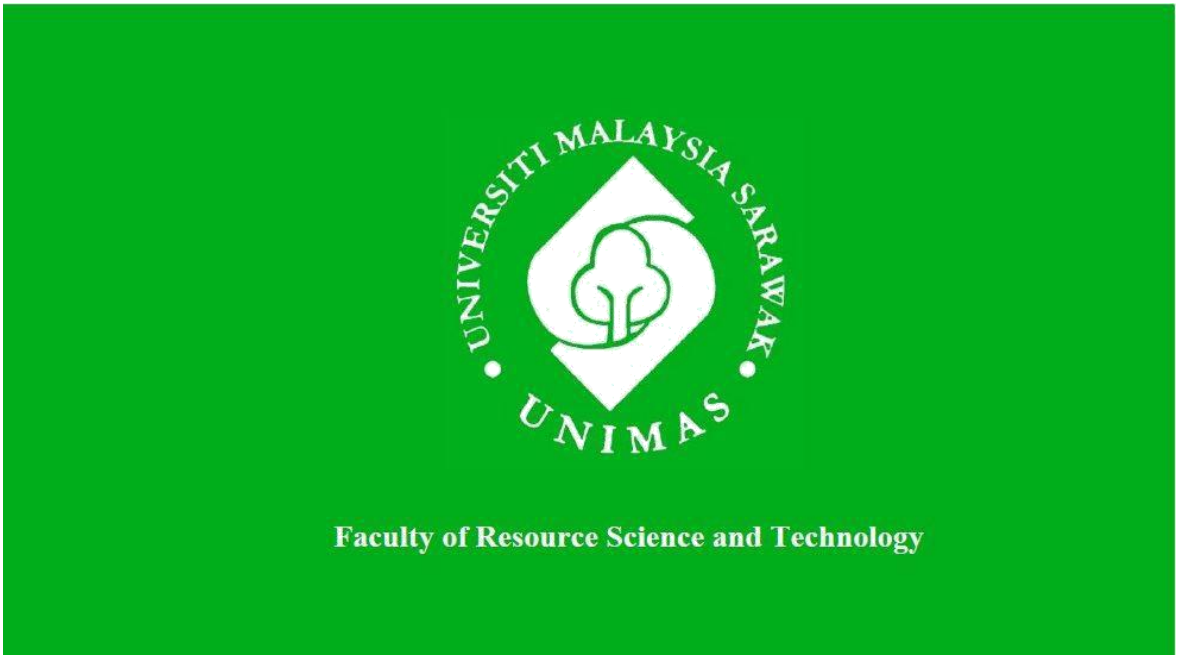

Comparison of the Growth of All Four Dengue Virus Serotypes in C6/36 and Vero Cell Lines

Rahmah Fadilah Sjam'un

Bachelor of Science with Honours
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RAHMAH FADILAH SJAM'UN

39663

This project is submitted in partial fulfilment of the requirement for the degree of

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Supervisor: Dr. Magdline Sia Henry Sum

Resource Biotechnology

Department of Molecular Biology

Faculty of Resource Science and Technology

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Declaration

I hereby declare that this study entitled ***'Comparison of the Growth of all Four Dengue Virus Serotypes in C6/36 and Vero Cell lines'*** is based on my original work except for quotation and citation, which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UNIMAS or other institutions.

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Rahmah Fadilah Sjam'un

Resource Biotechnology Programme

Department of Molecular Biology

Faculty of Resource Science and Technology

University Malaysia Sarawak

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List of Abbreviations

BGM	buffalo green monkey kidney cells
CPE	cytopathic effect
DENV	dengue virus
DENV-1	dengue virus serotype 1
DENV-2	dengue virus serotype 2
DENV-3	dengue virus serotype 3
DENV-4	dengue virus serotype 4
DF	dengue fever
DHF	dengue haemorrhagic fever
DMEM	Dubelco's modified eagle medium
DSS	dengue shock syndrome
EDTA	ethylenediaminetetraacetic acid
FBS/FCS	foetal bovine serum/ foetal calf serum
L15	Lebovitz 15
PFU	plaque forming unit
RT-PCR	reverse transcription polymerase chain reaction
TBE	tris-borate-EDTA
TPB	tryptose phosphate broth

