cDNA CLONES CONSTRUCTION OF DENGUE VIRUS TYPE-2 (NEW GUINEA C-STRAIN)

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cDNA CLONES CONSTRUCTION OF DENGUE VIRUS TYPE-2 (NEW GUINEA C-STRAIN)

by

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LIST OF ABBREVIATIONS

BCP	1-Bromo-3-Chloropropane
bp	base pair
BSA	Bovine Serum Albumin
$CaCl_2$	Calcium chloride
cDNA	Complementary DNA
CO_2	Carbon dioxide
CPE	Cytophatic effect
ddH_2O	deionized distilled water
DEN	Dengue
DEN-2	Dengue type 2
DEPC	Diethylpyrocarbonate
DF	Dengue fever
DHF	Dengue hemorrhagic fever
DME	Dulbecco's Modified Eagle's media
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
DSS	Dengue shock syndrome
E. coli	*Escherichia coli*
EDTA	Ethylene diaminetetraacetic acid
FBS	Fetal bovine serum
FCS	Fetal calf serum
H_2O	water
IPTG	Isopropyl-β-D-thiogalactopyranoside
kb	Kilobase pair
LB	Luria Bertani
MEM	minimum essential medium
Mg^{2+}	Ion magnesium
$MgCl_2$	Magnesium chloride
M-MLV	Molony murine leukemia virus
MOPS	3-[N-Mopholino] propanesulphonic acid

mRNA	Messenger RNA
NaCl	Sodium chloride
NCBI	National Center of Biotechnology Information
NGC	New Guinea C
PBS	phosphate-buffered saline
PCR	Polymerase Chain Reaction
pfu	plaque forming unit
RE	Restriction endonuclease
RNA	Ribonucleic acid
RT	Reverse transcription
RT-PCR	Reverse transcription-Polymerase Chain Reaction
SAP	Shrimp Alkaline Phosphatase
SDS	Sodium dodecyl sulfate
TAE	Tris-acetate-EDTA
Taq	*Thermus aquaticus*
T_m	Melting temperature
UV	Ultra violet
v/v	Volume/volume
vRNA	viral RNA
w/v	Weight/volume
X-Gal	X5-bromo-4-chloro-3-indolyl- β-D-galactopyranoside

PEMBINAAN KLON cDNA VIRUS DENGGI JENIS-2 (STRAIN C NEW GUINEA)

ABSTRAK

Denggi virus jenis-2 (DEN-2) merupakan salah satu ahli daripada famili virus *Flaviviridae*. DEN-2 mempunyai genom ribonuleik asid (RNA) bebenang tunggal kutub positif yang terdiri daripada 10,723 nukleotida dan mengekodkan precursor poliprotein tunggal. Dalam kajian ini, genom RNA DEN-2 strain C New Guinea telah diekstrak dari jangkitan ke atas sel Vero dan sel C6/36. Genom RNA virus DEN-2 telah ditranskripsikan secara terbalik dan diamplifikasikan sebagai tujuh fragmen bertindih, yang merangkumi keseluruhan genom, dengan menggunakan transkripsi terbalik-tindakbalas rantai polimerase (RT-PCR). Setiap fragmen telah berjaya diklonkan ke dalam plasmid salinan tinggi, iaitu vektor pGEM®-T, diikuti dengan transformasi ke dalam *Escherichia coli* (*E. coli*) strain JM109. Identiti setiap klon telah disahkan dengan menggunakan penjujukan DNA. Tujuh fragmen bertindih yang dihasilkan telah digabungkan di dalam vektor pGEM®-T dan ditransformasikan ke dalam *E.coli* strain JM109 pada suhu 25°C dengan kehadiran ampisilin (25 μg/ml) untuk menghasilkan satu klon cDNA yang membawa keseluruhan genom DEN-2.

cDNA CLONES CONSTRUCTION OF DENGUE VIRUS TYPE-2 (NEW GUINEA C-STRAIN)

ABSTRACT

Dengue virus type-2 (DEN-2) is a member of the *Flaviviridae* family. DEN-2 has a single-stranded positive polarity ribonucleic acid (RNA) genome of 10,723 nucleotides in length that encodes for a single polyprotein precursor. In this work, viral RNA genome was extracted from Vero and C6/36 cells infected with DEN-2 New Guinea C strain. The viral RNA genome of DEN-2 was reverse transcribed and amplified as seven overlapping fragments, covering the whole genome, by reverse transcription-polymerase chain reaction (RT-PCR). Each of these fragments was successfully cloned into a high copy number plasmid, pGEM®-T vector and transformed into *Escherichia coli* (*E. coli*) JM109 strain. The identity of each clone was verified using DNA sequencing. The seven overlapping fragments were successfully joined together in pGEM®-T vector and transformed into *E. coli* JM109 strain at 25°C with the presence of ampicillin (25 μg/ml) to produce a cDNA clone carrying the full-length genome of DEN-2.

1.0 INTRODUCTION

1.1 Brief introduction to viruses

Virus, the word itself is unadorned Latin for "poison". Most viruses were small and they could not be seen under the light microscope. Most virions fall into the range between 20 – 400 nm in sizes (Hurst, 2000). However, the largest virus, Mimivirus, is 400 nm in diameter for the mature particles and this virus can be observed under a light microscope (Scola *et al.*, 2003). Besides, viruses could not be cultivated on artificial media. They must be cultivated not only in a host organism, but within a host cell. Viruses are intracellular parasites that require the metabolic activities of the host cell to support their growth (Voyles, 2002).

