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COMPARATIVE TRANSOVARIAL TRANSMISSION OF DENGUE VIRUS SEROTYPES 1, 3 AND 4 IN WILD TYPE AND TRANSGENIC *AEDES AEGYPTI* FEMALE MOSQUITOES

By

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ABSTRACT

COMPARATIVE TRANSOVARIAL TRANSMISSION OF DENGUE VIRUS SEROTYPES 1, 3 AND 4 IN WILD TYPE AND TRANSGENIC *Aedes aegypti* FEMALE MOSQUITOES

Suria Marlina

Dengue, a viral fever transmitted by *Aedes* mosquito species, is the most rapidly spreading vector borne disease in the world with over 100 million cases per year in over 100 countries. There are no vaccines or antiviral drugs available to mitigate the disease and vector control remains the only option to control dengue. The sterile insect technique with Releasing Insect Carrying a Dominant Lethal (RIDL) is a new biotechnology approach for vector control. The aim of this study was to examine the transovarial transmission of dengue virus (DENV) serotypes 1, 3 and 4 in wild type (WT) and RIDL female *Aedes aegypti*. A total of 300 female mosquitoes from both strains were fed with blood containing dengue virus using an artificial membrane feeding technique and another 300 mosquitoes served as the non-infected control. Fully engorged mosquitoes were selected and separated into individual containers for egg laying. The viral load in the pools of larvae (20 larvae/pool) was determined by real-time RT-PCR for a period of 14 days post-infection.

Positive samples from the real-time RT-PCR analysis were further confirmed by virus isolation using the mosquito C6/36 cell line. The highest transovarial transmission rate was detected in DENV-1 (14% for RIDL; 17% for WT), followed by DENV-3 (10% for RIDL; 6% for WT), and DENV-4 with the lowest transovarial transmission rate (3% for RIDL; 2% for WT). The highest viral copy numbers recorded for DENV-1, -3 and -4 in the RIDL mosquitoes were $2.22x10^{9}$, $3.60x10^{1}$ and $4.82x10^{6}$ copies/pool, respectively. The corresponding values in the WT mosquitoes were $3.64x10^{9}$, $5.94x10^{4}$ and $1.32x10^{1}$ copies/pool, respectively. The results indicated that there was transovarial transmission in both RIDL and WT strains. The present study also showed that RIDL and WT mosquitoes were most susceptible to DENV-1. However, there were no significant differences ($p>0.05$) in transovarial transmission between RIDL and WT mosquitoes for all the dengue serotypes evaluated.

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APPROVAL SHEET

This dissertation entitled **"COMPARATIVE TRANSOVARIAL TRANSMISSION OF DENGUE VIRUS SEROTYPES 1, 3 AND 4 IN WILD TYPE AND TRANSGENIC *AEDES AEGYPTI* FEMALE MOSQUITOES"** was prepared by SURIA MARLINA BT. MANSOR and submitted as partial fulfillment of the requirements for the degree of Master of Science at Universiti Tunku Abdul Rahman.

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It is hereby certified that **Suria Marlina Bt. Mansor** (ID No: **0909159**) has completed this dissertation entitled "Comparative Transovarial Transmission of Dengue Virus Serotypes 1, 3 and 4 In Wild Type and Transgenic Aedes Aegypti Female Mosquitoes" under the supervision of Dr. Sit Nam Weng (Supervisor) from the Department of Biomedical Science, Faculty of Science, and Dr. Khoo Kong Soo (Co-Supervisor) from the Department of Chemical Science, Faculty of Science.

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DECLARATION

I hereby declare that the dissertation is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

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(SURIA MARLINA BT. MANSOR)

Date: 2 MAY 2014

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LIST OF ABBREVIATIONS

Bti	*Bacillus thuringiensis* H-14
Co.	Company
CPE	Cytopathic effect
DENV	Dengue Virus
DHF	Dengue Hemorrhagic Fever
DSS	Dengue Shock Syndrome
ELISA	Enzyme-linked immunosorbent assay
IF	Immunofluorescence
IgG	Immunoglobulin G
IgM	Immunoglobulin M
L-15	Leibovitz L-15 medium
mL	Milliliter
Nm	Nanometer
RIDL	Release of Insect Containing a Dominant Lethal
RNA	Ribonucleic acid
Rpm	Revolutions per minute
SIT	Sterile Insect Technique
Spp	Species
TPB	Tryptose Phosphate Broth
tTA	Tetracycline-controlled transactivator
µL	Microliter

CHAPTER 1

INTRODUCTION

1. Introduction

Dengue is one of the main mosquito-borne diseases in the world, mainly in countries situated in the equatorial region having high temperatures and humidity that are favorable for its transmission (Gubler, 1998a). According to World Health Organization (2010), in the last two decades, dengue has become endemic in more than 100 countries as well as in Africa, Southern America, Eastern Mediterranean, Southeast Asia, and Western Pacific (Figure 1.1). It is estimated that 2.5 billion people are at risk, with as many as 50 million cases worldwide each year. The disease is hyperendemic in Southeast Asian countries, as dengue hemorrhagic fever (DHF) is more severe and often a deadly disease in children. As Southeast Asia and the Western Pacific are the most severely impacted, the disease is quickly spreading to previously unaffected regions (WHO, 2010).

Gubler (1998a) reported that factors such as urbanization, poor sanitation in newly urbanized areas, climatic changes and viral evolution, which have

increased virus transmission have been linked to El Nino conditions, poor vector control, unplanned urban overpopulation of areas leading to inadequate housing and public health systems such as water system, sewerage and waste management and the increase of air travel.

All of these factors are implicated in the rapid spread of dengue and must be measured in order to combat dengue. Unplanned urbanization is believed to have had the largest impact on disease amplification in individual countries, whereas air travel is believed to have had the largest impact on global spread (Gubler, 1998a).

1.1 History of Dengue

Historically, *Aedes aegypti*, a native mosquito of Africa was introduced to the Americas in 1600s by the slave trade. It is an extremely competent vector of yellow fever and dengue. *Aedes aegypti* was dispersed around the world during the 18^{th} and 19^{th} centuries through the shipping trade, which invaded port cities and then moved inland along routes of transportation (Gubler, 1997). According to Gubler (2011), dengue epidemics happened at irregular periods because since the vectors relied on sailing ships to transport them between the continents. The consequence was major epidemics of dengue in Asian and American countries. In 1635, French West Indies was documented as the first epidemic of dengue,

although dengue had been described in China as early as 992 AD (Gubler, 1998b). The Chinese termed the disease as “water poison” as it was alleged to be related with flying insects allied with water (WHO, 2011).

During the period prior to World War II, *Aedes aegypti* prevalence and distribution throughout the Asian and Pacific Islands region had an enormous augmentation due to unrestrained development, mobility of humans that was expedited by aircraft, insufficient water and sewage management, as well as unmanageable vector control programs (Gubler, 1997).

During the war, water storage was increased for domestic use as well as for fire control, because the existing water system was destroyed. Large amounts of war equipment were abandoned as they were moved between cities and countries; therefore providing ideal habitat for *Aedes aegypti*, because this equipment collected rain water, resulting in the movement of mosquitoes to new geographical locations (WHO, 2011). Furthermore, consistently moving thousands of Japanese and allied soldiers between countries in Asia and the Pacific, caused most of them prone to dengue virus (DENV) infection. On the other hand, this provided ideal conditions for movement of viruses between cities, countries and other regions as well as susceptible individuals for epidemics transmission. The war was the likely cause for generating the conditions for the emergence of DHF in Southeast Asia (WHO, 2011).

Figure 1.1: Map of dengue distribution
(Center for Disease Control and Prevention, 2010)

1.2 Dengue Transmission

The main vectors of dengue are *Aedes aegypti* and *Aedes albopictus*. Both species show high vectorial competency for dengue viruses (WHO, 2012). *Aedes aegypti* shows higher vectorial capacity compared to *Aedes albopictus* in an urban epidemic cycle (Lambrechts et al., 2010). This is because *Aedes aegypti* is highly domesticated, strongly anthropophilic and requires numerous feedings for the completion of the gonotrophic cycle; cycle of taking a blood meal and then laid a batch of eggs (Lumsden, 1957; Nelson et al., 1978). In contrast, *Aedes albopictus* partially invades minor areas of urban cities and the species does not require a second blood meal to complete the gonotrophic cycle.

The transovarial transmission arises when the dengue virus is passed from infected female mosquito to its offspring (Suzana et al., 2011). Studies by Aitken et al. (1979) and Halstead (1988) indicated the incidence of transovarial transmission in several species of mosquitoes including *Aedes* for yellow fever and dengue viruses.

1.3 Problem Statement

About 25,000 fatalities occur annually as a result of dengue infection. Serious cases require hospitalization and intensive cared. At present, there is no vaccine available that confers protection from four serotypes of dengue viruses (Lee et al., 2007).

Conventional methods such as space spraying with insecticides and breeding site reduction are ongoing strategies to control *Aedes aegypti* and *Aedes albopictus* mosquitoes. Public education and 'COMmunication for Behavioural Impact' (COMBI) program involving the public are advocated to sustain breeding site reduction. According to Lee et al. (2007), neither breeding site elimination nor larviciding is practical for a large and diverse country like Malaysia.

Both dengue vectors are able to survive in artificial containers in and nearby the houses which are closer to human. According to Reiter et al. (1995), though space spraying has, at best, a limited impact, it may have long term consequences such as resistance and contamination in the environment. At present, it is not likely that an integrated vector control program could completely do away with space spraying. Field assessment of new control agents such as pyrethroid formulations and surfactant monomolecular films against *Aedes aegypti* adults and larvae have been conducted; but these are only effective under certain conditions.

Furthermore, microbial control agent such as *Bacillus thuringiensis* (Bti) has been formulated and used against container breeding *Aedes* larvae. Unfortunately, none of these methods can prevent dengue outbreak, mostly due to low threshold of transmission (Lee et al., 2007). This is because the threshold for dengue transmission is as low as 2 – 3 adult female mosquitoes to emerge every day in a neighborhood of 100 persons (Focks et al., 2000; Lee et al., 2007).

As the maintenance of dengue virus at a low level of transmission within the human population, transovarial transmission is considered to be a significant measure in its conservation during inter-epidemic phases. The part of viral conservation during this period in *Aedes albopictus* other than its primary vector has also been documented by Rodhain and Rosen (1997). It has clearly suggested that transovarial transmission may provide a mechanism for the virus to maintain itself in an area through the low mosquito season; this is further suggested by studies demonstrating the possibility of maintenance of the virus through several generations of *Aedes aegypti* (Joshi et al., 2002) and *Aedes albopictus* (Shroyer, 1990) without further exposure to the virus.

Research on the evaluation of new technologies that seem to be a promising biotechnology solution to suppress the *Aedes* population below the dengue transmission threshold has to be deployed. In addition to the environmental management, chemical and biological control, RIDL-based transgenic mosquito signifies an alternative approach of suppressing the dengue

vector. The potential application of transgenic mosquito in the field has received much attention from the public and scientific community with regards to its risk, efficacy, and impact to the environment. Scientific studies are obligatory to address all these issues prior to field release of the mosquito.