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Rahmah Fadilah Sjam'un (39663)

Resource Biotechnology
Faculty of Resource Science and Technology
Universiti Malaysia Sarawak

ABSTRACT

Dengue virus, which is transmitted by an infected *Aedes aegypti* mosquito, is one of the most rampant arboviruses that have been infecting millions of people around the world. Previous studies showed that the virus propagated in different cell lines showed diverse results in different experiments conducted. Hence, the aim of this study is to compare the growth of all four dengue virus serotypes in both C6/36 and Vero cell lines. Three main parameters that were observed and analysed were the differences in cytopathic effect, virus titers and RNA concentration obtained from the experiment. The results obtained showed that the growth of DENV was significantly higher in C6/36 cell line compared to Vero cell line for all four DENV serotypes. Hence, this study allowed a better understanding on the susceptibility differences that occurred where high rate of viral propagated indicated a high affinity of the cell line towards dengue virus.

Keywords: Dengue virus, C6/36 cell line, Vero cell line

ABSTRAK

Virus denggi yang menular melalui nyamuk Aedes aegypti, merupakan salah satu arbovirus yang paling berleluasa dan telah menjangkiti berjuta-juta orang di seluruh dunia. Kajian terdahulu menunjukkan bahawa virus yang dibiakkan dalam sel yang berbeza menunjukkan keputusan yang pelbagai. Oleh itu, tujuan kajian ini adalah untuk membandingkan pertumbuhan empat serotaip virus denggi dalam sel C6/36 dan sel Vero. Tiga parameter utama yang diperhatikan dan dianalisis adalah perbezaan dalam kesan cytopatik, titer virus dan kepekatan RNA yang diperolehi daripada eksperimen. Keputusan yang diperolehi menunjukkan bahawa pertumbuhan DENV adalah lebih tinggi dalam sel C6/36, berbanding dengan sel Vero untuk keempat-empat serotaip. Oleh itu, kajian ini membolehkan pemahaman yang lebih baik tentang perbezaan kecenderungan yang berlaku di mana kadar pertumbuhan virus yang tinggi menunjukkan pertalian yang tinggi antara sel terhadap virus denggi.

Kata kunci: Virus denggi, sel C6/36, sel Vero

1.0 Introduction

Dengue virus (DENV) infection is one of the most rampant arthropod-borne viral infections in subtropical and tropical regions worldwide. The two competent vectors of DENV are *Aedes aegypti* and *Aedes albopictus*. Over 100 countries and around 40% of the world's population are threaten by DENV with approximately 100 million of new infections are reported annually all around the globe (*World Health Organization*, 2000). There are four serotypes of DENV, which are DENV-1, DENV-2, DENV-3 and DENV-4 whereby the degree of virulence varies among them. The increase in the spread of mosquito vectors and viruses in recent years has been due to globalization as well as the growing in viral diversity since several DENV serotypes can co-circulate in the same location within a short period of time. Despite its worldwide morbidity neither pathogenesis nor the tropism of DENV is well characterized due to the complexity of dengue virus (Diamond *et al.*, 2000).

Some of the cell lines which are used for *in vitro* virus inoculation and isolation are susceptible to several types of viruses while others are quiet specific. Examples of commonly use cell lines are African green monkey kidney cultures, such as Vero and BSC-1 (Payment & Trudel, 1993). This experiment used two of the most commonly utilized cell line for *in vitro* propagation of DENV which are the C6/36 cell line from the larvae of *Aedes albopictus* and Vero cell line. Both of these cell lines were chosen due to their high permissiveness and infectivity which aids in a better proliferation of viral particles. Comparison of the growth of all four DENV serotypes in both C6/36 and Vero cell lines were done via three main approaches which were the differences in cytopathic effect (CPE), the differences in virus titers for both cell lines and the RNA concentration obtained from the experiment. CPE were observed under light microscope, RNA concentrations were obtained through RNA quantification and virus titers were obtained

via viral plaque assay. Viral plaque assay was used to measure the concentration of dengue virus (DENV) in the form of plaque forming unit (PFU). Through the comparison of the cytopathic effect (CPE) formed, detection and evaluation of the plaques, and RNA quantification, all four DENV serotypes' effectiveness and their susceptibility towards corresponding cell lines were determined.