All viruses have the same basic structure: a core nucleic acid surrounded by protein. Individual viruses contain only a single type of nucleic acid, either deoxyribonucleic acid (DNA) or ribonucleic acid (RNA). The DNA or RNA genome may be linear or circular, a single-stranded or double-stranded. Viruses are frequently classified by the nature of their genomes. Almost all viruses form a protein sheath, or capsid, around their nucleic acid core. Capsids are made up of protein subunits. Thus, unrelated viruses such as a phage, an animal virus, and a plant virus may have capsids that are similarly built and almost indistinguishable morphologically. In some viruses specialized enzymes are stored within the capsid (Raven *et al.*, 2005). Many animal viruses and some plant viruses form an enveloped around the capsid. The essential structure of the envelopes is that of all biological membranes, a phospolipid bilayer in which are embedded specific proteins (Luria *et al.*, 1978).

The Baltimore classification is based upon the relationship between the viral genome and the messenger RNA (mRNA) used for translation during

expression of the viral genome. There are six classes of the Baltimore classification (Voyles, 2002). Nevertheless, the classification was revised based on the fundamental importance of mRNA in the replication cycle of viruses. For viruses with RNA in particular, genome replication and the expression of genetic information are inextricably linked. Therefore, both of these criteria are taken into account of the new classification scheme (Figure 1.1) (Cann, 2001; Dimmock *et al.*, 2001).

The seven classes of the revised Baltimore classification scheme (Voyles, 2002; Cann, 2001) are:

1. Class I viruses: The genome is double-stranded DNA. The mRNA is synthesis in the normal fashion using negative-strand DNA as template.
2. Class II viruses: The replication occurs in the nucleus, involving the formation of a double-stranded intermediate which serves as a template for the synthesis of single-stranded progeny DNA.
3. Class III viruses: The genome is double-stranded RNA. These viruses have segmented genome. Each segment is transcribed separately to produce individual monocistronic mRNAs.
4. Class IV viruses: The genome is single-stranded positive-sense RNA. The viruses in Class IV can be subdivided into two groups: (a) viruses with polycistronic mRNA and, (b) viruses with complex transcription.
5. Class V viruses: The genome is single-stranded negative-sense RNA. These viruses can be divided into two types: (a) Non-segmented genomes and, (b) segmented genomes.
6. Class VI viruses: The genome is single-stranded positive-sense with DNA intermediate.

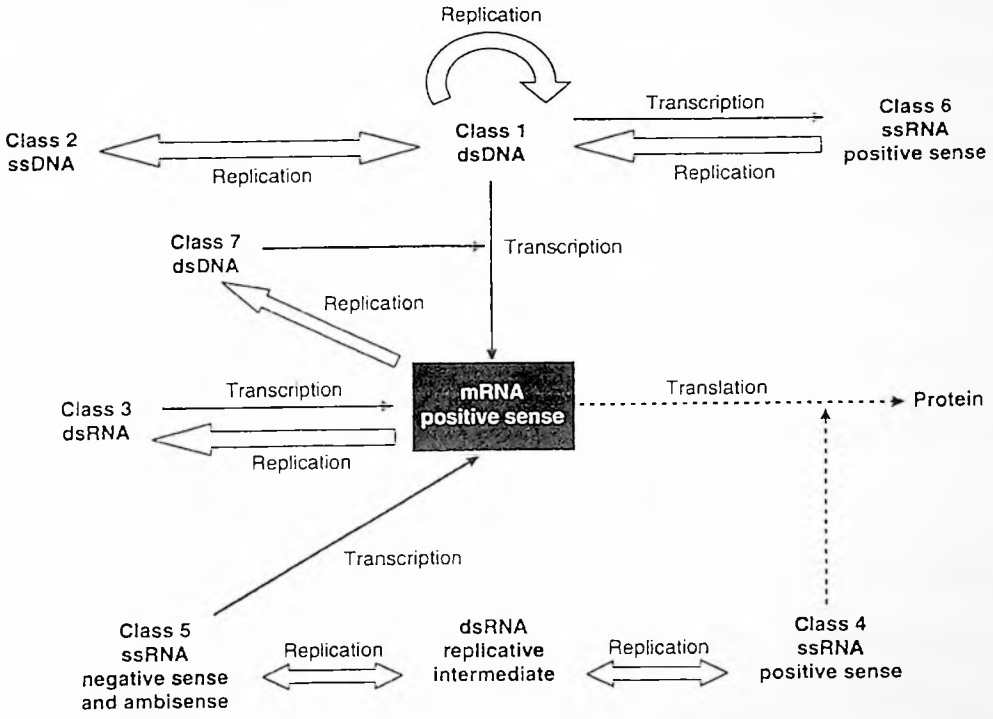

Figure 1.1 The revised Baltimore classification. Viral genomes are classified based on the fundamental importance of mRNA in the replication cycle of viruses. Solid arrows represent transcription; open arrows represent replication and dash arrows represent translation. [Adopted from (Dimmock *et al.*, 2001)]

7. Class VII viruses: The genome is double-stranded DNA with RNA intermediate.

1.2 The family of *Flaviviridae*

The *Flaviviridae*, developed from Latin *flavus*, or "yellow", referring to the prototype virus, yellow fever virus (Kuhn *et al.*, 2002). *Flaviviridae* is classified in Class IV viruses in the Baltimore classification. The viruses classified in this family are enveloped 40 – 60 nm particles with an isometric nucleocapsid of 25 – 30 nm and one molecule of linear positive-sense single-stranded RNA of 9500 – 12,500 nucleotides (Dimmock *et al.*, 2001; Mayo and Pringle, 1998). The majority of *flaviviridae* are arthropod-borne, transmitted to vertebrates by chronically infected mosquito or tick vectors.

