveloped virus that contains a single-stranded, positive sense (messenger) RNA genome packaged inside a core protein, which is surrounded by an icosahedral scaffold and encapsidated by a lipid envelope (Noisakran et al., 2010). All four serotypes of DENV can be found worldwide.

The relationships between the serotypes and transmission efficiency or disease expression are uncertain, but DENV-2 and DENV-3 are likely to contribute the most to disease severity and mortality (Guzman and Istúriz, 2010). Studies on the outbreaks in endemic areas, such as South East Asia revealed that a primary infection with DENV-1 or DENV-3 frequently resulted in a more severe disease than if DENV-2 or DENV-4 were the primary infection (Tang et al., 2012). Dengue virus also causes a wide range of clinical manifestations ranging from inapparent or mild febrile illness to severe and fatal haemorrhagic disease.

1.1.4 Genome structure and viral proteins

The distinction between dengue serotypes is based on their antigenicity. Flavivirus particles are spherical in shape, with a lipid envelope approximately 50 nm in diameter (Halstead, 2008). The genome is approximately 11 000 base pairs long, with 5’ capped and 3’-end usually not polyadenylated. The 5’ and 3’ non-coding regions are important for regulating viral replication. The termini of the genome contain untranslated regions (UTRs) that have key roles in the regulation of translation and genome replication. The 5’
UTR is relatively short and has a type I cap structure; the 3’ UTR contains several conserved RNA structures and lacks a terminal polyadenate tract (Murphy and Whitehead, 2011). The gene order for structural proteins from the 5’ terminus is C-prM-E (Figure 1.2).

There are three structural proteins encoded in the 5’-one third of the viral genome: the capsid (C) protein forms the nucleocapsid shell protecting the viral genome, and the premembrane (prM), and envelope (E) proteins, both virion surface proteins embedded in the virion envelope. The main biological properties of the viruses are located in the E protein, including receptor binding, haemagglutination of erythrocytes, neutralising antibody induction, protective immune response, membrane fusion and virion assembly (Schoub and Blackburn, 2004). The M- protein, which consists of seven antiparallel β-strands, is important in the formation and maturation of the viral particles (Lai et al., 2008).

Seven non-structural (NS) proteins are encoded in the 3’-two thirds of the viral genome: NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5 (WHO, 2007). The structural proteins form the viral particle while the non-structural proteins participate in the replication of the RNA genome, virion assembly and invasion of innate immune response.

Figure 1.2: Schematic representation of the organisation of the genes for Flavivirus
1.1.4.1 NS-1 protein

NS-1 the first non-structural protein is synthesised in the rough endoplasmic reticulum as a hydrophilic, water-soluble, monomeric glycoprotein. It is a 48 000 molecular weight glycoprotein containing two signals of the type, Asn-X-Ser/Thr, used for addition of N-linked carbohydrate. The NS-1 glycoprotein is produced by all flaviviruses and is secreted from mammalian cells. Dengue virus non-structural protein-1 (NS1) is a secreted glycoprotein that is absent from viral particles but accumulates and resides in the supernatant and on the plasma membrane of cells during infection. It plays a critical role in viral RNA replication and has a central position in DENV pathogenesis.

NS1 is strongly immunogenic and type-specific anti NS1 antibodies play a role in protection against disease and produces a very strong humoral response (WHO, 2007). Some of the NS1 protein is expressed as a soluble secreted form, which has been implicated to contribute to dengue viral propagation and the amount secreted is closely related to dengue viral titre. NS1 has an important yet unclear role in RNA replication, localizes to sites of RNA replication, and mutation of the N-linked glycosylation sites in NS1 can lead to dramatic defects in RNA replication (Lindenbach and Rice, 2007).