Pervin *et al.* (2003) stipulated that DENV propagation was significantly higher in C6/36 inoculation compared to those of Vero cell line which contradicts with the earlier finding of Gubler in 1984. Throughout the decades, numerous experiments conducted showed different results. Hence, the aim of this study was to compare the growth of all four DENV serotypes propagated in both C6/36 and Vero cell lines. It was necessary to do the comparison because for all four DENV serotypes, different patterns of CPE were formed in different cell lines. The different patterns of DENV infection reflected the different degree of the cell line's permissiveness towards DENV infection. Besides this, the comparison for the growth of DENV had shown which cell line has a higher sensitivity towards DENV, thus indicating which cell line was more suitable for the propagation of DENV. At end of this study, we had determined the differences in the formation of CPE, the titer of viable virus and the RNA concentration that occurred during the growth of all four DENV serotypes propagated in both C6/36 and Vero cell lines.

The objectives of this study were as follows:

1. To compare the CPE formed for all four DENV serotypes in C6/36 cell line and Vero cell line.
2. To compare the different titer for all four DENV serotypes propagated in both cell lines.
3. To analyse RNA concentration of all four DENV serotypes.

2.0 Literature Review

2.1 Dengue virus

2.1.1 Genome of dengue virus

Dengue virus (DENV) is a small enveloped virus that consists of a positive sense single stranded RNA genome. DENV is classified under family *Flaviviridae* and genus *Flavivirus*. It is also referred to as arthropod-borne or arbovirus due to its mode of transmission which is via an arthropod vector such as mosquitoes. Its size is approximately 50 to 60 nm and there are ten proteins that encodes for its genome. The coat of the virus is formed by three of these structural proteins which also aids in delivering RNA to the target cells. The three structural proteins are the envelope protein (E), capsid protein (C) and membrane-associated protein (prM) (Chambers *et al.,* 1990). Figure 1 shows the illustration of DENV genome. Among the *Flavivirus,* the E protein is structurally conserved and it is made up of three distinct domains. The first domain, Domain I (DI), involves in the conformational changes which is crucial during viral entry and the escape of nucleocapsid from the endosomal compartment. Besides this, the second domain, Domain II (DII) and the third domain, Domain III (DIII) consists of the fusion loop and involve in the binding of cellular receptors respectively (Rey *et al.*, 1995). Meanwhile, the productions of new viruses once they enter the cells are orchestrated by seven other non-structural proteins, which are the NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (Chambers *et al.,* 1990). *Flavivirus* envelope is the defining features of the family which distinguish itself from the other family of viruses. DENV is enveloped by lipid membrane where a short transmembrane segment attached 180 identical copies of the envelope protein to the surface of the membrane. The envelope protein will attach to a cell surface and initiate the infection process (Chambers *et al.,* 1990). In order to study different aspect

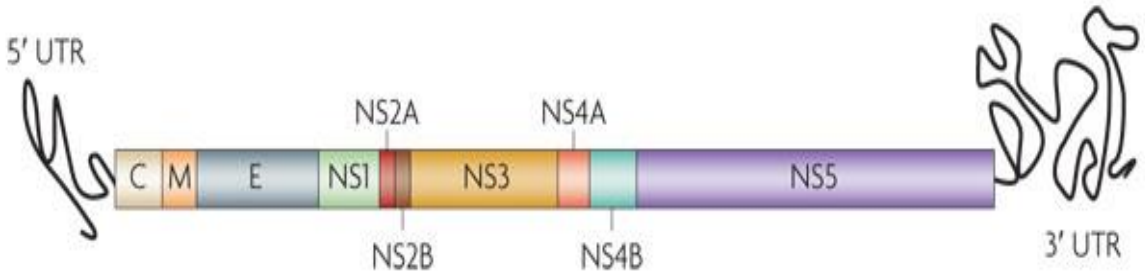

Figure 1. Dengue virus genome. The single open reading frame encodes three structural proteins which are the envelope protein (E), capsid protein (C) and membrane-associated protein (prM) as well as seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5).