This virus family contains three genera: *Flavivirus, Pestivirus and Hepacivirus* (Dimmock *et al.*, 2001; Leyssen *et al.*, 2000). Flavivirus is a large group of viruses that multiply in the vertebrate host and insect or tick vector, e.g. yellow fever virus, tick-borne encephalitis virus group, dengue virus group and Japanese encephalitis virus group (Dimmock *et al.*, 2001).

The genus *Flavivirus* contains 70 recognized species that are antigenically related and occur worldwide (Dyer *et al.*, 2007). The Flavivirus genus includes viruses transmitted by mosquitoes and ticks, as well as zoonotic agents with no known arthropod vector (Clyde *et al.*, 2006).

1.3 Dengue virus

Dengue virus is a member of the *Flavivirus* (Filomatori *et al.*, 2006; Leyssen *et al.*, 2000). Dengue viruses are transmitted in a human-mosquito-human

cycle, with *Aedes aegypti* and *Aedes albopictus* as the principal arthropod vectors (Zhang *et al.*, 2006; Munoz *et al.*, 1998).

A primary infection with any of the four genotypes of dengue viruses usually results in subclinical or self-limited febrile disease (Wu *et al.*, 2001). Dengue viruses (genotypes 1 – 4) cause dengue fever (DF), the most prevalent arthropod-borne viral disease of humans (Bente and Rico-Hesse, 2006; Takhampunya *et al.*, 2006). DF is characterized by biphasic fever, headache, myalgia, eye pain and rash (Guy and Almond, 2008). Recovery is usually completed in 7 – 10 days but prolonged asthenia is common (Guy and Almond, 2008). The severe form of the disease is commonly referred as dengue hemorrhagic fever / dengue shock syndrome (DHF / DSS) and it is often associated with secondary infections (Osman *et al.*, 2008; Jimenez and Fonseca, 2001). These diseases occurs in over 100 countries, with more than 3 million people in the tropical and subtropical world living in areas at risk of infection (Figure 1.2) (Guy and Almond, 2008; Twiddy *et al.*, 2002). At present, no vaccine has been approved for human use and treatment is only supportive to reduce the severity of the diseases (Whitby *et al.*, 2005; Jimenez and Fonseca, 2001).

Dengue infection occurs only in tropical and subtropical areas where insect vectors are present (Izquierdo *et al.*, 2008). Dengue is endemic in Malaysia (Fong *et al.*, 1998). Dengue 2 and 3 viruses are currently the predominant genotypes in Malaysia (Fong *et al.*, 1998).

1.3.1 Dengue virus genome

Dengue viruses have a spherical shape of approximately 40 to 60 nm diameter that contain a single-stranded positive-sense RNA genome (Blaney *et al.*, 2008). The

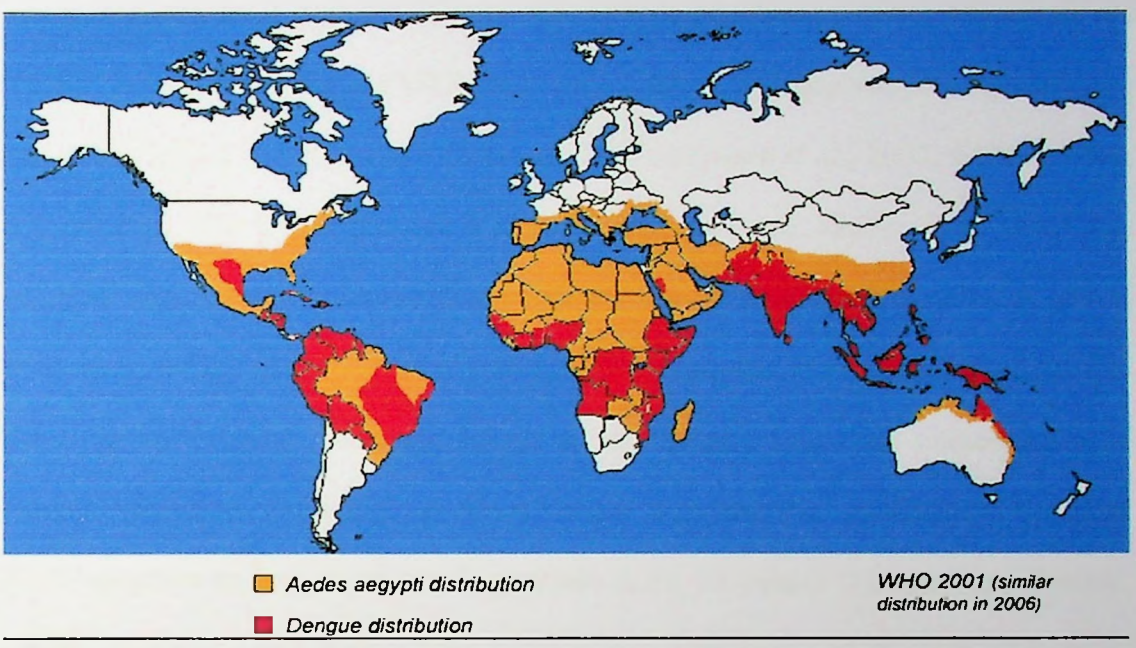

Figure 1.2 Worldwide distribution of *Aedes aegypti* and dengue fever. WHO had reported these distribution in 2001 and a similar distribution has been observed through 2006. [Adopted from (Guy and Almond, 2008)]