The flavivirus nonstructural protein NS1 is expressed as three discrete species in infected mammalian cells: an intracellular, membrane-associated form essential for viral replication, a cell surface associated form that may be involved in signal transduction, and a secreted form (sNS1), the biological properties of which remain elusive. The DENV NS-1 protein has also been intensively investigated as a potential target for vaccines and antiviral drugs (Amorim et al., 2014).
1.1.5 Virus replication

The initial step of viral replication is the attachment of infectious viral particle to host cell. Recognition of the appropriate target cell and binding is mediated by viral surface or envelope proteins interacting with one or more cellular membrane proteins or other attachment factors on the plasma membrane. There are two methods by which attached virus may penetrate the host cells, the virion envelope may fuse with the plasma membrane with immediate deposition of the nucleocapsid into the cytoplasm, or the plasma membrane may invaginate, forming an endocytic vesicle around the still enveloped virus (Halstead, 2008).

Since dengue viral genome can function as mRNA, if the viral RNA can be delivered into a cell’s cytoplasm through biologically active vesicles, translation and genome synthesis can occur accordingly (Noisakran et al., 2010). Two conditions are necessary for dengue virus fusion; an acidic environment and the presence of a negatively charged membrane (Zaitseva et al., 2010). Acidic conditions have been shown to activate a fusion protein, which leads to the deposition of the nucleocapsid within the cytoplasm.

Since the dengue virus RNA genome has a positive sense, it must first be translated to make the RNA polymerase required for its replication. The polymerase transcribes the positive-strand RNA to negative-strand RNA, which then serves as template for additional positive strands. Viral RNA is then translated into a polyprotein that is processed by viral and cellular proteases and the viral NS proteins replicate the genome RNA (van Cleef et al., 2013)
Flavivirus assembly takes place at the endoplasmic reticulum (ER) where translation of the viral RNA and production of the viral polypeptide occurred. The structural glycoproteins prM and E localize to the luminal side of the ER and form an immature particle with prM and E in a heterodimeric complex. The cell’s protein synthesis produces new viral proteins that replicate the viral RNA and begin to form viral particles. Furin-mediated proteolysis of prM in the trans-Golgi network triggers rearrangement, homodimerization of E, and formation of the mature viral particle before release from the infected cell (Whitby et al., 2005).

The new mature viruses bud on the surface of the infected cell and are released by exocytosis and they are able to enter other white blood cells, such as monocytes and macrophages (Rodenhuis-Zybert et al., 2010). Released virus contain little, if any, prM; therefore, cleavage of prM must occur before or during exit from the cell. As the virus is transported through exocytic vesicle, and immediately prior to release, the prM protein is cleaved by a furin like protease to its mature M protein form, thus allowing the formation of E protein homodimer and activating the E protein for the pH dependent conformational changes which occur during subsequent attachment and entry into cells (Halstead et al., 2005). Immature, prM containing flavivirions are about 60-fold less infectious than mature virus. PrM may maintain the virion in a highly stable but relatively inert state. The final cleavage step makes the virus competent for infection but more labile. The processes of dengue viral replication have been simplified as shown in Figure 1.3.
Figure 1.3: Dengue virus replication. Virions are first adsorbed onto host cellular membranes and enter the cell by receptor mediated endocytosis. Fusion with lysosomes release the nucleocapsid, which disassembles to release the capped viral genomic RNA followed by translation and polyprotein processing which generate individual viral proteins that participate in viral replication or nucleocapsid formation. Virus particle formed following virion budding from the endoplasmic reticulum (ER). Host furin cleaves prM to generate a mature virion in the ER and Golgi complex. The virions is released following visicle fusion. (Adapted from Mukhopadhyay et al. (2005)).
1.2 Dengue transmission

Principle hosts for dengue viruses are human and mosquito; mosquito remains infected throughout its life but human develops illness once they are infected. The four dengue viruses originated in monkeys and independently jumped to humans in Africa or Southeast Asia between 100 and 800 years ago (Halstead, 2008).

Dengue viruses can be transmitted through the bite of female mosquitoes of *Aedes aegypti, Aedes albopictus, Aedes polynesiensis* and several species of the *Aedes scutellaris* complex. Each of these species has a particular ecology, behaviour and geographical distribution. *Aedes aegypti* is one of the most efficient vectors for arboviruses because it is highly anthropophilic, frequently bites several times before completing oogenesis, and thrives in close proximity to humans (WHO, 2009).