(Adapted from "Dengue: A continuing global threat" by M. G. Guzman *et al*., 2010, Nature Reviews Microbiology, 8, p. 9.).

of the life cycle of dengue virus (DENV), cryoelectron microscopy has been used. Figure 2 shows the low resolution image of DENV obtained by electron microscope, whereby, in order to generate the final model, the atomic structures of the individual pieces are fit together into the image. Through detailed analysis of the structure, researchers have revealed that the antibodies block the normal action in DENV infection by distorting the arrangement of the envelope proteins (Kuhn *et al.*, 2002).

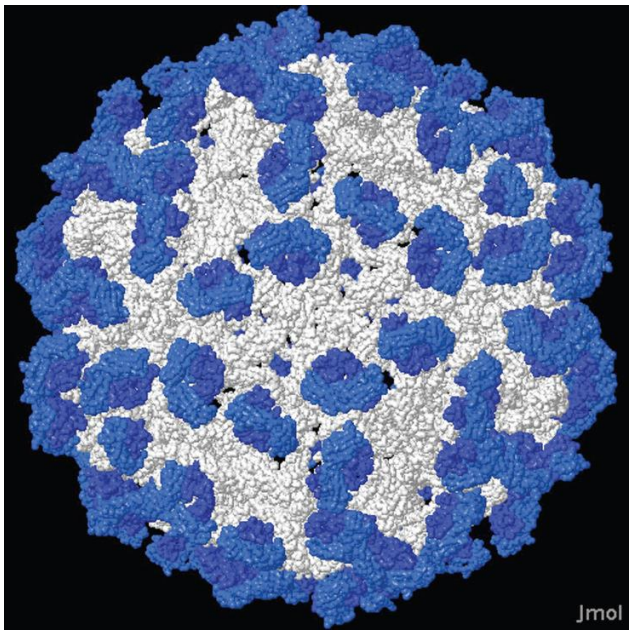

Figure 2. The low resolution image of DENV by electron microscope. The image shows that on the surface of the virus, the envelope protein (white) has numerous antibody Fab fragments (blue) bound to the viral proteins.

(Adapted from "Dengue Virus" by Protein Data Bank, 2008, retrieved from www.pdb.org).

2.1.2 Epidemiology of dengue virus

Dengue virus (DENV) is one of the most prominent threats to the public health as it affects subtropical and tropical regions worldwide, especially in the semi urban and urban areas. The incidences, severities and geographical spreads of dengue fever (DF) and dengue haemorrhagic fever (DHF) are rising in the Western Pacific, Eastern Mediterranean, Americas and South-East Asia region (*World Health Organization*, 1997). Moreover, there are about 2 500 million to 3 000 million people live in the DENV-transmittable areas, which eventually increase the risk of occurrence for dengue virus infection among the populations. Annually, there are around 500,000 cases of DHF and 50 to 100 million cases of dengue fever with 24,000 deaths have been reported (*World Health Organization*, 1997). About two-fifths of humanity or approximately 2.5 billion people living in sub-tropical and tropical regions are at risk of DENV infection (Gubler & Clark, 1995). Furthermore, this virus, which is one of the most crucial arthropod-borne arboviral diseases, has also been declared as endemic in South East Asia region by the World Health Organization.

In Malaysia, dengue fever was first reported in 1902 by Skae following the outbreak that had occurred in Penang between Novembers to December 1901 (Skae, as cited in Abubakar & Shafee, 2002). Since then, among the urban dwellers of Kuala Lumpur and Penang, a few other outbreaks had occurred, which was later followed by its widespread throughout Malaysia. Around that time, the local *Aedes albopictus* had been replaced by *Aedes aegypti* as the main carrier of dengue viruses. In 1960s, dengue virus had become endemic in Malaysia and in 1962, in Penang, the first laboratory-confirmed DHF case had been reported (Rudnick, as cited in Abubakar & Shafee, 2002). In Malaysia, throughout the years, the rate of incidence shows an upward trend whereby in 1999, there were 44.3 cases per 100,000 populations and increased towards 181 cases per 100,000