In the previous study, female RIDL and WT *Aedes aegypti* mosquitoes were infected with DENV-2. The result showed that transovarial transmission had occurred in both females (Ali et al., 2010). Since the RIDL progeny will die before emerging as adults and being able to reproduce or bite; it is very unlikely that the strain would play any role in the maintenance of a virus in the field. Consequently, the lethal gene insertion does not affect the transovarial transmission when it occurs. This study has also showed the importance of larval control since the immature stages may become the reservoir of the virus during the inter-epidemic period. It is clearly revealed that the suppression of the early phase of *Aedes* mosquito and the eradication of breeding sources should be emphasized.

The present study aimed to assess the risk of transovarial transmission of three different serotypes of dengue virus which have not been investigated in RIDL *Aedes aegypti* mosquitoes. In this project, when female *Aedes aegypti* mosquitoes from a cohort are subjected to infected blood meal with DENV-1, DENV-3 and DENV-4, statistically significant difference in transovarial transmission rates of dengue virus between a transgenic strain and the unmodified wild type strain will be detected.

1.4 Research Objectives

1.4.1 General Objective

To compare the transovarial transmission of DENV-1, DENV-3 and DENV-4 in wild type (WT) versus transgenic RIDL *Aedes aegypti* female mosquitoes.

1.4.2 Specific Objectives

1. To determine and quantify the infection rate in larvae by real-time RT-PCR.
2. To compare and determine the number of eggs laid and number of larvae hatched by both RIDL and WT strains.

CHAPTER 2

LITERATURE REVIEW

2.1 Dengue Viruses

Dengue is a human disease caused by a virus transmitted by *Aedes* spp. The dengue virus (DENV) comprises four immunologically related, single positive-stranded ribonucleic acid (RNA) viruses named DENV-1, DENV-2, DENV-3 and DENV-4 that occur in several tropical regions of the world (Gubler, 1997). The virus belongs to genus *Flavivirus* and family Flaviviridae, which contains approximately 70 viruses (Westaway and Blok, 1997). The flavivirus is sphere-shaped with a diameter of 40-50 nm and covered with a lipid envelope. The virus genome is estimated at 11 kilobase pairs and encodes three structural and seven nonstructural proteins (Gubler, 1998b). Flaviviruses have similar clutch epitopes on the envelope protein that produce cross reactivity in serological tests. These make unequivocal serological diagnosis of flaviviruses including dengue very difficult. Moreover, each serotype is also composed of different genotypes, epitomizing the widespread genetic variability of the dengue serotypes (WHO, 2011).

The first dengue viruses were isolated from soldiers who fell sick in India (Calcutta), New Guinea and Hawaii in 1945 (Sabin, 1952). The viruses from India, Hawaii and one serotype from New Guinea were antigenically similar, while three other serotypes from New Guinea appeared to be different. They were designated as prototype viruses, i.e., DENV-1, Hawaii and DENV-2, New Guinea-C. Two more serotypes, DENV-3 and DENV-4 were subsequently isolated from patients with hemorrhagic disease during an epidemic in Manila in 1956 (Gubler, 1998b).

Primary infection with DENV provides permanent resistance to the infecting serotype but does not confer long term cross-protective immunity to other serotypes (Halstead, 1974). Once infected, an individual acquires lifetime immunity to a specific serotype of the virus, but may become more susceptible to the other three serotypes (Guzman and Vazquez, 2010). As the secondary infection progresses, with more severe clinical manifestations, it can be correlated with higher viremia (Cordeiro et al., 2009). This is because the antibodies raised against the primary infecting dengue virus serotype may cross-react with the subsequently infecting dengue virus serotype and may function to enhance the ability of the second dengue virus serotype to infect the host, in a process called antibody-dependent enhancement of infection (Porterfield, 1986; Halstead, 2002).

In India, DENV-3 was the most prevalent serotype followed by DENV-1 during the 2006 outbreak (Preeti et al., 2008). All the four dengue virus serotypes were found to co-circulate in the outbreak of 2006 as DENV-3 endured the

predominant serotype as reported in 2003–2004 (Dar et al., 1999; Dash et al., 2005). In Nicaragua, DENV-3 was found circulating from 1994 until 1998 (Guzman et al., 1996; Harris et al., 2000), while in 1999–2001, DENV-2 was identified as the most predominant dengue virus (Balmaseda et al., 2006). However, in 2003, DENV-1 was reported as the most prevalent dengue serotype. When the two periods were compared, more shock and internal hemorrhaging were observed in the period when DENV-2 predominated, whereas increased vascular permeability was associated to a greater degree with DENV-1 (Balmaseda et al., 2006). This indicated that DENV-2 produced more severe disease than DENV-1. In a study covering 25 years of dengue cases in Thailand, DENV-2 was found to be the most frequent serotype isolated from dengue hemorrhagic fever or dengue shock syndrome cases (35%), followed by DENV-3 (31%), DENV-1 (24%), and DENV-4 (10%) (Nisalak et al., 2003).

In Malaysia, for a three decade period from the 1960's to the 1990's, DENV-2 was the most prevalent serotype (Rudnick et al., 1965; Chee and Abubakar, 2003). In contrast, Lam (1993) reported that from 1973 till 1992, DENV-3 was the most predominant virus in dengue outbreak in Malaysia followed by DENV-2 and DENV-1. The Ministry of Health (2010) of Malaysia had reported that all four serotypes can be isolated at any one time but the predominant circulating dengue virus showed a sinusoidal pattern (Figure 2.1). For example, DENV-3 was the predominant serotype in the early 90s which peaked in 1993, and subsequently declined. It then reemerged, reaching the peak in 2001.

A national data had showed that the dominant DENV serotypes circulating in Malaysia had changed persistently from year 2000 until 2012, from DENV-2 in 2000, to DENV-3 in 2001-2002, DENV-1 in 2003-2005, DENV-2 in 2006-2009 and DENV-1 in 2010-2011 (Yusoff, 2008; Mohd-Zaki et al., 2014) (Figure 2.2). On the other hand, DENV-4 was less prevalent and established less than 20% of infection throughout the periods compared to the other three serotypes of dengue viruses (Yusoff, 2008; Mohd-Zaki et al., 2014). In 2008, a new serotype of dengue virus, named DENV-5 was discovered in Sarawak and it was phylogenetically distinct from the other four serotypes (Vasilakis et al., 2011). To date, DENV-5 has only been related to an outbreak in humans and it was believed that the virus is circulating, perhaps between the monkeys in the forests of Sarawak (Vasilakis et al., 2011).

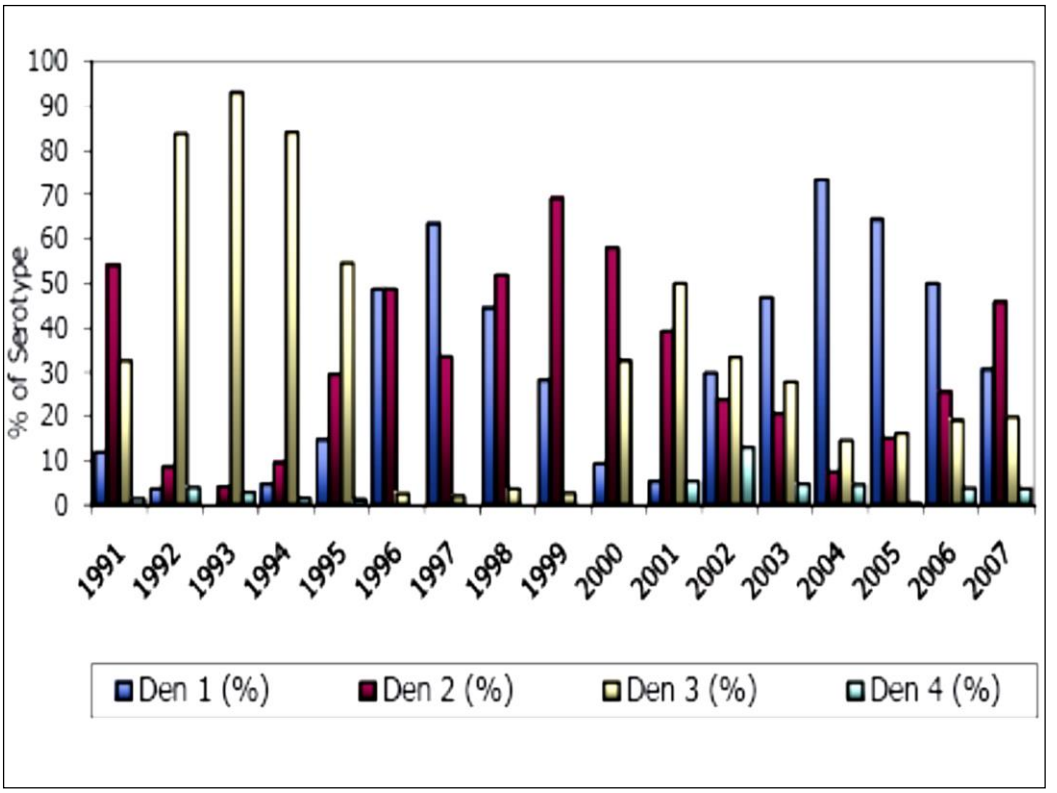

Figure 2.1: Percentage of dengue serotypes in Malaysia (1991 – 2007) (Ministry of Health, 2010. p. 3)

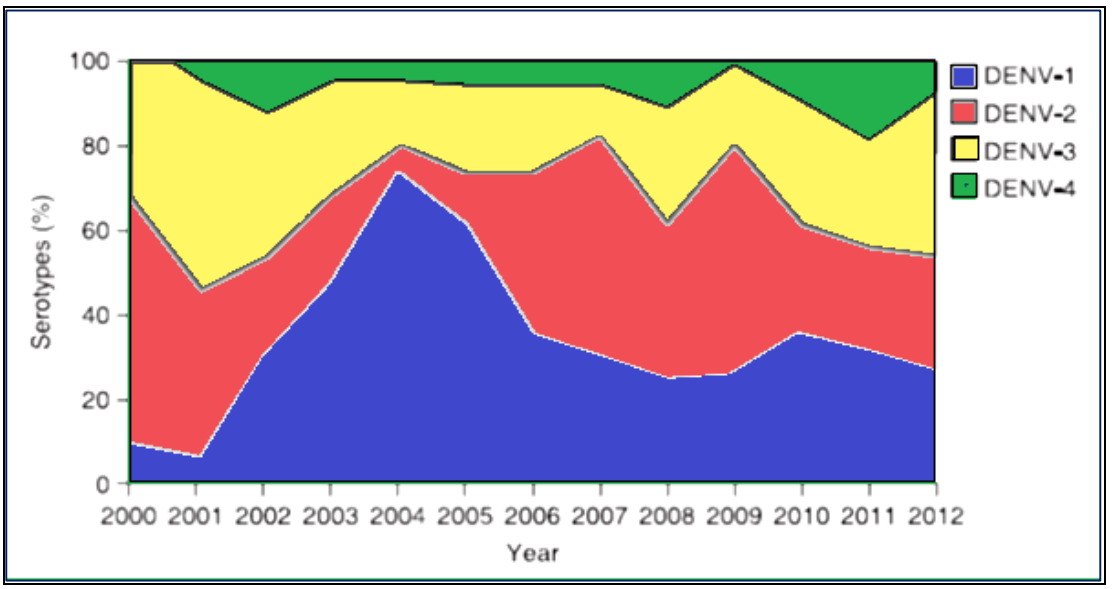

Figure 2.2: Circulation of dominant DENV serotypes in Malaysia throughout the period of 2000 to 2012 (Mohd-Zaki et al., 2014).