In mosquito, after ingestion of a blood meal containing virus, the virus infects the epithelial cells lining the midgut, then escapes from the midgut epithelium into the haemocele and infects the salivary gland. The virus is secreted in the saliva, causing infection during probing (Chawla et al., 2014). The mosquito’s saliva containing dengue virus are transferred through the bite site when the infected mosquito is taking a blood meal and then spreads to possible target tissues such as lymph nodes, spleen, bone marrow and liver (Halstead, 2008).

A female mosquito that takes blood meal from a person infected with dengue fever, during the initial 2-10 days febrile period becomes itself infected with the virus in the cells lining its gut (Vassil St. Goergiev, 2009). The mosquito may be infected with 2 different viruses without affecting the yield of either virus.
1.2.1 The vector

Generally, mosquitoes spend the aquatic phase in immature stages and the terrestrial phase in the adult stage during which the events of mating, blood feeding and ovipositing take place. The life span of the adult mosquito usually depends on several factors: temperature, humidity, sex of the mosquito and time of the year (Alameda, 2001). The adult life of *Aedes aegypti* can range from 2 weeks to a month depending on environmental conditions and the life cycle can be completed within one and a half to three weeks as shown in Figure 1.4 (D.A. Bleijs, 2013).

Larvae are mostly found in containers that may hold water, such as discarded tyres, buckets, flowerpots, wading pools and blocked rain gutters, and they can also be found in natural sites such as bromeliads, treeholes, and discarded coconut shells (Rigau-Pérez *et al.*, 1998). In addition, the species prefers dark places with moisture for blood feeding and resting, such as in closets and under beds (Higa, 2011). They typically bite during the day, particularly in the early morning and in the evening (WHO, 2012). The feeding behaviour of the mosquito is characterised by easily interrupted feeding and repeated probing of one or several hosts. Because of this behaviour, the mosquito, if infective, may transmit DENV to multiple persons in a short time, even if they only probe without taking blood (Gubler, 1998).
Figure 1.4: Life cycle of *Aedes aegypti* from egg to adult mosquito (adapted from D.A. Bleijs (2013)).
1.3 Clinical features of dengue

Infection with a DENV can produce a spectrum of clinical illness which ranges from a non-specific viral symptom to a severe and fatal haemorrhagic disease. Upon DENV infection, some individuals may develop mild disease with flu-like symptoms, whereas a few individuals may develop severe disease. Typically, people infected with dengue virus are asymptomatic or only have mild symptoms such as an uncomplicated fever. Young children may have an undifferentiated febrile disease with maculopapular rash while older children and adults have either a mild febrile syndrome or the classical incapacitating disease.

After incubation period, the illness begins abruptly followed by the phases of febrile, clinical and recovery. The acute febrile phase of the illness, characterized by fever and myalgia, lasts 2-7 days and is often accompanied by facial flushing, skin erythema, generalised body ache and headache. Back pain, arthralgias and conjunctivitis may also occur (Whitehorn and Farrar, 2011). Fever, chills and malaise are common but nonspecific (Rothman, 2011). The earliest abnormality in the full blood count is a progressive decrease in total white cell count, which should alert the physician to a high probability of dengue.

Around the time of defervescence, an increase in the capillary permeability in parallel with increasing haematocrit levels may occur, and this marks the beginning of the critical phase. Progressive leukopenia followed by a rapid decrease in platelet count usually precedes plasma leakage. The degree of increase above the baseline haematocrit often reflects the severity of plasma leakage. Shock occurs when a critical volume of plasma is lost through leakage and it often preceded by warning signs (WHO, 2009).
A gradual reabsorption of extravascular compartment fluid takes place in the following 48-72 hours if the patient survives the critical phase. The patient’s general wellbeing improves, appetite returns, gastrointestinal symptoms abate and haemodynamic status stabilizes. The haematocrit stabilizes or may be lower due to the dilution effect of reabsorbed fluid. White blood count usually starts to rise soon after defervescence but the recovery of platelet count is typically later than that of white blood cell count (WHO, 2009). Convalescence is accompanied by asthenia, and a full recovery often takes several weeks (Lim et al., 2013).