populations in 2007 (*Ministry of Health*, as cited in Nizal *et al.*, 2012). The causes for the remarkable emergence of dengue fever (DF) and dengue haemorrhagic fever (DHF) are too complex to be taken as a single entity as they involve a combination of interrelated factors to form the conditions that favour the transmission of dengue virus by the mosquito vector. The factors which may induce the transmission of DENV over the past few decades includes high population density, increase in domestic and international travel, rural urban migration as well as rapid urbanization which includes massive economic and industrial developments that creates a man-made environment. This environment is suitable for the mosquitoes to breed due to the inadequacies in some infrastructure such as lacking in proper solid-waste disposal. This eventually causes cities such as Melaka, Seremban, Kuala Lumpur and Penang to be hyper endemic to the transmission of dengue virus where there are more than one circulating virus serotypes appears at a certain time (*Ministry of Health*, as cited in Kumarasamy, 2006). Neither pathogenesis nor the molecular virology of dengue virus is well characterized despite its worldwide morbidity due to the complexity of dengue virus (Diamond *et al*., 2000).

2.1.3 Clinical symptoms of dengue virus infection

There are four different spectrums of illnesses caused by dengue virus (DENV) infection, which are the undifferentiated fever, classic dengue fever, DHF and dengue shock syndrome (DSS) (Rigau-Pérez *et al.,* 1998). Since dengue virus infection is associated with varieties of interrelated clinical illnesses, it is unfavorable for dengue fever to adopt a detailed clinical definition. As asserted by Rigau-Pérez *et al.* (1998), the clinical diagnosis for dengue virus infection may include a few of the following criteria, such as a continuous high fever for three days or more, retro-orbital pain, that is the backache and headache, spontaneous bleeding or petechial haemorrhage, hepatomegaly, reduction in platelet count that followed by an increment in the haematocrit, shock, vomiting and abdominal pain.

Moreover, all dengue cases need to be confirmed by laboratory test. Rigau-Pérez *et al.* (1998) reported that for dengue haemorrhagic fever (DHF), all the criteria must present, which includes, history of acute fever or fever that lasts between two to seven days, tendencies of haemorrhagic, thrombocytopenia and plasma leakage as a result of high vascular permeability, which can be marked by ascites, hypoproteinaemia and pleural effusion. On the other hand, dengue shock syndrome (DSS) is the more dangerous form of DHF, where it includes all four characteristics of DHF, together with the evidence of failure in circulatory system. There are four stages of DSS, ranging from fever together with non-specific symptoms and spontaneous bleeding for DSS Grade I and Grade II respectively, towards failure in circulatory in Grade III and finally intense shock whereby the pulse or blood pressure is undetectable in Grade IV (Rigau-Pérez *et al.,* 1998).

2.1.4 Laboratory diagnosis of dengue virus infection

Generally, the incubation period of dengue virus (DENV) is between three to ten days or an average of four to six days (*World Health Organization*, 1997). The Primary and Secondary immune response are the two patterns of host serological responses towards DENV infections. There are four serotypes of DENV, which are DENV-1, DENV-2, DENV-3 and DENV-4 that are classified based on their immunological and biological criteria. Infection with one serotype does not confer immunity to the other serotypes. Instead, successive infection with a different DENV serotype will leads to a more severe outcome due antibody-dependent enhancement (ADE) (Halstead, 1988). In ADE, the second infecting DENV serotype and the pre-existing sub-neutralizing antibodies from the primary infection will form complexes that bind to the cells, thus, eventually causes an increase in DENV uptake as well as its replication (Halstead, 1988). Figure 3 shows the model for ADE of DENV replication. In different geographical regions but at the same