2.2 Reemergence of Dengue and Dengue Hemorrhagic Fever

There are three types of dengue fever, namely classical dengue fever (DF), dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) (Gubler, 1998b; Johnson et al., 2005).

Dengue fever is seen in syndromes that are age-dependent. Infants and children may have undistinguishable febrile illness or mild febrile disease with maculopapular rash. Older children and adults usually have an obvious illness characterized by fever, headache, myalgia, and gastrointestinal symptoms, often terminating with a maculopapular rash.

Dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS) proceeds through two stages (Halstead, 1980; Gubler, 1998b). The illness begins with sudden onset of fever accompanied by dengue-like symptoms. DHF and DSS are characterized by a reduced immunoglobulin M (IgM) response, an increase in vascular permeability and hemorrhage, followed by vascular collapse, which may cause fatality. In a crucial form of DHF, blood vessels begin to leak and lead to bleeding from the nose, mouth and gums. Deprived of quick treatment, the blood vessels can breakdown, causing shock (dengue shock syndrome) and eventually death (Gubler, 1998b).

2.2.1 Worldwide Distribution of Dengue

Dengue fever is the most common arthropod-borne disease; it is spreading rapidly in the world, and 55% of the world's population is at risk for dengue (WHO, 2006). According to WHO (2010), the incidence has increased 30-fold, with increasing geographic expansion to new countries and also from urban to rural settings in the last 50 years. A number of 1.8 billion of the population at risk for dengue worldwide live in member states of the WHO South-East Asia Region and Western Pacific Region, which bear nearly 75% of the current global disease burden due to dengue (WHO, 2010). At present, dengue is considered as reemergent disease especially in the Eastern Mediterranean region (Rathor, 2000).

Countries situated in the equatorial region, having high temperature and humidity are ideal grounds for dengue transmission (Rohani et al., 2009). In the 1970s, epidemic activity increased in the Pacific Islands and Americas since dengue was reintroduced into the region in 1970 (Gubler, 1998b). Epidemic dengue transmission intensified since the 1980s due to the expansion of the geographic dispersal of the vectors as well as the viruses. It now causes not only a global resurgence of dengue fever, but also the emergence of DHF in many new countries (Rosen, 1982; Halstead, 1992; Gubler and Clark, 1995; Pinheiro and Corber, 1997).

The main epidemiological changes of dengue in America occurred during the 1950s until 1970s (Figure 2.3). Epidemic dengue was uncommon in America, as the main dengue vector, *Aedes aegypti* had been eradicated from most of Central America and South America regions. The eradication program was discontinued in the beginning of 1970s, and as a consequence, this species then began to reinvade the countries from which it had been eradicated (Pinheiro and Corber, 1997; Gubler, 1998b).

Hyperendemicity (the co-transmission of various dengue virus serotypes) was established in Southeast Asian capitals with the increase of epidemic transmission and epidemic dengue hemorrhagic fever (DHF) had emerged (Halstead, 1980; Halstead, 1992; Gubler, 1998a). The current emergence of dengue creates a serious public health risk, and now the disease is considered as

one of the most important arthropod-borne human viral diseases in terms of morbidity and mortality (WHO, 2009). An estimation of 50-100 million new infections occurs yearly, with 200,000 to 500,000 cases of potentially life-threatening DHF or DSS (Luplertlop et al., 2006; Srikiatkhachorn and Green, 2010).

In Southeast Asia, the major event in the history of dengue was the appearance of epidemics of dengue hemorrhagic fever. First in Manila, Philippines, in 1956, then more widely in Luzon, in 1958, followed by Bangkok, Thailand, in 1958 and 1961, in Singapore in 1960-61, Penang, Malaysia, in 1962-64 and finally in Kuala Lumpur, Malaysia, in 1963 (Smith, 1986). In 1956, the first ever known epidemic of DHF arose in Manila, Philippines. Within 20 years it had spread through the entire of Southeast Asia; and DHF had become a prominent source of hospitalization and mortality among the descendants of the area in the mid-1970s (Rudnick, 1986). In Asia, epidemic DHF has expanded geographically from Southeast Asian countries west to India, Sri Lanka, the Maldives, and Pakistan, and east to China (Gubler and Clark, 1995). Niue, Palau, Yap, Cook Islands, Tahiti, New Caledonia, and Vanuatu were the numerous island countries of the South and Central Pacific that have proficient either major or minor DHF epidemics (Gubler, 1997).

Factors that contributed to the reemergence of dengue were the rapid growth and urbanization of populations, the increased travel of people, which

facilitates distribution of dengue viruses, the circulation of all four dengue serotypes thus increasing the risk of DHF, and the lack of the vector control programs (Gubler, 1997). The practice of water storage in domestic settings due to the problems of water supply and the exponential growth of containers that can hold water such as tyres and disposable containers greatly contribute to the increase of vector densities favoring virus transmission (Gubler, 1997).

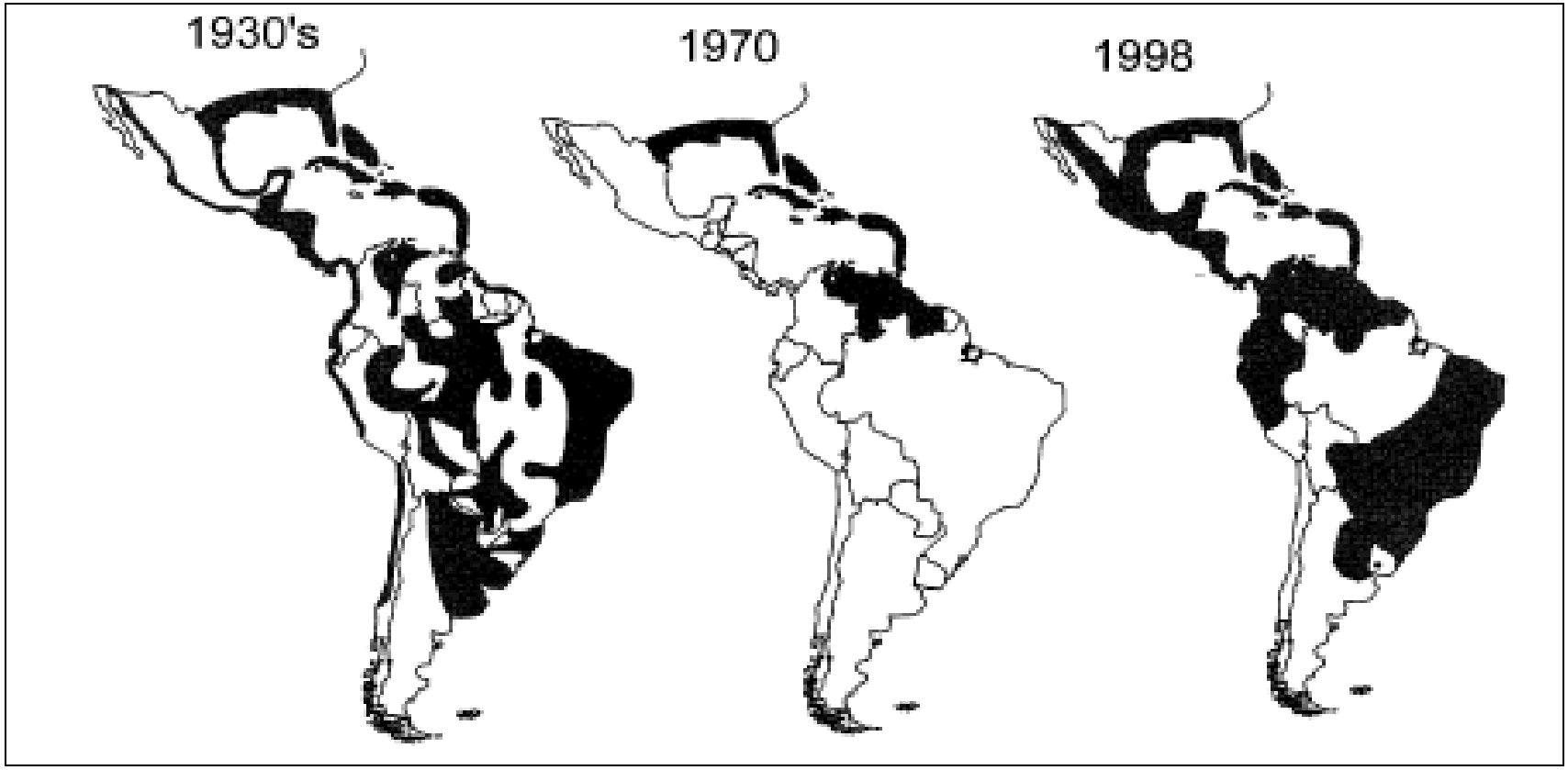

Figure 2.3: *Aedes aegypti* dispersal in the America throughout the 1930s, 1970 and 1998 (Gubler, 1998b. p. 481)

2.2.2 Malaysia

In Malaysia, dengue is sustained by the native *Aedes albopictus* before the introduction of the alien *Aedes aegypti* during the turn of the 20th century (Smith, 1956). In early 1900s, *Aedes aegypti* was reported in Port Swettenham (Port Klang) and Klang, but not in inland of peninsular Malaysia (Daniels, 1908; Leicester, 1908). By 1910s, *Aedes aegypti* had replaced *Aedes albopictus* as the main species in Kuala Lumpur (Stanton, 1920). It was more or less confined to the towns; where it was not found in many inland villages. A survey on 49 towns in West Malaysia by Macdonald (1956) discovered that zero or almost zero *Aedes aegypti* indices were recorded for a large number of towns comprising Sungkai, Kampung Kerdas, Pedas, Dusun Tua, Mentakab and Kuala Kubu Bharu which are all situated inland. Cheong (1974) made a survey of 157 towns and found that *Aedes aegypti* had made inroads in these towns. This supported the suggestion that *Aedes aegypti* was introduced from the wild populations of Africa forests to Malaysia seaports by shipping.

Dengue fever had been reported in Malaysia as early as 1902, but the more threatening dengue infection with hemorrhagic sign was first reported in Penang in 1962 (Rudnick et al., 1965). Penang General Hospital reported 62% of cases with hemorrhagic manifestations and 45% cases presented with shock syndrome fever. In between November 1962 to July 1963, 41 pediatric cases with a provisional

diagnosis of dengue hemorrhagic fever were admitted to the Penang General Hospital of which five were fatal (Rudnick, 1986).

From 1975 to 1980, though the number of reported cases increased, due to the improved surveillance and public health education, dengue remained at an endemic level with the classical dengue fever syndrome most regularly diagnosed compared to dengue hemorrhagic fever (Rudnick, 1986). During these five years, reports of dengue fever from small towns and populations centers in rural areas increased. These were associated with the apparent spread and increase of *Aedes aegypti* population in many parts of the peninsular (Rudnick, 1986). The number of DF cases had significant intensified in following year of 1974, 1982, 1987, 1991 and 1998 with a longer silence period of 8 and 7 years in DF/DHF incidence between 1974 to 1982 and 1991 to 1998, respectively. Smaller incidence peaks of 5 and 4 years was prominent between 1982 to 1987 and 1987 to 1991, respectively (Abubakar and Shafee, 2002). The total annual number of dengue disease cases reported for both DF and DHF had increased from 7,103 cases in year 2000 to 46,171 cases in year 2010, with the majority of the cases were reported as DF (Mohd-Zaki et al., 2014).