single and positive-stranded RNA is approximately 11kb in length (Munoz *et al.*, 1998). The genomic RNA presents a single open reading frame which encodes a precursor polyprotein and is flanked by two non-translated regions (5' and 3' NTR) (Leitmeyer *et al.*, 1999). Co- and posttranslational proteolytic cleavage of the precursor results in the formation of three structural proteins, followed by at least seven non-structural proteins (Figure 1.3) (Clyde and Harris, 2006; Munoz *et al.*, 1998). Dengue virus has a 5'UTR containing a type I $m^7GpppN_1mpN_2$ cap structure but devoid of a 3' poly(A) tail (Chiu *et al.*, 2005; Leyssen *et al.*, 2000; Gubler, 1998; Kapoor *et al.*, 1995; Wengler and Wengler, 1993; Irie *et al.*, 1989). The limited size of RNA virus genomes necessitates maximization of the coding capacity of genes; thus, many of the dengue virus structural and non-structural proteins serve multiple functions in the viral life cycle (Clyde *et al.*, 2006). Since the nucleocapsid of dengue viruses is a tenuous structure which is permeable to RNases, the lipid envelope plays an important role in genomic RNA protection (Henchal and Putnak, 1990).

Flaviviruses replicate in the cytoplasm of susceptible cells like other positive-strand RNA viruses (Perera *et al.*, 2008). Replication of dengue viruses proceeds along a two-step pathway in the host cell (Alwarez *et al.*, 2005). After the synthesis and maturation, the nonstructural proteins and the viral RNA form a replication complex that catalyzes the synthesis of the negative-strand RNA, which in turn is used as template to amplify new strands of genomic RNA (Alwarez *et al.*, 2005).

Dengue viruses surveillance and disease diagnosis typically rely only on serologic assays such as ELISA, which are often unable to determine the infecting serotype, especially after secondary infections (Klungthong *et al.*, 2008).

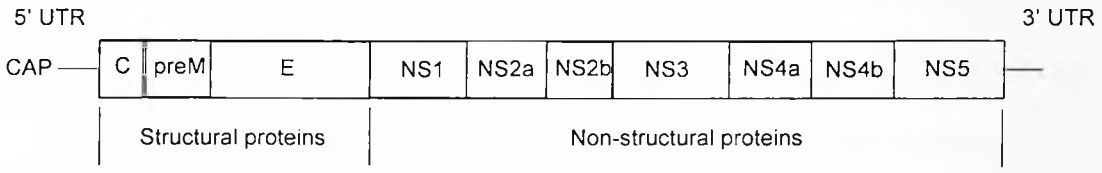

Figure 1.3 Genomic organization of the dengue virus. The viral genome consists of a single-stranded RNA molecule of positive polarity which is capped. UTR are present at the 5' and 3' ends of the genome. Boxes indicate mature proteins generated by proteolytic processing [Modified from (Leyssen *et al.*, 2000)].

Subgenomic replicon constructs of the *Flaviviridae* contain all of the genetic elements needed for amplification in susceptible host but lack the major part of the genes encoding the structural proteins (Ng *et al.*, 2007). The RNAs consequently replicate but are not packaged into viral particles. Because of the non-infectious nature, replicons are important tools in the study of viral replication (Ng *et al.*, 2007).

1.3.2 Dengue virus structural proteins

The mature dengue virion (Figure 1.4) contains three structural proteins: C, the non-glycosylated nucleocapsid or core protein (14 kDa); M, a membrane-associated protein (8 kDa); and E, the major glycosylated envelope protein (51 – 59 kDa) (Figure 1.5). Immature mainly intracellular virus contains a protein known as prM (sometimes, preM), a precursor of M (Munoz *et al.*, 1998; Henchal and Putnak, 1990). The gene order for the structural proteins from 5' terminus is C-prM-E (Li *et al.*, 1999; Henchal and Putnak, 1990). The proteins are derived from a single, long, precursor polyprotein (Henchal and Putnak, 1990).

The C protein is the first viral polypeptide synthesized during translation, has a molecular weight of about 13,500 Dalton, and is rich in lysine and arginine residues (about 25%). C protein lacks an N-terminal, hydrophobic signal sequence, which suggest that its synthesis is on non-membrane-bound ribosomes (Henchal and Putnak, 1990).

Specific proteolytic cleavage of a glycosylated prM precursor during virus maturation results in the formation of the M protein. The role that plays in the mature virion is not known (Henchal and Putnak, 1990).