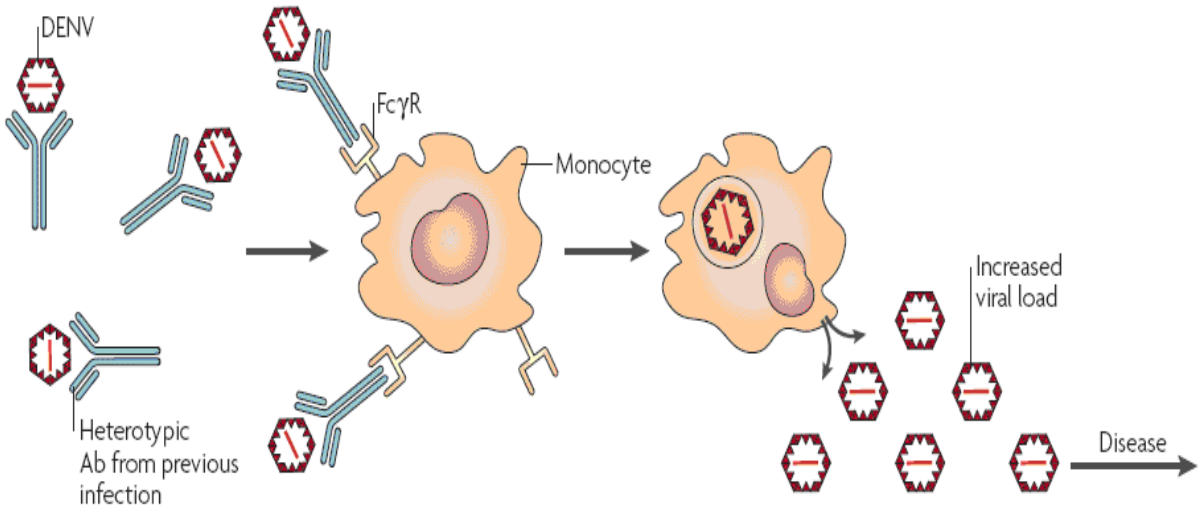

Figure 3. Illustration for antibody-dependent enhancement of dengue virus replication.

(Adapted from "Prospects for a dengue virus vaccine" by S. S. Whitehead *et al.*, 2007, Nature Reviews Microbiology, 5, p. 520.).

season, all four serotypes of DENV can circulate concurrently, where depending on the immunity or susceptibility of the population, one serotype may predominate over the other serotypes (*World Health Organization*, 1997). For instance, in Malaysia, DENV-3 was predominant in 1990 to 1995 followed by DENV-2 from 1997 to 2000 (Kobayashi *et al.*, 1999).

Laboratory diagnosis of dengue virus (DENV) infection can be generally divided into three methods, which are serological methods, virus isolation and molecular techniques. The most conventional serological method is the traditional haemagglutination inhibition (HI) test (Clarke & Casals, 1958). However, it is labour-intensive as it needs three days to be carried out with both convalescent and acute serum samples. Therefore, the much faster and simpler serological test, the enzyme linked immunosorbent assay (ELISA), has been used in most hospital laboratories in this country. Another method for the laboratory diagnosis of DENV infection is via molecular technique. This technique involves the amplification and detection of DENV RNA by reverse transcription polymerase chain reaction (RT-PCR). Besides this, another method for the laboratory diagnosis of DENV infection is the virus isolation. Although it is time consuming, costly and laborious, which causes it to be performed in only a few research laboratories, it is by far the most definite method.

2.2 Cell lines

2.2.1 Types and selection of cell lines

Generally, there are three types of cell cultures that are used for virus inoculation and isolation in the laboratory which includes primary cultures, diploid cell cultures and continuous cell line cultures (Payment & Trudel, 1993). Some of the cell cultures are susceptible to several types of viruses while others are quiet specific. Examples of

commonly used cell lines are African green monkey kidney cultures, such as Vero, BGM and BSC-1 as well as Rhesus monkey kidney (RhMK) primary cultures. Besides this, WI-38 (Wistar-38) human embryonic lung fibroblast and Hep-2 cells which is the human larynx carcinoma are also used (Payment & Trudel, 1993). Another example of cell line is the mosquito cell line, for instance AP-61 from *Aedes pseudoscutellaris,* C6/36 from *Aedes albopictus,* and TRA-284 from *Toxorhynchites amboinensis* (Kuno *et al.,* 1985).

There are wide varieties of cell lines which can be infected by dengue virus (DENV) where the virus has different consequences towards different cells lines. Shafee and Abubakar (2011) reported in their study that there were different patterns of DENV infection in different cell lines which reflected different degree of the cell lines' permissiveness towards DENV infection. Furthermore, through the microscopic and immunostaning experiments done in their study, it also showed that there were different levels of apoptosis induced by DENV in different cell lines, which imply that the programmed cell death was dependent on the unique characters of each cell line (Shafee & Abubakar, 2011).