The factor accountable for the exaggerated recovery and rise of epidemic dengue over the past 50 years is closely associated with demographic and social changes. Sudden worldwide population evolution and related unplanned and uncontrolled urbanization are identified as the major factors for humid and

evolving countries, such as Malaysia (Gubler, 1998a). Study conducted in Sarawak by Chang et al. (1997) indicated that forest clearance switching to oil palm plantation led to the change of species composition of malaria to dengue vectors in relation to an increase in both their relative density and incidence.

2.3 Mosquitoes

Mosquitoes belong to the family Culicidae, comprising 3,100 species from 34 genera within the two-winged flight of the Diptera order (Goma, 1966; Service, 1996). They are one of the most primitive families of Diptera as being more closely related to midges, gnats, and crane flies. These insects can be found all over the world except for the cold places. The habitat for three quarters of all mosquito species can be found in the tropics and subtropics, as the warm moist climate is suitable for rapid development and adult survival (Service, 1996). Mosquitoes demonstrate ‘complete metamorphosis’, in which they have to stay in different habitats and consume different types of nutrients. This process starts from first instar larvae to adult including the non-feeding pupal stage (Williams and Feltmate, 1992).

2.3.1 Differentiation of *Aedes aegypti* and *Aedes albopictus*

Aedes mosquito is generally recognized by its black and white striped legs and small, black and white body. *Aedes aegypti*, also known as *Stegomyia aegypti* or yellow fever mosquito, is a mosquito that can transmit dengue, chikungunya, yellow fever, West Nile and other viruses (Reinert et al., 2004). It is a tropical mosquito that originates from Central Africa (Smith, 1956). This domestic breeder has been dispersed to all parts of the world, as it was found to breed on sailing ships (Chandler, 1945; Smith, 1956; Gubler, 1997).

Aedes albopictus, also known as the Asian tiger mosquito or forest day mosquito, is originated from the tropical forest of Southeast Asia (Smith, 1956). *Aedes albopictus* arises throughout the Southeast Asia as it has been found abundantly in city, countryside, farm or jungle (Hawley, 1988). *Aedes albopictus* can breed in tree holes, even after being brought out to the different area (Hawley, 1988). *Aedes albopictus* has a rapid bite, thus allowing it to escape from most attempts by people to kill it. As it is closely associated with humans, this mosquito has become a significant pest in many communities (Hawley, 1988).

Both *Aedes aegypti* and *Aedes albopictus* are quite alike; however there are some dissimilarities in the white scales pattern on the body. Figure 2.4 shows the presence of white scales on the clypeus of *Aedes aegypti* but none on *Aedes*

albopictus. Both of the adult species can be identified by the arrangement of silvery bands of the thorax. *Aedes aegypti* has lyre shaped markings; two curved white strips on both side of the frontal thorax (Figure 2.5), while in *Aedes albopictus*, there is only a single silvery line originating from the head and running through the middle of the frontal thorax (Figure 2.6). Both *Aedes aegypti* and *Aedes albopictus* larvae appear similar, but differences can be observed under the microscope (Hawley, 1988). The comb scales of *Aedes aegypti* larvae are three pronged, with a longer central prong, similar to a pitch fork (Figure 2.7). The comb on the 8^{th} segment has 8 to 12 teeth which have well developed lateral denticles. *Aedes albopictus* has comb scales, which are not forked and has 8 to 12 teeth without denticles (Figure 2.8).

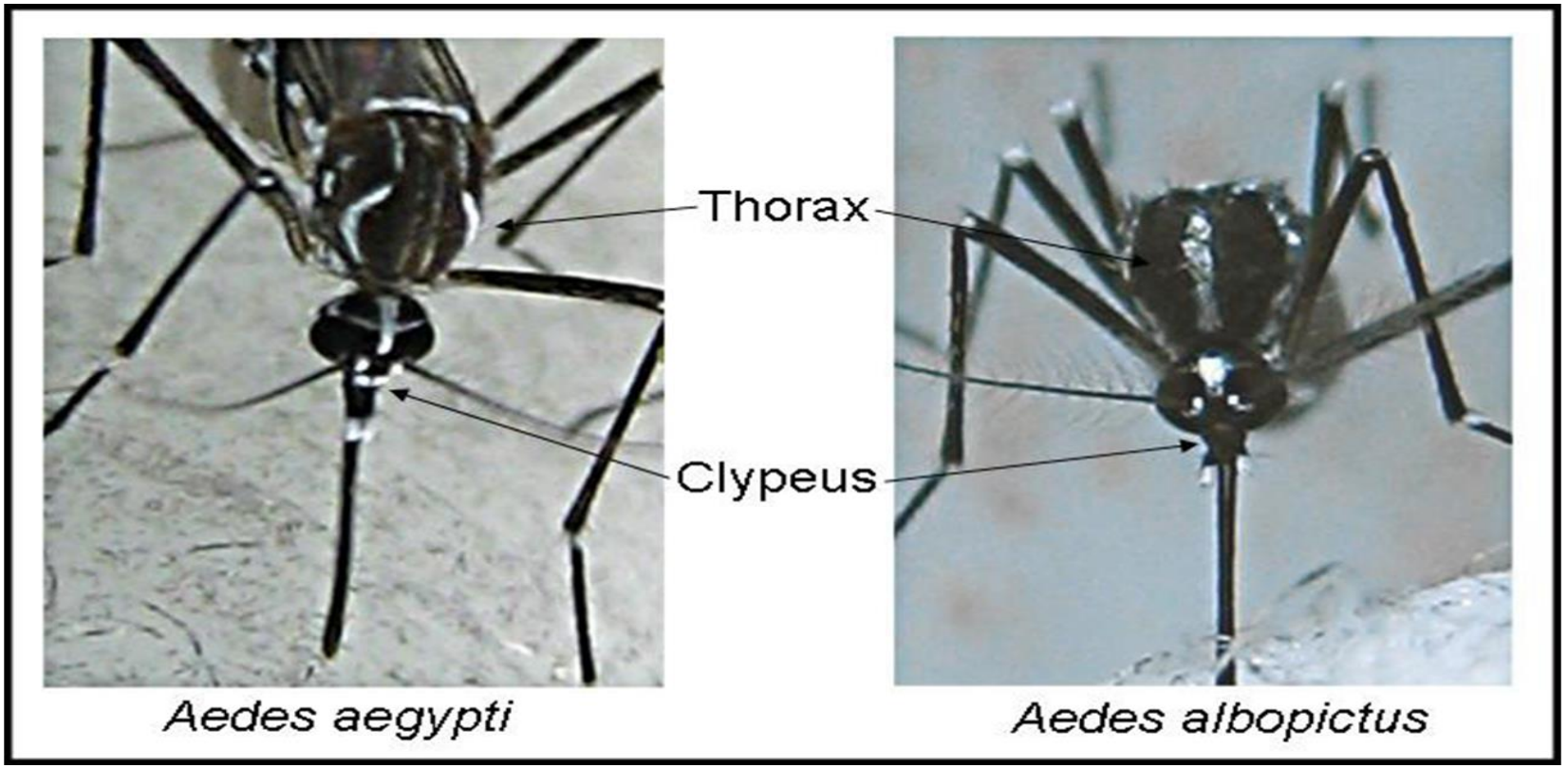

Figure 2.4: Anatomy identification of *Aedes aegypti* and *Aedes albopictus*
(Nimmo, 2007. p. 22)

2.4 Distribution of *Aedes albopictus* and *Aedes aegypti*

Aedes aegypti is commonly an indoor breeder while *Aedes albopictus* is an outdoor breeder (Lo and Narimah, 1984). *Aedes aegypti* has established predominantly in urban areas, both inside and outside houses. Subsequently, *Aedes aegypti* generally related with dengue hemorrhagic fever in urban areas. However, *Aedes albopictus* is more widespread in urban areas, and is associated with dengue fever. A mosquito survey in Penang Island found that *Aedes aegypti* was present in the urban areas, whereas *Aedes albopictus* was present in the urban, rural and forested areas (Rudnick et al., 1965).

Through the ovitrap surveillance, *Aedes aegypti* was found to be densest in the crowded poorer central city of Penang Island compared to *Aedes albopictus* where the densities were higher than those of *Aedes aegypti* both inside houses and outdoors (Yap and Thiruvengadam, 1979). Another ovitrap surveillance conducted in Penang Island showed that both *Aedes aegypti* and *Aedes albopictus* are relatively common in most areas; but *Aedes aegypti* was more abundant in low cost housing and in more crowded, central areas of city, whereas *Aedes albopictus* was more abundant in the middle and high cost housing, in the gardens, parks and in outer urban as well as suburban areas. In some of these latter areas, *Aedes aegypti* was present in very low numbers or even some could not be detected (Rudnick, 1986).

Figure 2.5: Thorax of *Aedes aegypti* (Sivanathan, 2006)

Figure 2.6: Thorax of *Aedes albopictus* (Sivanathan, 2006)

Figure 2.7: Comb scales structure of *Aedes aegypti* larvae (WHO, 2011)

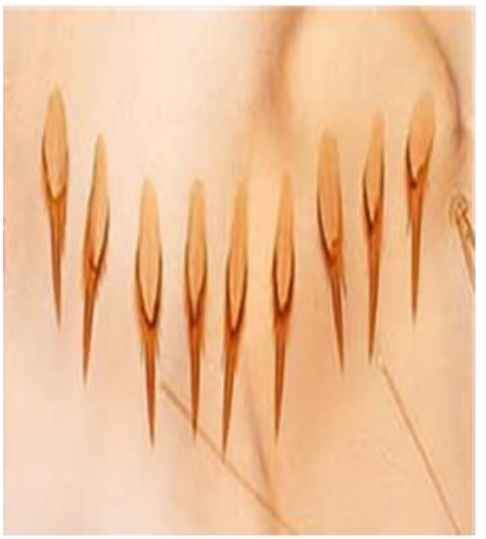

Figure 2.8: Comb scales structure of *Aedes albopictus* larvae (WHO, 2011)

In Sarawak, *Aedes aegypti* was found in 30% of houses in Kuching during the ovitrap surveillance but *Aedes albopictus* bites man more frequently than *Aedes aegypti* (Macdonald et al., 1967). A survey regarding the distribution and relative prevalence of *Aedes aegypti* in Sabah, Brunei and Sarawak was conducted by Macdonald and Rajapaksa (1972). The relative prevalence recorded for *Aedes aegypti* in Sabah, Brunei and Sarawak were 0.74, 0.69 and 0.62 respectively. In their study, *Aedes aegypti* was found in the east coast whereas no *Aedes aegypti* was found in the west coast of Sabah. There was no apparent reason for this as the types of housing and the customs of the people were similar throughout the surveyed areas. In Brunei, the survey showed that the *Aedes aegypti* was established in coastal towns such as the villages where large and scattered houses were built on piles on mud flats of the river.