The E glycoprotein appears as a homotrimer on the surface of mature virions. Functional domains responsible for neutralization of the virus, fusion and

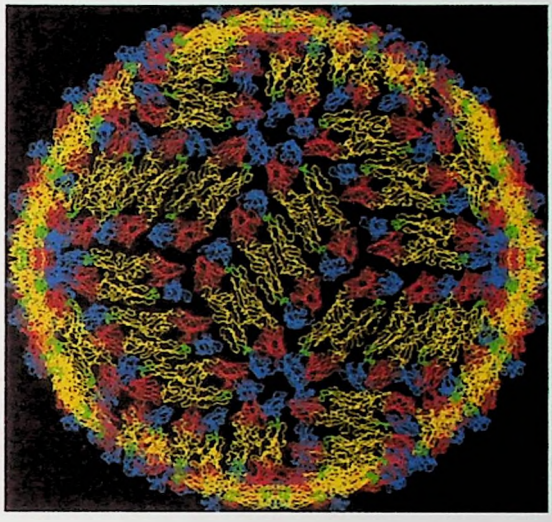

Figure 1.4 Mature dengue particle. Structure of the whole virus showing each monomer with domains I, II and III in red, yellow and blue, respectively. The fusion peptide is shown in green. [Adapted from (Kuhn *et al.*, 2002)]

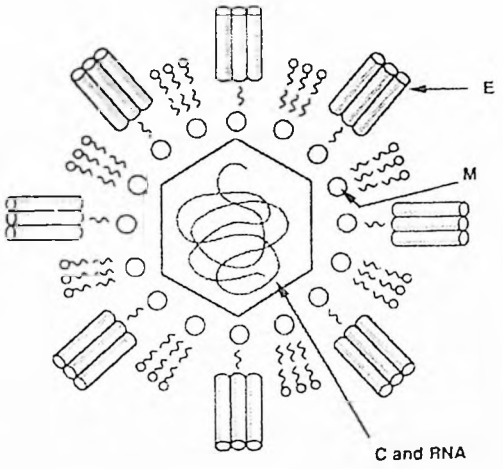

Figure 1.5 Schematic representation of mature virion. E represents envelope protein; M represents membrane-associated protein; and C represents core protein. [Adopted from (Henchal and Putnak, 1990)]

interaction with specific cell surface virus receptors are associated with the E protein (Henchal and Putnak, 1990).

1.3.3 Dengue virus nonstructural proteins

Dengue viruses have seven non-overlapping nonstructural proteins namely NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5 (Leitmeyer *et al.*, 1999; Henchal and Putnak, 1990).

NS1 is a ~48,000-Dalton glycoprotein. It is synthesized in the rough endoplasmic reticulum as a hydrophilic, water-soluble, monomeric glycoprotein. The role of NS1 in virus replication is not known (Henchal and Putnak, 1990).

NS2 coding region consists of two proteins, NS2a and NS2b (Leitmeyer *et al.*, 1999). NS2a is a ~20,000-Dalton hydrophobic protein and NS2b is a ~14,500-Dalton hydrophobic protein without any known function in virus replication (Henchal and Putnak, 1990).

NS3 is a ~70,000-Dalton hydrophilic protein (Henchal and Putnak, 1990). NS3 is the second largest nonstructural protein specified by the virus (Matusan *et al.*, 2001). NS3 is known to posses multiple enzymatic activities, including serine proteinase located in the N-terminal region and an NTPase-helicase in the remaining 70% of the protein (Matusan *et al.*, 2001).

NS4a and NS4b are hydrophobic proteins with molecular weight ~16,000 Dalton and ~27,000 Dalton, respectively (Henchal and Putnak, 1990). Their roles in virus replication are unknown but they might be RNA replication complex cofactors along with the putative viral RNA dependent RNA polymerase (Henchal and Putnak, 1990).

NS5 is the largest nonstructural protein in dengue virus with molecular weight ~105,000 Dalton (Henchal and Putnak, 1990). NS5 posses RNA-dependent RNA polymerase (RdRp) activity (Matusan *et al.*, 2001). The NS5 protein is predicted to contain at least two distinct domains: the N-terminal region is predicted to be a S-adenosyl-methionine (SAM) transferase domain; whilst the C-terminal domain of NS5 contains eight highly conserved sequence motifs that have been recognized in many RdRp (Johansson *et al.*, 2001).

1.4 Dengue virus replication cycle

Infection with one of the arthropod-borne flaviviruses begins when the vector takes a blood meal and the virus is introduced into the host (Clyde *et al.*, 2006). The initial site of interaction of dengue virus with the host cell is heparan sulfate, which allows the virus to concentrate on the surface of the cell (Leyssen *et al.*, 2000). The virus binds to and enters a permissive host cell via receptor-mediated endocytosis (Figure 1.6) (Clyde *et al.*, 2006). Endosomatic pH changes trigger the fusion between viral envelope and the endosomal membrane (Clyde *et al.*, 2006; Leyssen *et al.*, 2000). The fusion allows the entry of the nucleocapsid into the cytoplasm and genome uncoating (Clyde *et al.*, 2006).

After release of the genome into cytoplasm, the 5' untranslated region (5'UTR) directs the RNA to the ribosomes and the translation of the input strand takes place (Clyde *et al.*, 2006; Leyssen *et al.*, 2000). The viral polyprotein is processed co- and posttranslationally into individual and functional viral proteins (Leyssen *et al.*, 2000). After that, the virus switches from translation to synthesis of a negative-strand intermediate, which serve as template for the production of

multiple copies of positive-strand viral RNA (vRNA) (Clyde *et al.*, 2006; Leyssen *et al.*, 2000).

After replication, the viral genome is encapsidated in the nucleocapsid proteins and directed to the endoplasmic reticulum (Leyssen *et al.*, 2000). The envelope proteins become glycosylated after passing through the secretory pathway and the mature viruses are released into the extracellular space (Leyssen *et al.*, 2000).