1.4 Dengue case classification


Symptomatic dengue virus infections were grouped into undifferentiated fever, dengue fever (DF) and dengue haemorrhagic fever (DHF). Difficulties in applying the criteria for DHF in the clinical situation, together with the increase in clinically severe dengue cases which did not fulfil the strict criteria of DHF, has led to the request for the classification to be considered (WHO, 2009).
1.4.1 The 1997 dengue case classification

Since the 1970s, clinical dengue has been classified according to the WHO guidelines as DF and DHF. The 1997 WHO guidelines (Figure 1.5) classified dengue into DF, DHF (Grades 1 and 2) and dengue shock syndrome (DSS) (DHF Grades 3 and 4). DF is defined as a febrile illness with at least 2 clinical findings, including nausea, vomiting, headache, arthralgia, retro-orbital pain, rash, myalgia, haemorrhagic manifestations and leukopenia. The definition of DHF consists of 4 clinical criteria: fever, a haemorrhagic tendency (spontaneous bleeding or a positive tourniquet test result), thrombocytopenia (platelet count, ≤ 100 000 cells/mm³) and plasma leakage as shown by pleural effusion, ascites, or ≥ 20% haemoconcentration (WHO, 1997).

Experience with this classification system has exposed a number of limitations and studies have demonstrated an overlap between case definitions of DF, DHF and DSS (Deen et al., 2006). The case definition fails to identify a significant proportion of severe dengue cases, for example, severe manifestations such as encephalopathy and hepatic failure which are not included in the DHF case definition. Reports have argued that the case definition of DHF is too rigid and too difficult to apply in primary care or resource-limited settings (Balmaseda et al., 2005; Bandyopadhyay et al., 2006).
Figure 1.5: Manifestations of dengue virus infection (adapted from WHO (1997)).
1.4.2 The 2009 dengue case classification

It then became apparent that the 1997 classification system is not universally applicable for appropriate clinical management, and in the 2006, the WHO Dengue Scientific Working Group recommended additional research into dengue diagnostics and triaging of patients for optimised clinical management and this has led to the re-classification of dengue into dengue with and without warning signs and severe cases published in 2009 (Barniol et al., 2011; Hadinegoro, 2012)

The 2009 classification (Figure 1.6) differs significantly from the previous classification in both conceptual and practical levels. The 2009 WHO dengue case classification classify dengue according to the levels of severity: dengue without warning signs; dengue with warning signs (abdominal pain, persistent vomiting, fluid accumulation, mucosal bleeding, lethargy, liver enlargement and increasing haematocrit with decreasing platelets); and severe dengue (dengue with severe plasma leakage, severe bleeding, or organ failure). Patients who recover following defervescence are considered to have non-severe dengue and those who deteriorate tend to manifest warning signs. Further deterioration is classified as severe dengue, although recovery is possible if appropriate and timely treatment is given (WHO, 2009).
Figure 1.6: The 2009 revised dengue case classification (adapted from WHO (2009)).
1.5 Host immune response

To establish infection and replication in the hosts, flaviviruses have evolved a variety of strategies to modulate the host’s immune responses (Ye et al., 2013). They must evade or inhibit important elements of the innate immune system, namely the type I interferon (IFN) response, which negatively influences the subsequent development of antigen-specific adaptive immunity against those viruses.

Innate immunity and type I IFN responses function as the first line of defence against viral infections, and responds by immediate protective defence mechanisms (Morrison et al., 2012). It involves the rapid recognition of pathogen associated molecular patterns (PAMP) in non-immune cells or cells of the innate immune system such as monocytes or macrophages, dendritic cells (DC), and natural killer (NK) cells. Hence the first barrier to overcome for successful viral infection is the rapid innate immune responses of the host, including type I IFNs, inflammatory cytokine, complement response, NK cell immunity, apoptosis and autophagy (Ye et al., 2013). Most viruses target these important elements to avoid being sensed or recognised in infected cells and to efficiently establish infection in the host.