A nationwide pupal survey was done in the urban towns of Peninsular Malaysia (Lee, 1991). Both *Aedes aegypti* and *Aedes albopictus* were found breeding indoor and outdoor in a variety of containers. *Aedes aegypti* appeared to be the leading indoor breeder, whereas both species showed no difference in preference for outdoor containers. The use of household insecticide products such as mosquito coil, liquid vaporizer, mat and aerosol to reduce the number of the mosquitoes indoor appeared to force *Aedes aegypti* to breed outdoor rather than indoor (Lee, 1991). Hence, the competition between *Aedes aegypti* and *Aedes albopictus* continued.

2.5 Life Cycle of *Aedes aegypti*

Aedes aegypti has a composite life-cycle with affected changes in shape, function, and habitat. *Aedes aegypti* is also a holometabolous insect, indicating that the insect has a complete metamorphosis, which includes the egg, larva, pupa, and adult stages. There is an aquatic phase which comprising the larva and, pupa stages and a terrestrial phase covering the egg and, adult stages in the *Aedes aegypti* life-cycle (Figure 2.9). The entire life cycle of *Aedes aegypti* is completed within one-and-a-half to three weeks at room temperature, depending on the level of feeding (WHO, 2011). The period of egg hatching to adult emergence may vary, as under the optimal conditions the cycle for would be a week. It may take a longer period for adults to emerge at lower room temperature (WHO, 2011).

2.5.1 Eggs

The female *Aedes aegypti* frequently takes multiple blood meals within a single gonotrophic cycle (McClelland and Conway, 1971; Trpis and Hausermann, 1986; Scott et al., 1993), which increases dispersal of their progeny. Gonotrophic cycle is the process of blood-feeding, egg maturation and oviposition is repeated several times throughout the life-cycle of the female mosquito.

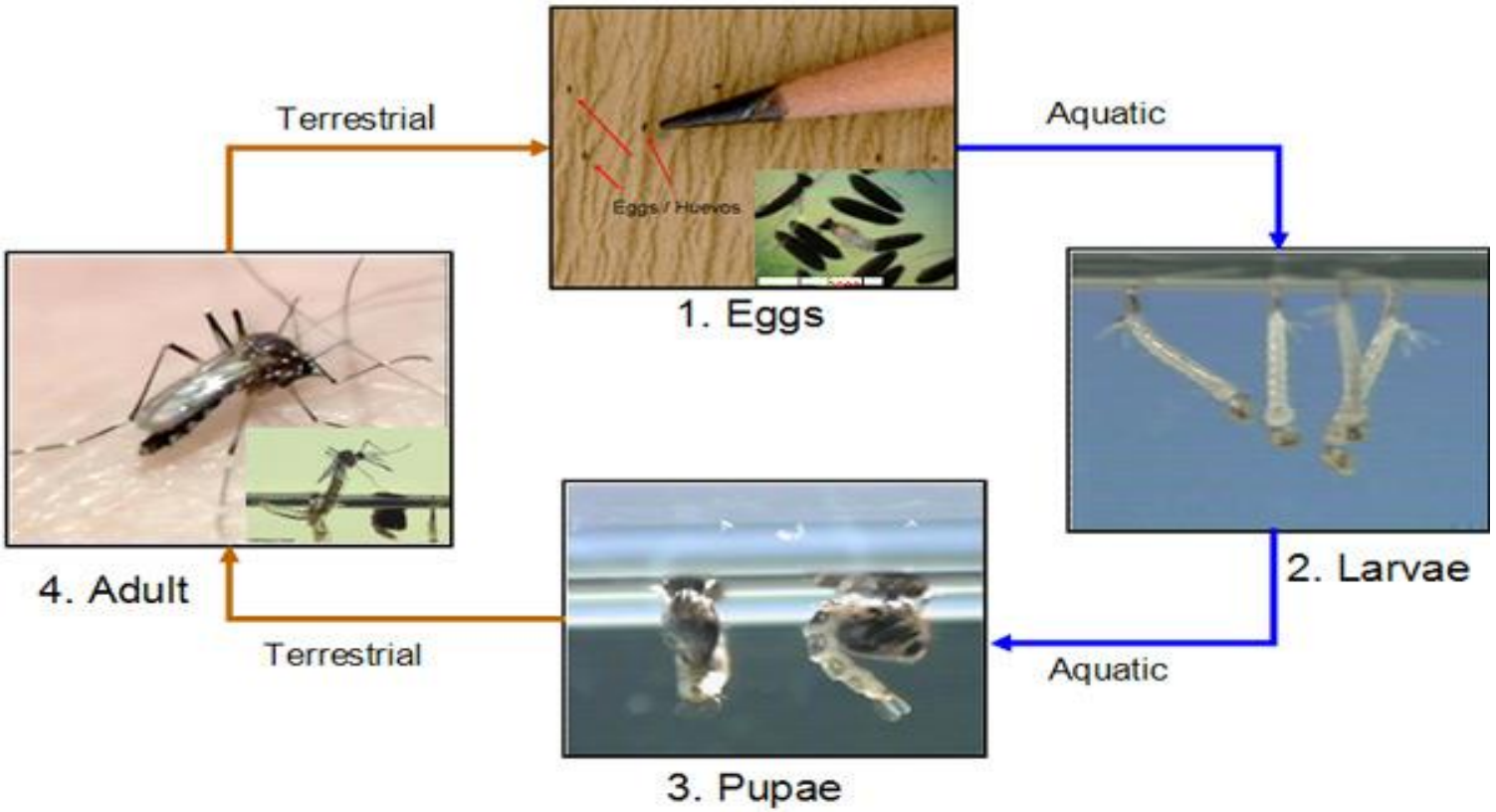

Figure 2.9: Life cycle of *Aedes aegypti*
(Center for Diseases Control and Prevention, 2010)

The amount and quality of blood meal will contribute to the number of eggs, including the effects of sugar meal taken before the blood meal (Wieslaw and Woodbridge, 2004). The females produce an average of 100 to 200 eggs per batch after consuming a complete blood meal. The number of eggs produced depends on the size of the blood meal taken. A smaller blood meal produces fewer eggs (Nelson, 1986). In a lifetime, female mosquitoes are capable of laying up to five batches of eggs.

Eggs are laid individually rather than in a bulk on the moist surfaces, such as in tree holes and man-made containers. Some eggs were laid over hours or even days, but some can be laid at one time, accountable to the accessibility of exact substrates (Clements, 1999). Regularly, a female will lay its eggs over two or more sites instead a single bunch of eggs at a single site only. The eggs are deposited at distances on the water line (Foster and Walker, 2002).

The egg shell of *Aedes aegypti* has a mosaic pattern. It is smooth, oval shaped, and around one millimetre long (Foster and Walker, 2002). Eggs are white when first laid but later turn into glossy black. Embryonic growth is generally accomplished in two days in a tropical environment, but can be slowed up to seven days in chill moderate climates (Foster and Walker, 2002).

Most eggs hatch into larvae within 48 hours if submerged in water. However, the eggs are resistant to desiccation and can withstand without water up to a year, which means that even if all larvae, pupae, and adults are eliminated at some point in time, repopulation will occur as soon as the eggs in the containers are flooded with water (Christophers, 1960; Nelson, 1986).

2.5.2 Larvae

Larvae are legless and the body is divided into three parts, the head, the thorax, and the abdomen. Most *Aedes* larvae can be distinguished from other genera by their small siphon (Nelson, 1986). Larvae feed on organic particles in the water, such as algae. *Aedes aegypti* larvae spent most of the time at the water's surface but move to the base of the container when they need to feed or upon being disturbed (Nelson, 1986).

The larvae must obtain oxygen from the air, which they obtain via breathing spiracular tubes that have direct contact with the air when the larvae are at the water surface (Becker et al., 2003). Due to this, larval control of mosquitoes can be accomplished using degradable oils that disrupt the surface tension of water and penetrate the tracheal systems of larvae, resulting in suffocation (Foster and Walker, 2002).

There are four instar stages of larvae, by which larvae will spend short period of time in the first, second and third instars while in the fourth instar, it will spend up to three days and will grow up to approximately eight millimeters long. Larvae go through developmental stages in which they molt, or shed their skin up to three times to allow them to grow from first to fourth instars. At each molt, the head is increased to the full size characteristic of that instar while the body grows continuously (Becker et al., 2003).

Nearly all the breeding sites of *Aedes aegypti* larvae are man-made containers. Larvae are often found nearby the houses, in pools, tires, or in other water holding objects. Lee and Cheong (1987) reported that larvae of *Aedes aegypti* showed preference for drum (20%), earthen jar (21.3%) and concrete tank (33.3%).

Larval development is temperature dependent. Commonly, the male will pupate earlier; therefore males will develop faster than females. In a moderate temperature and with adequate water supply, *Aedes aegypti* larvae can survive in the larval stage for many months (Foster and Walker, 2002).

2.5.3 Pupae

The fourth instar larvae will then enter the pupal stage. This stage of the mosquito's life is also aquatic. Pupae are mobile and sensitive to stimulation. They do not consume food and take between two to three days to mature, during which metamorphosis takes place and larval features are lost as the body of the adult takes form (Becker et al., 2003).

The body of a pupa consists of a cephalothorax (fused head and thorax) and the abdomen. The cephalothorax has a pair of respiratory trumpets that provide the developing adult with oxygen. The newly formed adult emerges from the water after breaking the pupal skin. During the emergence, the adults had enlarged the abdomen by ingesting the air, hence splitting open the pupal case and the head will emerge first. Pupae are also resistant to desiccation, and adults can emerge even if breeding sites have almost dried out (Becker et al., 2003).

2.5.4 Adult

Adult mosquitoes emerge from the pupal stage at the water surface by increasing their hydrostatic pressure, causing the cuticle of the pupa to split (Becker et al., 2003). The legs and wings are stretched as the mosquito increases

the hemolymph pressure, and the soft tissue becomes sclerotized within a few minutes, allowing the mosquito to fly (Becker et al., 2003).

The life span of adults is depends on the environmental circumstances whereby it can range from two weeks to a month (Becker et al., 2003). At emergence, male mosquitoes are not sexually mature; they emerge a few days earlier than the females and thus reach sexual maturity at the same time, which synchronizes mating compatibility (Becker et al., 2003). After emergence from pupa, adults mated and the fertilized female consumed a blood meal within 1-2 days, which is important to provide nutrients to the growth of eggs (Becker et al., 2003).

Most females oviposit in multiple sites. The gonotrophic cycle lasts one to two days in about 1% of an assessment population of *Aedes aegypti*, while 51% lasted two to three days, 32% lasted three to four days and 4% lasted four to five days. The remaining mosquitoes still oviposit more than five days after the blood meal (Macdonald, 1956).

2.6 Dengue Transmission

Epidemic dengue and hyperendemic dengue are two common arrays of dengue virus transmission trails. The former transmission arises when DENV is

presented in an area by means of remote occasion that implicates a solitary viral strain. Enormous transmission can occur when the proportion of vectors and adult hosts is adequate (Gubler, 2004). In addition, vector control efforts, fluctuations of climate, and crowd resistance convey to the regulator of the epidemics. Transmission accomplishes in the middle of a city and then extents to the remaining part of the country which turn out to be the present array of transmission in certain areas of Asia, South America, Africa and small island nations (Gubler, 2004). Tourists visiting the regions are at augmented risk of contracting dengue throughout the phases of transmission (Gubler, 2004).