1.5 Dengue virus detection

The development of a more rapid diagnostic assay for dengue virus detection with high sensitivity and specificity will be very useful for the management and treatment of patients and for epidemiological surveillance.

A molecular hybridization technique with radiolabeled, strand-specific RNA probes was developed to detect dengue virus type 2 RNA in pools of infected *Aedes albopictus* mosquitoes but the convenience of RNA-RNA hybridization as a surveillance diagnostic tool can be further improved by developing nonradiolabeled probes (Olson *et al.*, 1988). Virus identification was simplified serotype-specific monoclonal antibodies were prepared by Henchal *et. al.* (Henchal and Putnak, 1990). However, faster techniques are needed for the early diagnosis of dengue fever and dengue hemorrhagic fever during the acute viremic phase of infection. An isothermal nucleic acid sequence-based amplification (NASBA) assay was developed and reported by Wu *et. al.* (2001). This assay was optimized to amplify viral RNA of all four dengue virus serotypes by a set of universal primers and to type the amplified products by serotype specific capture probes that utilized electrochemiluminescence (Wu *et al.*, 2001).

Molecular techniques based on genomic sequences by RT-PCR, nested PCR and real-time PCR have made possible the rapid diagnosis of dengue virus infections. Identification of dengue virus serotypes by using Polymerase Chain Reaction (PCR) was developed and demonstrated with four sets of specific primers. All procedures of rapid RT-PCR were completed within 2 hours and the results of the serotypes were consistent. The method was shown to be useful for clinical and field specimens (Morita *et al.*, 1991). A novel single-tube nested PCR which is less prone to cross-contamination and reduces reaction cost and time was developed and reported by Gomes *et. al.* This method was less sensitive than the conventional two-step RT-PCR for the detection of virus in serum samples (Gomes *et al.*, 2007). In order to detect and identify dengue serotypes in serum samples effectively, a single-step quantitative RT-PCR (Q-RT-PCR) assay was developed and reported in year 2008 (Sadon *et al.*, 2008). Sets of primers were selected from the capsid region of the viral genome. Dengue serotypes were detected using the specific primers and fluorogenic *TaqMan*™ probes. The results from this method shown that the Q-RT-PCR is a rapid, sensitive and reproducible tool for the detection and quantitation of the four dengue serotypes in clinical samples (Sadon *et al.*, 2008).

1.6 Potential treatments for dengue virus

Little is known about the pathogenic mechanisms that lead to dengue fever and its severe form, dengue hemorrhagic fever. This is due to the fact that only humans show signs of disease. A broad range of animals, namely chickens, pigeons, other fowl, goats, dogs, pigs, lizards, guinea pigs, rabbits, rats, mice and hamsters show no signs of dengue disease, even after inoculation of high virus doses and by different routes (Bente and Rico-Hesse, 2006). There is no treatment for

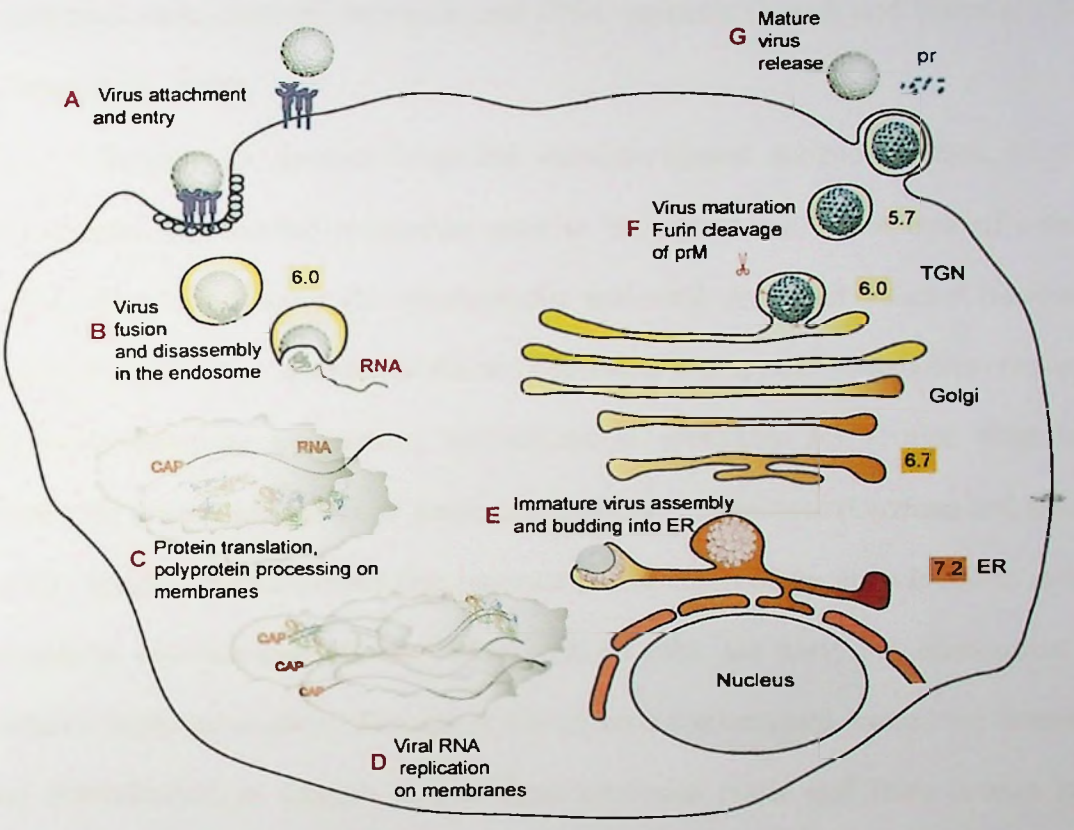

Figure 1.6 The flavivirus life cycle. Numbers shown in colored boxes refer to the pH of the respective compartments. [Adopted from (Perera *et al.*, 2008)]