Several factors should be considered when selecting the most suitable cell line for DENV inoculation. The types of cell lines, either finite or continuous, plays a crucial role since technically, continuous cell lines grows faster and easier to be maintained. Moreover, it can produce a higher yield per flask and easier to be maintained when compared to the finite cell lines. In addition, the species origin of the cell line is also pivotal as there are lesser biohazard restrictions for non-human cell lines. Furthermore, the stability of the cell lines and the characteristics of the virus growth are also other factors that should be considered during the selection process. In this study, two types of cell lines were used, which were C6/36 cell line and Vero cell line.

2.2.2 C6/36 cell line

The most common method for isolation of viruses is through the use of mosquito cell cultures (Pant, as cited in Samuel & Tyagi, 2006). In 1978, C6/36 cell line had been initially derived from the larval tissue of *Aedes albopictus* mosquito. C6/36 cell line and two other mosquito lines which are the AP-61 from *Aedes pseudoscutellaris* and TRA-284 from *Toxorhynchites amboinensis* are used as sensitive assays for the isolation of viruses from patients as they are highly susceptible to dengue virus (DENV) infection (Gubler, 1998). Besides this, they can also support other arboviruses multiplication such as sindbis and chikungunya viruses as well as being highly stable since their optimal growth occurs at lower temperature when compared to other mammalian cells. Furthermore, the use of C6/36 cell line for the isolation of DENV has been proven to be economical, highly sensitive and rapid whereby in a relatively short time, large number of samples can be processed (Samuel & Tyagi, 2006). The mosquito cell lines' sensitivity may vary depending on the virus strain inoculated. Nevertheless, mosquito cell cultures have a higher sensitivity for the isolation of DENV when compared to those of mammalian cell cultures. C6/36 cell line is also mainly used for virus isolation during the periods of epidemic.

2.2.3 Vero cell line

Another permissive cell line for DENV propagation is Vero cell line. In 1962, this Vero cells were extracted from African green monkey, *Cercopithecus aethiop,* and were isolated from its kidney epithelial cells. There were varieties of results reported from numerous experiments over the past few decades on the infectivity of DENV towards Vero cell line. Boege (as cited in Anderson et *al.*, 1992) asserted that the dimeric form of the non-structural-1 protein (NS1) as well as viral structural proteins, E, C and M will be secreted

by the Vero cells that had been infected by DENV-4. On the contrary, there were no forms of NS1 secreted by C6/36 upon the infection of DENV-4 although it did secreted viral structural protein. The susceptibility is affected by several factors, where one of it is the virus serotype. This was shown in the experiment conducted by Anderson *et al.* (1992), who reported that Vero cells were more permissive to DENV-4 infection when compared to other cell lines since Vero cells that displays viral antigen were more than 85% of the total cell. Compared to other cell lines, viral propagations on Vero cell line are influence by the external factors such as the presence of chemicals, additive and the ambient temperature (Stim, 1970). Therefore, a distinct condition is required for the optimum utility in Vero cell line.

2.3 Previous research of DENV infection on C6/36 cell line and Vero cell line

There have been numerous reports on the effectiveness of dengue virus (DENV) isolated in different cell lines. As mention earlier, *in vitro* propagation of DENV commonly use either mosquito or monkey cell lines due to their high permissiveness and infectivity when compared to human cell lines (Wikan, as cited in Shafee & Abubakar, 2011). Although technically both cell lines should show approximately the same end titer of dengue virus in responds to DENV infection, but, experimentally, both cell lines showed different result in different experiment conducted. In 1969, Singh (as cited in Pervin *et al.,* 2003) reported that there were frequent viral growths in both C6/36 and Vero cell lines, while in 1984, Gubler (as cited in Pervin *et al.,* 2003) explained that apparently, some viruses grow slowly in C6/36 cells. Nevertheless, Syndow *et al.* (2000) explained that most of the experiment reported throughout time showed that there was a higher rate of viral infection for C6/36 cells compared to those of Vero cells in a tendency line of linear regression. Nonetheless, Syndow *et al.* (2000) added that both cell lines still showed a high and approximate rate of infectivity towards DENV. At the same time, Pervin *et al.* (2003) also