Hyperendemic dengue transmission is caused by co-circulation of various viral serotypes in a region where large group of susceptible hosts and a proficient vector are continuously extant (Wilder-Smith and Gubler, 2008). This is the main pattern of worldwide transmission. In regions of hyperendemic dengue, antibody occurrence increases with the phase and majority of adults are resilient. Hyperendemic transmission seems to be a crucial hazard for dengue hemorrhagic fever (Wilder-Smith and Gubler, 2008). Travelers to this region have more possibility to be infected compared to the travelers in the region of epidemic transmission (Wilder-Smith and Gubler, 2008).

2.6.1 Sylvatic Transmission

The sylvatic cycle, also called enzootic transmission cycle, is a portion of the natural transmission cycle of a pathogen. The sylvatic cycle is the fraction of the pathogen population's lifespan spent cycling between wild animals and vectors. Humans are usually an incidental or dead-end host, infected by the vector.

Dengue viruses circulate in both a sylvatic cycle involving non-human primates and various species of *Aedes* mosquitoes such as *Aedes furcifer*, *Aedes luteochephalus* and *Aedes taylori* and in a human (endemic) cycle primarily vectored by *Aedes aegypti* (Wang et al., 2000; Wolfe et al., 2001; Vasilakis et al., 2007). The only sylvatic DENV serotype that has been isolated in Africa is DENV-2 (Salluzo et al., 1986; Rodhain, 1991; Diallo et al., 2003). Sylvatic DENV-1, DENV-2 and DENV-4 have been isolated in Malaysia, though the last isolation of a sylvatic virus namely DENV-4 arose in 1975 (Wang et al., 2000). Sylvatic DENV-3 has not been isolated but is assumed to exist in Malaysian sylvatic cycle on the basis of seroconversions in sentinel monkeys (Vasilakis et al., 2007). All four serotypes of dengue are maintained in canopy-dwelling *Aedes niveus* mosquitoes and non-human primates (Rudnick et al., 1967; Rudnick, 1978). Rudnick (1986) suggested an association between *Macaca* and *Presbytis* spp. monkeys with *Aedes niveus* mosquitoes as the main vector in Malaysia.

Smith (1956) reported the infection of dengue virus in forest animals. Of a large number of animals tested, several species of tree-dwelling mammals were positive for dengue antibody as opposed to very few ground-dwelling animals. In a sequel study, Smith (1958) reported that monkeys, of the tree-dwelling mammals could be confirmed serologically as dengue-positive. In addition, *Aedes albopictus* may be the bridge vector among non-human primates in the forest and human in rural areas (Smith, 1958).

The emergence process, as sylvatic cycle interacts with human populations in the rural regions in West Africa and Southeast Asia was supported over the vector substituting, from arboreal *Aedes* mosquitoes to peridomestic (*Aedes albopictus*) and domestic (*Aedes aegypti*) mosquitoes and shifting of reservoir hosts from non-human primates to humans (Figure 2.10). The occurrence of the existent, divergent human DENV strains from the sylvatic transmission cycle was best defined by the allopatric and possibly ecological segregating of inherited sylvatic DENV strains in dissimilar species of non-human primate populations (Chen and Vasilakis, 2011).

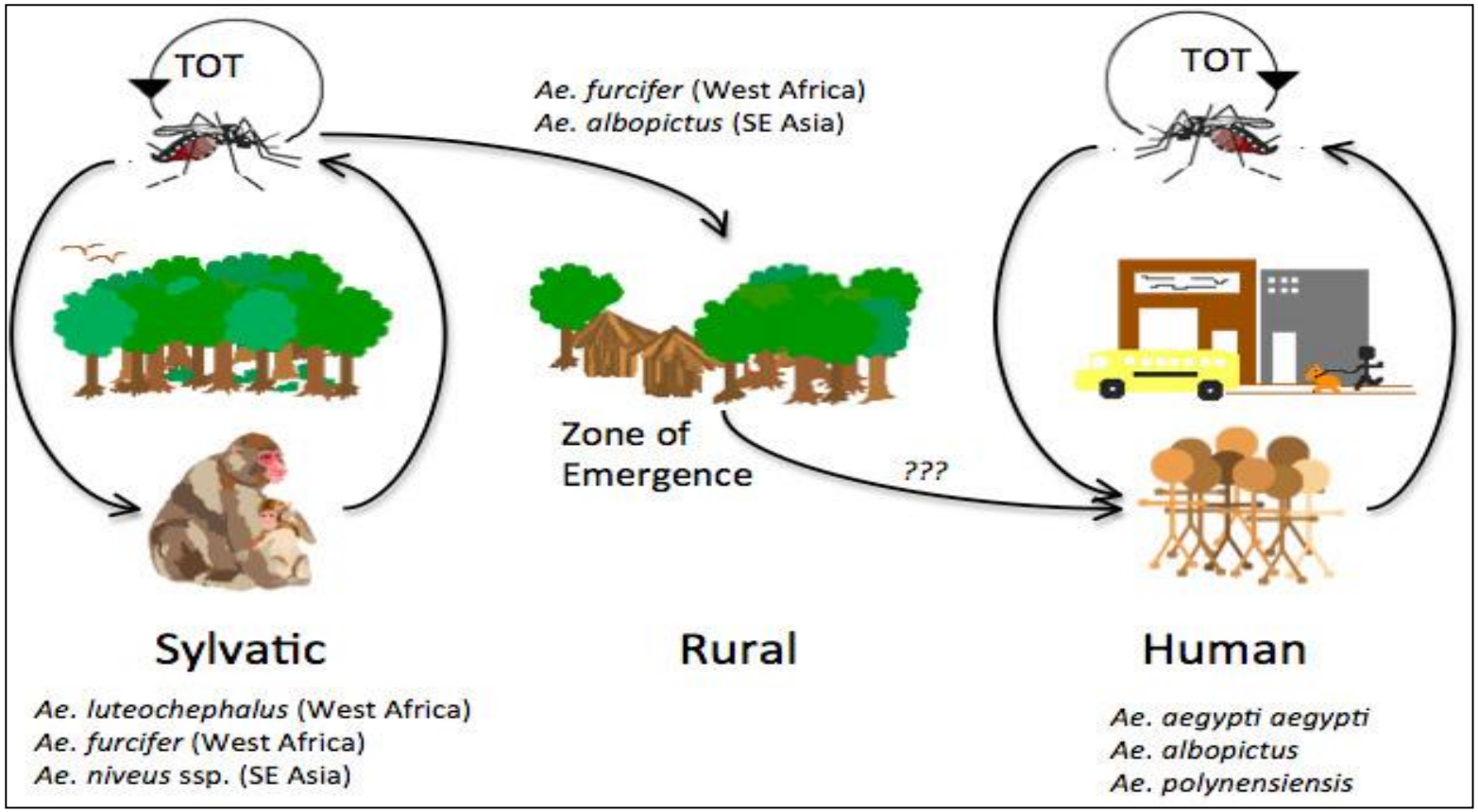

Figure 2.10: The transmission cycles of dengue virus (DENV).
(Chen and Vasilakis, 2011. p 1563)

Infected humans are the main carriers and multipliers of the virus, and serving as a source of the virus for uninfected mosquitoes. Its natural history suggested that biologically these viruses are highly adapted to their mosquito hosts and they were most likely mosquito viruses prior to becoming adapted to non-human primates and to human as well (Chen and Vasilakis, 2011).

2.6.2 Transovarial Transmission

Viral transmission occurs horizontally through mosquitoes as the vector. The dengue virus presents two main modes of transmission which is human to mosquito and mosquito to human (Patricia, 2010). As shown in Figure 2.10, dengue virus is maintained in a "human-mosquito-human" cycle (Chen and Vasilakis, 2011). The female mosquitoes generally acquire the virus while feeding on the blood of an infected person. The susceptible humans cannot contract the virus by coming into contact with infected humans. After virus incubation for eight to ten days, an infected mosquito is capable, during probing and blood feeding, of transmitting the virus for the rest of its life (Maidana and Yang, 2008).

Transovarial transmission is vertical transmission, in which the infected mother passes the virus to its offspring (Maidana and Yang, 2008). Transovarial transmission of dengue virus infection has been reported in *Aedes* mosquito vectors and has become a focus of research globally. It has been revealed that the

transovarial transmission of dengue viruses over several generations of *Aedes aegypti* mosquitoes could occur under laboratory conditions (Joshi et al., 2002; Wasinpiyamongkol et al., 2003; Rohani et al., 2008). The transmission of each of the four dengue serotypes has been demonstrated experimentally in *Aedes albopictus* (Rosen et al., 1983) and for DENV-2 in *Aedes aegypti* (Jousset, 1981).

The first study of transovarial transmission of dengue virus in Malaysia was reported by Lee et al. (1997), involving DENV-2 in *Aedes aegypti*. The incidence of transovarial DENV-2 in wild mosquito populations has been confirmed by Rohani et al. (1997). Lee and Rohani (2005) showed that the dengue outbreak in a suburban residential area in Kuala Lumpur was related to transovarial transmission of DENV-2. A laboratory study in Brazil demonstrated the transovarial transmission of DENV-2 and DENV-3 in the females of *Aedes aegypti* and *Aedes albopictus* respectively (Martins et al., 2012). A field study in an urban district of Bangkok (Thailand) reported that the infected *Aedes aegypti* transovarial transmission were found to be more predominant four months before a high incidence of human infections whereby all four dengue serotypes were identified, with DENV-4 was the most prevalent, followed by DENV-3, DENV-1, and DENV-2, respectively (Thongrungkiat et al., 2011). In both studies in Brazil and Bangkok, the immature *Aedes* mosquitoes were collected from the field site and were reared until adulthood in the laboratory.

A positive correlation was discovered between high transovarial transmission of dengue virus in emerged adults from field-collected larvae and the high incidence rate of human dengue cases. The results showed that dengue virus-infected field-caught *Aedes aegypti* were detected from six to eight weeks before the initiation of a dengue outbreak among humans in the raining season (Chow et al., 1998; Urdaneta et al., 2005; Angel and Joshi, 2008). Furthermore, a study reported that DENV-2 infected *Aedes aegypti* females that were reared in laboratory condition had transovarially transmitted the virus to their offspring and the study also found that there was a raise in the infection rate of transovarial transmission due to longer period of eggs gestation at room temperature (Mourya et al., 2001b). Therefore, it could have important epidemiological consequences for dengue transmission as the possibility that these events might occur in nature (Mourya et al., 2001b). DENV-2 and DENV-3 were isolated from the field caught *Aedes aegypti* males in India which signified the occurrence of transovarial transmission and it was observed that the transmission was higher in the summer months whereby the reported dengue cases in human were low (Arunachalam et al., 2008). This indicates that DENV have adapted with the hostile climatic changes and thus will be preserved in the environment throughout the time.

2.7 Vector Control

Preventing or reducing dengue virus transmission depends entirely in controlling the mosquito vectors or interruption of human–vector contact. WHO

(2012) introduced the strategic approach known as Integrated Vector Management (IVM) to control mosquito vectors. This approach is the decision making process for the optimal use of resources for vector control effectively and less cost. Furthermore, vector transmission can be decreased through the use or combination of various methods namely environmental management, chemical control and biological control (WHO, 2012).