disease caused by dengue viruses and a licensed vaccine against dengue is not yet available (Yauch and Shresta, 2008; Shresta *et al.*, 2006; Stephenson, 2005). This is not surprising knowing the fact that there is no animal models that could be used to understand the pathogenic mechanism of the virus. Nevertheless, potential vaccines against dengue are in various stages of development including live attenuated virus, inactivated virus, subunit, vectored, and DNA vaccines (Yauch and Shresta, 2008; Jaiswal *et al.*, 2004).

In order to discover drugs and vaccines against dengue diseases, a lot of fundamental and applied researches need to be carried out. The lack of animal models does not hamper the attempts for anti-viral drug and vaccine discovery against dengue virus. Because of the technical skill and special containment required for direct mosquito inoculation, cell culture is preferable for routine diagnosis, despite the greater sensitivity of methods employing mosquitoes (Guzman and Kouri, 2004). Singh and Paul (1969) first reported the isolation of dengue viruses in *Aedes albopictus* cell cultures (White, 1987). C6/36 cells are easy and economical to maintain in the laboratory. The use of C6/36 cells has centered around the isolation and identification of dengue viruses from mosquito pools and from human sera collected in the acute stage of febrile illnesses (White, 1987).

Using the cell culture, dengue virus was able to propagate and study. This is shed some insights into the virus replication process. Because of this, few anti-viral drug targets have been recognized. Among them are inhibition of 5'-end genome capping, protease inhibitor, RNA-dependent RNA polymerase (RdRp) and helicase (Leyssen *et al.*, 2000).

In term of prevention, vaccine against dengue virus would be sought for. The use of synthetic peptides for the antigenic characterization of dengue pr and M

protein was reported. The immunological activities of five synthetic peptides of the prM protein of dengue-2 virus containing B cell epitopes were evaluated in BALB/c mice and two peptides elicited neutralizing antibodies against all four dengue serotypes (Vazquez *et al.*, 2002). DNA shuffling and screening technologies were applied to develop a single recombinant dengue envelope (E) antigen capable of inducing neutralizing antibodies against all four antigenically distinct dengue serotypes (Apt *et al.*, 2006). RNA-dependent RNA polymerase and E protein are two targets for antiviral drug design (Malet *et al.*, 2008; Perera *et al.*, 2008).

Dengue virus was found involved in inducing the expression of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), in immune cells and human umbilical vein endothelial cells (HUVECs) at the mRNA and protein levels. A decrease in dengue virus RNA was seen in recombinant TRAIL-treated monocytes. Recombinant TRAIL inhibited dengue virus titers in dengue virus-infected dendritic cells by an apoptosis-dependent mechanism. The data suggest that TRAIL plays an important role in the antiviral response to dengue virus infection and is a potential candidate for antiviral against dengue virus (Warke *et al.*, 2008).

1.7 Vector control

One way to prevent dengue diseases is by control the vector for the virus. Active disease surveillance is an important component of a dengue prevention program. Three proactive surveillance components include a sentinel clinic and physician network, a fever alert system that uses community health workers, and a sentinel hospital system. All these components require a good public health laboratory to provide diagnostic support in virology, bacteriology and parasitology (Gubler, 1998).

Mosquito control is one of the disease prevention approach but space sprays with insecticides to kill adult mosquitoes are not usually effective unless they are used indoors. The most effective way to control the mosquitoes that transmit dengue is larval source reduction, i.e., elimination or cleaning of the water-holding containers that serve as the larval habitats for mosquitoes. Community participation and ownership of prevention program require extensive health education and community outreach (Gubler, 1998).

1.8 Objectives of the study

As mentioned earlier, there is no treatment for dengue diseases and licensed vaccine against dengue is not yet available. Many research works been done in order to design antiviral drugs and vaccine development against dengue. However, this work was not easy due to RNA genome of dengue virus. RNA is unstable and difficult to work with. Alternatively, cDNA clone could be used for further manipulation of the viral RNA genome.

The aim of this study is to construct cDNA clones of dengue virus type-2 (New Guinea C-strain) using high copy number plasmid. The construction of cDNA clones could facilitate further understanding of the virus and manipulation of the virus genome.

2.0 MATERIALS AND METHODS

2.1 Materials

The materials used were purchased from the suppliers shown in Table 2.1.