Adaptive immunity is triggered when a pathogen evades the innate immune system and generates a threshold level of antigen. The system consists of the humoral immune response (production of antibodies by B cells) and the cellular immune response (activities carried out by CD4+ and CD8+ T cells) (Ye et al., 2013). The lymphocytes of the adaptive immune system evolved to provide a more versatile means of defence which, in addition, provides increased protection against subsequent reinfection with the same pathogen.
Conversely, it may also play a critical role in the enhancement of disease severity in most patients with DHF/DSS (Murphy and Whitehead, 2011).

The cells of the innate immune system, however, also play a crucial part in the initiation and subsequent direction of adaptive immune responses, as well as participating in the removal of pathogens that have been targeted by an adaptive immune response.

1.5.1 Primary and secondary dengue infections

Two types of infections are caused by DENV, primary and secondary infection. Primary infection causes acute febrile illness known as DF and secondary infection causes more severe cases and results in DHF (Idrees and Ashfaq, 2013). Primary infection with any of the four serotypes results in a lifelong immunity to that serotypes, and temporary immunity to the others. However this temporary immunity usually wanes after 6 months, at which point an individual is susceptible to the other three DENV serotypes. Subsequent infections often leads to more severe secondary infection in the presence of heterologous dengue antibodies, which attributed to antibody dependent enhancement (Murrell et al., 2011).

The diagnosis of dengue fever can be made serologically by detecting anti-dengue IgM and/or IgG antibodies. Primary infections are characterized by an increase in dengue-specific IgM antibodies four to five days after the onset of fever and by an increase in IgG antibodies (Chawla et al., 2014). In patients experiencing a primary infection, anti-dengue antibodies, initially of the IgM class, evolve relatively slowly. The presence of IgM without IgG is suggestive of DENV primary infection. After a primary infection IgG reaches peak levels in the blood after 14-21 days.
Secondary responses are IgG antibodies which appear early, often during the febrile period, rise rapidly and are referred to as anamnestic (memory) responses. In subsequent re-infections, level peak earlier and the titres are usually higher. Both IgM and IgG provide protective immunity to the infecting serotype of the virus. IgM antibody is transient and generally disappears 30-90 days after onset of illness in primary infections while IgG antibody by contrast, persists for at least 60 years and probably for the life of the patient (Gubler, 2010).

Figure 1.7: An Immune response to dengue infection (adapted from Guzman et al. (2010))
1.6 Laboratory diagnosis of dengue

Currently, dengue diagnosis is based on serology, viral isolation and viral RNA detection. Proof of a dengue infection depends on confirmatory reverse transcriptase-polymerase chain reaction (RT-PCR), dengue serology, specific NS-1 antigen detection or viral isolation, if available (Whitehorn and Farrar, 2011). During the early stages of the disease, virus isolation, nucleic acid or antigen detection can be used to diagnose the infection. At the end of the acute phase of infection, serology is the method of choice for diagnosis. Enzyme linked immunosorbent assays (ELISA) are still the most widely used technique for serological diagnosis (De Paula and Fonseca, 2004).

The differential diagnosis is extensive and varies depending on where the patient is seen, but would include malaria, typhoid, leptospirosis, scrub and murine typhus, septicaemia, other viral haemorrhagic fevers (eg. Ebola, Lassa fever), Chikungunya, and Rift Valley fever (usually without a rash) (Whitehorn and Farrar, 2011).

1.7 Management and prevention of dengue

To date, there are no available vaccine, chemoprophylactic or effective antiviral treatment for dengue. Thus far, the current prevention for dengue virus is by the prevention of its vector which is mosquito while medical supportive care is the recommended primary treatment for infected patients. Patients with DF require rest, oral fluids to compensate for losses via diarrhoea or vomiting, analgesics, and antipyretics for high fever.

Emergency control measures are based primarily on application of insecticides, and it is essential to monitor periodically the vector’s susceptibility to the insecticides