2.7.1 Environmental Management

Environmental management is about making changes in the environment in order to minimize the vector breeding sites and therefore reduces the human contact with vector-pathogen by abolishing, changing, eliminating or reutilizing the potential containers (Figure 2.11). The enormous populations of *Aedes aegypti* are often related to the poor water supply, insufficient sanitation and waste disposal services, thus, urban groundwork and basic services that offer to the reduction in larval habitats should be improved and well maintained (Chang et al., 2011). In Malaysia, breeding sites are often discarded and neglected containers rather than domestic water storage containers (Chang et al., 2011). Modifications to prevent *Aedes* breeding may be long lasting measures and involve modifying building design such as roof gutter as shown in Figure 2.12.

Figure 2.11: Modified water storage (Evenproducts Ltd., 2011)

Figure 2.12: Modified roof gutter (Brookes, 2013)

2.7.2 Chemical Control

Chemical control approaches comprise larviciding with the use of chemicals such as synthetic insecticides, insect growth regulators and microbial insecticide while the adulticiding includes personal protection measures such as household insecticide products, repellents for long-term control and space spray (both thermal fogging and ultra-low volume sprays) as short-term epidemic measures (Yap et al., 1994).

2.7.2.1 Larviciding

Larvicides are generally used in domestic mosquito breeding containers or tanks that cannot be destroyed, eliminated or where other mosquito control

methods are impossible or too expensive to use. Larvicides must be in low harmful to other species and must not substantially alter the flavor, odor or color of the water (WHO, 2012). Through the Pesticide Evaluation Scheme, WHO (2006) recommended several larvicides that are safe for drinking-water treatment such as temephos, insects growth regulator methoprene and *Bacillus thuringiensis israelensi* (Bti) (Figure 2.13).

In Australia, methoprene is generally used in discarded and unused containers, roof gutters and rainwater-collecting containers to reduce overall mosquito populations seasonally and for outbreak control (Ritchie, 2005). These larvicides can also be used in cement or concrete-lined drains where drainage is difficult. However, application of chemical larvicides on a long-term basis allowed the vector to develop resistance to the insecticides. In addition, high cost and harmful effects to the environment are also the disadvantages (Gubler, 2011).

2.7.2.2 Adulticiding

Adulticides are used to decrease the disease transmission by lowering the adult vector population and targeting infectious adults through reducing their longevity which function as remaining exterior control as well as space controls (WHO, 2011).

According to WHO (2012), space spraying is suggested only in emergency circumstances for suppressing a continuing epidemic or to avoid an initial one. Space spraying causes an enormous, prompt demolition of the adult vector population (Figure 2.14). Therefore, abrupt space spraying can be commenced at the similar period as basis decline methods such as larviciding can be increased as if the illness observation is sufficient to track cases at early phase of transmission.

Figure 2.13: *Bacillus thuringiensis israelensi* (Bti) granules (Deschamp, 2012)

Figure 2.14: Thermal fogging (Suriyanto, 2013)

2.7.3 Biological Control

Biological regulator is based on the technique of introducing the living things which target, strive with or else decrease the densities of the aim organism. A selection of fish type was used to eradicate mosquitoes from bigger portable container for water storage. In Cambodia, introduction of a larvivorous guppy fish,

Poecilia reticulata (Figure 2.15) into water-filled containers was successful in reducing mosquito larvae (Seng et al., 2008).

Numerous predatory copepod species have been proven to be effective against dengue vectors (Figure 2.16). A vector control program in Vietnam expending copepods in massive water pools, pooled with decreasing of source, effectively eradicated *Aedes aegypti* in several communities by which prohibited dengue transmission for few years (Nam et al., 2000; Kay and Vu, 2005).

Figure 2.15: Larvivorous guppy fish (Doggett, 2008)

Figure 2.16: Copepod (40x) (Wagner, 2012)

2.8 Sterile Insect Technique (SIT)

Sterile insect technique (SIT) is an economical method for the suppression of target pest inhabitants. Conventional SIT uses radiation to sterilize insects that

have been reared in a factory. As soon as released, the sterile males compete with wild males for the females but no viable offspring results from the mating (Dyck et al., 2005). Two American entomologists Bushland and Knipling proposed and used SIT in the 1950s and were awarded the 1992 World Food Prize (Lee et al., 2007). The first SIT program was to control the New World screw-worm fly (*Cochliomyia hominivorax*), a parasitic fly that lays its eggs in the living tissue of warm-blooded animals, such as livestock and humans in the island of Curacao in 1954. In 1982, the United State of America formally eliminated the screw-worm with SIT and the elimination program relocated gradually south through Central America until the entire area was confirmed screw-worm inhibited in 2001 (Vargas-Teran et al., 2005).

The SIT method uses the insects to find their own mating native females. Therefore it works better against targets which are difficult to find. The SIT is species-specific with slight non-targets effects. However, the dose of radiation required to sterilize insects is damaging their fitness and this has prevented SIT from being applied to other species such as mosquitoes. The problems with mass rearing, sex-separation, sterilization, distribution, and maintaining the competitiveness of the insects tend to reduce the cost-effectiveness of an SIT program (Wilke et al., 2009).

2.9 Release of Insect Containing a Dominant Lethal (RIDL) – SIT

Oxford Insect Technologies Co. has developed genetically sterile male *Aedes aegypti* mosquitoes using its proprietary Release of Insect containing a Dominant Lethal (RIDL) technology which are meant to yield offspring that will die as larvae or pupae (Phuc et al., 2007). It is a genetic enhancement of the well-established sterile insect technique (SIT) (Wilke et al., 2009). RIDL mosquitoes have been created using recombinant deoxyribonucleic acid (DNA) technology; inserting the RIDL gene system and a fluorescent marker into the mosquitoes (Wilke et al., 2009). As RIDL mosquitoes contain a homozygous genetic modification, their offspring will inherit the RIDL gene and will not survive to adulthood. However, the RIDL mosquitoes can live and reproduce normally when they are fed a diet containing a supplement, tetracycline that represses the RIDL gene expression (Wilke et al., 2009).

The fitness of genetically sterile *Aedes aegypti* has been independently verified in laboratory studies by Institut Pasteur in Paris (Jain, 2006). The RIDL mosquitoes have been reported to be fitter and competitive because RIDL uses genetic methods instead of radiation to achieve sterility (Jain, 2006; Phuc et al., 2007). Releases of RIDL males in massive numbers over an adequate time will suppress, or even eliminate, the *Aedes aegypti* population.

2.9.1 Molecular Biology of RIDL

A protein called tTA was produced by a lethal gene, which is capable to act as a switch to regulate the activity of other genes. The gene does not yield any toxic protein, it will interrupt normal cell function causing the mosquitoes to die. In the laboratory, to prevent the tTA protein from working, a supplement is provided which acts like an antidote. This supplement is tetracycline, an antibiotic, which binds to the tTA protein and stops it from interacting with other genes. When released into the wild, the RIDL adult mosquitoes can survive without the supplement but their offspring will die before reaching adulthood (Figure 2.17). This is because they cannot obtain the antibiotic in the quantities needed to survive during larval stage (Wilke and Marrelli, 2012).

The positive feedback system in RIDL is comprised of a tetO binding domain, a minimal promoter and the tTA gene. The minimal promoter activates the construction of a small amount of tTA protein in the absence of the repressor (tetracycline), which then binds to the tetO binding sites (Figure 2.18). The binding of tTA will boost more tTA, which then binds to more tetO binding sites. This positive feedback loop results in high level of tTA, which is lethal to mosquito cells. In contrast, the tetracycline is capable of binding to tTA protein and will prevent the binding of tTA to the tetO binding domain, switching off the positive feedback loop and hence mitigating harm to the cells (Wilke et al., 2009).

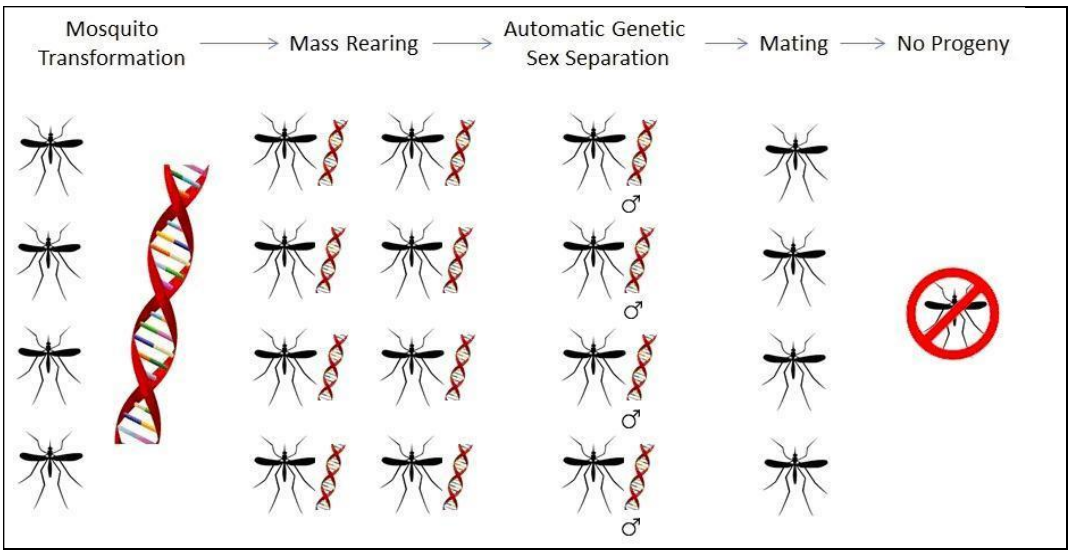

Figure 2.17: RIDL system
(Wilke and Marrelli, 2012. p. 289)

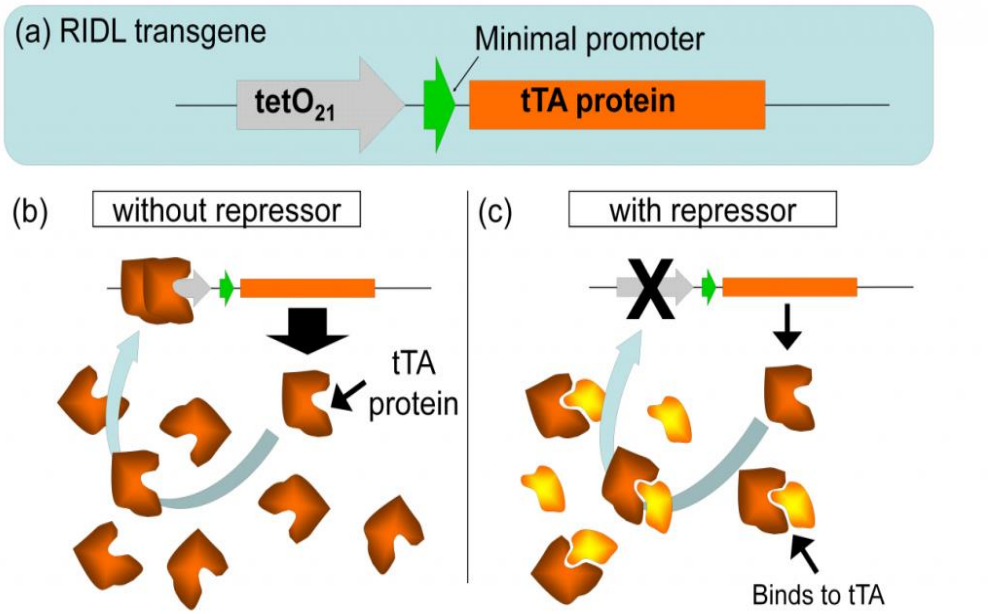

Figure 2.18: Positive feedback system in RIDL
(Wilke et al., 2009. p. 66)

2.10 Virus Detection

Virus isolation by cell culture with fluorescent-antibody staining and serological examination of antibodies are the two conventional methods that have been used to evaluate dengue virus infection (Gibbons and Vaughn, 2002; Kong et al., 2006). Virus isolation is widely used as the gold standard for diagnosing DENV infection (Kao et al., 2004). Serotypes of DENV are easily determined using immunofluorescence (IF) staining in infected cells with serotype-specific monoclonal antibodies (Henchal et al., 1982; Gubler et al., 1984).