Table 2.1 Materials used and their suppliers

Materials	Suppliers
QIAEX II Gel Extraction Kit, QIAamp® Viral RNA Mini Kit	Qiagen
Taq DNA Polymerase, PCR Nucleotide Mix, 1 kb DNA Ladder, restriction endonucleases, T4 DNA Ligase, pGEM-T Vector System, IPTG, X-Gal, RNAse A Solution, M-MLV Reverse Transcriptase, Recombinant RNasin® Ribonuclease Inhibitor, glycerol, agarose, 6X loading dye, Shrimp Alkaline Phospatase (SAP)	Promega
Restriction endonucleases	Fermentas, New England Biolabs
Tri Reagent, 1-Bromo-3-Chloropropane (BCP)	Molecular Research Center
C6/36 mosquito cell line, Vero cell line	University Malaya
Dengue-2 virus (New Guinea C strain)	University Malaya
MEM/EBSS medium, DME/High Glucose medium, sodium bicarbonate, sodium pyruvate, fetal bovine serum (FBS), penicillin-streptomycin, trypsin-EDTA, L-glutamine	Hyclone®
DMSO, PEG 8000	Sigma
Ethanol, ethidium bromide, isopropanol, ampicillin, formaldehyde, MOPS (sodium salt), EDTA, sodium chloride, sodium hydroxide, phosphate-buffered saline (PBS), bromophenol blue, calcium chloride, sodium acetate, Tris-Cl, sodium dodecyl sulfate (SDS), tryptone, yeast extract, agar-bacteriological, bacto-tryptone,	Amresco
Oligonucleotides	1st Base

2.2 Culture media and stock solutions

2.2.1 Media

Luria-Bertani (LB) medium and LB agar were prepared using the compositions shown in Table 2.2 (Sambrook and Russell, 2001).

Table 2.2 Composition of LB medium and LB agar (per liter)

Media	Compositions
LB medium	10 g tryptone, 10 g sodium chloride (NaCl), 5 g yeast extract
LB agar	10 g tryptone, 10 g sodium chloride (NaCl), 5 g yeast extract, 15 g agsr-bacteriological

2.2.2 Stock solutions

All stock solutions used are shown in Table 2.3 and 2.4.

Table 2.3 Solutions for electrophoresis of DNA

Solution	Compositions
50X TAE (1 liter)	242 g of Tris base, 57.1 ml of glacial acetic acid, 100 ml of 0.5 M EDTA

Table 2.4 Solutions used in blue-white selection of bacterial transformants

Solution	Compositions
X-Gal	100 mg X-Gal dissolved in 2 ml N,N'-dimethyl-formamide (covered with aluminum foil and stored at -20°C)
0.1 M IPTG	1.2 g IPTG in 50 ml water, filter-sterilized and stored at 4°C

2.2.3 Antibiotic

Ampicillin stock solution (100 mg/ml) was filter-sterilized using 0.22 μm filter (Sartorius) and stored in aliquots at -20°C.

2.3 Host strain and vector

The bacterial host strain used in this study and its genotypes are presented in Table 2.5. Circular map of the plasmid used in this study are shown in Figure 2.1.

Table 2.5 Genotype of *E. coli* strain used

Strain	Genotype
JM 109	*rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsd*R17 (r_K-, m_K+), *rel*A1, *sup*E44, Δ (*lac-pro*AB), [F', *tra*D36, *pro*AB, *lacI*^{q}ZΔM15]

2.4 Preparation of glassware and plastic ware

All glassware and plastic ware were autoclaved at 121°C for 15 minutes at the pressure of 975 kPa prior to use.

2.5 Outline for study

The outline for this study is shown in Figure 2.2.

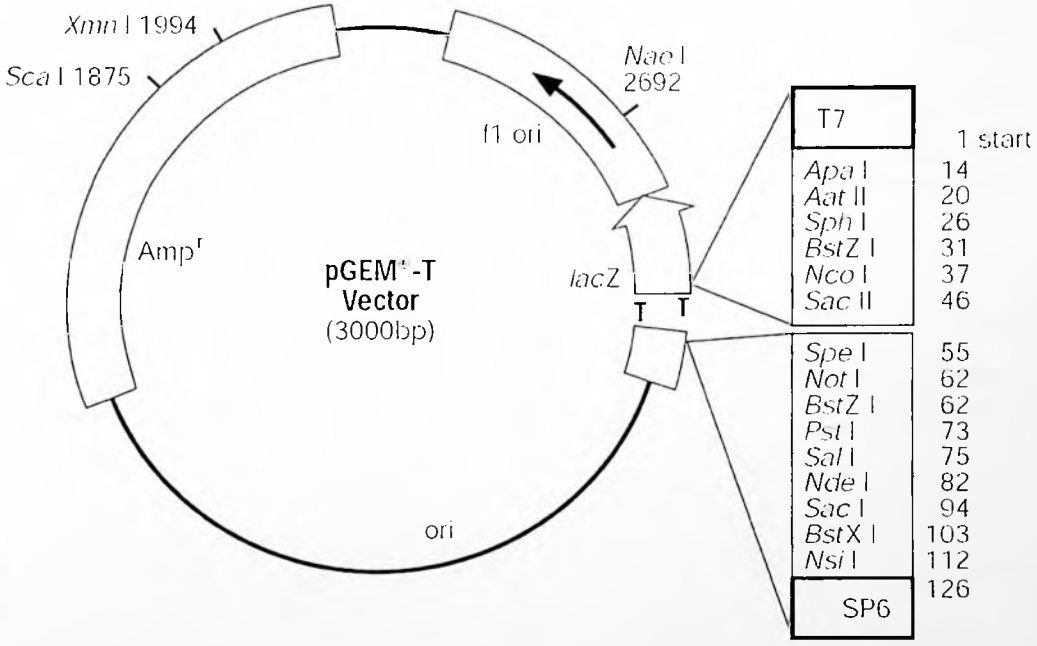

Figure 2.1 **Vector used in this study.** pGEM®-T vector (Promega).