Typical serological methods such as hemaglutination inhibition, complement binding, immunofluorescence and enzyme-linked immunosorbent assays (ELISA) are simple and can be done starting from day 2 until more than day 15 after the onset of fever (Thein et al., 1992). However, it takes about 7 to 10 days for serological diagnosis, and it could be confusing due the antibodies that cross-react with other flavivirus (Kong et al., 2006). Although virus isolation is the gold standard method in dengue diagnosis, it is uncommon in practice due to the long time required, higher cost and laborious (Paudel et al., 2011). Therefore, polymerase chain reaction (PCR) is a more practical way to diagnose dengue virus infection.

2.10.1 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Molecular analysis provides a more sensitive and rapid detection than conventional virus isolation methods as it amplifies nucleic acid even for inactivated virus. The use of RT-PCR in molecular analysis has been popularized throughout the global and is an important tool in research (Kong et al., 2006). RT-PCR is highly capable of detecting dengue virus of a severe infection in human serum (Lanciotti et al., 1992; Chang et al., 1994). RT-PCR is sensitive during the early viremic phase from day zero to day four (Pfeffer et al., 2002; Pastrino et al., 2005).

2.10.2 Real-time Reverse Transcriptase Polymerase Chain Reaction

A one-step real-time RT-PCR assay has been developed for rapid detection, serotyping and quantification of dengue virus using TaqMan probes (Kong et al., 2006). Real-time PCR assay is more rapid to perform the reaction compared to conventional nested PCR as the latter needs one more step (reverse transcriptase and then nested) to obtain the result (Paudel et al., 2011).

TaqMan RT-PCR is more specific and encompasses specific primer pair for each serotype. In addition, TaqMan RT-PCR assay uses fluorogenic probes to differentiate the four dengue serotypes (Sadon et al., 2008). The benefits of

TaqMan technology over standard RT-PCR methods are that the assay is a single-tube method which greatly reduces the risk of contamination and a quantitative measurement is made when fluorogenic signal is produced in positive samples that are monitored in real time (Callahan et al., 2001). The real-time RT-PCR must not be used as a substituting method for the virus isolation as the viability of the virus is crucial for the virus survey and pathogenesis study (Gubler, 1998a).

CHAPTER 3

MATERIALS AND METHODS

3.1 Propagation of Dengue Viruses

Dengue virus 1 (Hawaiian strain), dengue virus 3 (New Guinea-C strain) and dengue virus 4 (Hawaiian strain) were obtained from Department of Virology, Institute for Medical Research (IMR) and used as stock viruses. The viruses were inoculated in confluent monolayer of *Aedes albopictus* (C6/36) cell line (Rohani et al., 2009). The cells were maintained at 28°C in L-15 medium (Biowest, Nuaille, France) supplemented with 2% fetal calf serum (FCS) and 10% (v/v) of tryptose phosphate broth (TPB). The infected culture fluids were harvested once the prominent cytopathic effect (CPE) was observed approximately four to five days post infection. The cells were removed by centrifugation at temperature of 4°C for 4,000 rpm. The supernatant was then filtered through 0.22 μm cellulose acetate membrane filter units and kept in 1.5 mL sterile microcentrifuge tubes (Rohani et al., 2009). The virus was stored in a -70°C freezer for future use. All the tubes were labeled with the virus name, virus passage and date of storage.

3.2 Pre-Infection

3.2.1 Colonization of Wild Type *Aedes aegypti* Mosquitoes

The wild type (WT) *Aedes aegypti* colony is a disease-free laboratory strain maintained in the insectarium of IMR. The insectarium was controlled at 26 ±1°C, 80 ± 5% relative humidity, a 12 hour light cycle and constant air flow to prevent temperature fluctuations (Rohani et al., 2009). Filter papers (Whatman 1) that contained eggs were submerged in plastic trays (32 cm x 40 cm x 10 cm) filled with 2.55 L of overnight dechlorinated tap water (without tetracycline). These trays were labelled with the name of mosquito's strain, number of generation and date of eggs being submerged. After three days, the filter papers were removed from the trays to prevent larvae from getting stuck in the filter papers. The first and second instar larvae were fed with 1.42 g of cow liver powder mixture per tray while for the third instar larvae, they were fed on a small piece of partially cooked cow liver cubes (1.17 g per tray).

The larvae pupated between seven to ten days after submersion of the filter papers. Trays containing pupae were covered with mesh to ensure that the eclosed adults remain confined within the trays. Pupae were removed daily using a plastic pipette into a plastic container (10 cm x 17 cm x 5 cm) filled with overnight dechlorinated tap water. These plastic containers were transferred into wooden

cages (46.5 cm x 46.5 cm x 46.5 cm) for emergence of the adults. The cages were labelled with mosquito strain, setting up date and blood feeding date. A cage can host up to 1,000 mosquitoes at one time. The newly emerged adults were provided with a mixture of 10% sucrose and 1% vitamin B complex solution inside the cage (soaked in lint cloth).

For female adults to lay eggs, they need to be blood-fed. The females were allowed to blood-fed on a guinea pig placed in a special trap for overnight (Deng et al., 2012). Three days after the blood meal, they were provided with a piece of moist filter paper (Whatman No. 1, 19 cm in diameter) in a bowl (12.5 cm in diameter) for oviposition. Three or four pieces of filter paper were placed in each cage according to the number of adults in the cage. The filter papers were labelled with the strain of mosquito, number of generation and the date of eggs being laid. All the filter papers containing eggs were removed after seven days of oviposition and were then air dried at room temperature before storage.

3.2.2 Colonization of RIDL *Aedes aegypti* Mosquitoes

IMR established an Arthropod Containment Level-2 (ACL-2) facility to fulfill the pre-requisite for RIDL mosquito's colonization (Figure 3.1). Oxitec's Standard Operating Protocol (SOP) for rearing RIDL *Aedes aegypti* (Nimmo, 2007) was revised to comply with the government regulations. RIDL *Aedes aegypti* was

reared and colonized in the ACL-2 facility at 26 ± 1ºC, 80 ± 5% relative humidity with a 12 hour light cycle and constant air flow to prevent temperature fluctuations (Nimmo, 2007). RIDL mosquito's eggs were supplied by Oxitec. The microinjection of the lethal gene into the RIDL eggs was done in Oxitec laboratory.

For egg hatching purpose, filter papers (Whatman No. 1) carrying eggs were submerged in plastic trays (29 cm x 37.5 cm x 7.5 cm) containing 1.7 L of dechlorinated tap water with 17 mL of tetracycline solution (3 g/L) and 10 drops of fish food (Liquifry No. 1, Nottinghamshire, UK) to induce the egg hatching. Tetracycline is necessary to ensure that the lethal gene is repressed during the developmental stages of RIDL *Aedes aegypti* mosquito. The trays were labelled with the name of mosquito's strain, the number of mosquito's generation and date of submersion. Three days after hatching, the filter papers were removed from the trays to prevent larvae from being stuck in the filter papers. The larvae were fed with 0.15 g - 0.20 g of ornamental fish flakes (Tetramin®, Virginia, USA).

The larvae pupated after seven to ten days. For quality control, the pupae were screened for a fluorescence marker using a fluorescence microscope (Nikon SMZ-1000, Tokyo, Japan). The RIDL *Aedes aegypti* strain has an Actin5C-dsRed marker (Phuc et al., 2007), which will determine whether the fluorescence profile is consistent with the strain.

The pupae were removed from the trays and transferred into wooden cages (25 cm x 25 cm x 25 cm) for adult emergence as described for the laboratory strain (Section 3.2.1). The wooden cages were locked in a stainless steel cabinet (Figure 3.2) as requested by the Genetic Modification Advisory Committee (GMAC) of the Ministry of Natural Resources and Environment Malaysia. The cages were labelled with the mosquito strain, date of setting up and blood feeding date. The newly emerged adults were provided with a mixture of 10% sucrose and 1% vitamin B complex solution (soaked in lint cloth) inside the cages.

In order to collect the eggs, female RIDL *Aedes aegypti* were blood-fed with white mice placed in a special trap overnight (Jahangir et al., 2008). After three days, the females were provided with plastic containers (8 cm deep and 13 cm in diameter) containing 200 mL of dechlorinated tap water and lined with a piece of filter paper (Whatman No. 1) for egg laying. The filter papers were labelled with the strain of the mosquito, the date and batch number of the generation. The filter papers were removed after seven days and were dried at room temperature before storage.

Figure 3.1: RIDL colonies were reared in Arthropod Containment Level-2

Figure 3.2: Cages containing RIDL mosquitoes were kept in a stainless steel cabinet

3.2.3 Pupae Sex-Sorting

Male and female pupae were separated from each other as well as from larvae using the Hock's machine (Figure 3.3). Hock's machine is a mechanical separator based on two plates of glass set at an angle to trap pupae of different sizes (Focks, 1980). The pupae were sorted by pouring them onto the screen and washing with a gentle stream of water where females were retained on the screen and males were collected in the container below (McCray, 1961). The Hock's machine separated the male pupae, female pupae and larvae according to their size. Six hundreds male pupae and 600 female pupae of each strain were introduced into separate cages. Pupae can be sexed based on the genital lobe on the eighth pupal abdominal segments. A visual inspection by using dissecting microscope (model Raxvision ZTX-E, Kansas, USA) was conducted to ensure there was no mixing of different sexes of pupae and the mosquitoes were reared as virgins.

Figure 3.3: Pupae were sex sorted by using Hock's machine

3.2.4 Mating

Six hundred virgin male wild type *Aedes aegypti* mosquitoes and 600 virgin female wild type *Aedes aegypti* mosquitoes aged three to four days old were allocated for the mating experiment. The virgin female mosquitoes were separated into six cages labelled as A1, B1, C1, A2, B2 and C2 with 100 females each for triplicate respectively. After two days, 100 virgin male mosquitoes were introduced into each cage for mating purposes. The male mosquitoes were aspirated out after 24 hours. The mating experiment was repeated with RIDL *Aedes aegypti* mosquitoes.

3.3 Infection

3.3.1 Artificial Blood Feeding

Female mosquitoes were subjected to artificial blood feeding based on the techniques proposed by Graves (1980). In each experimental run, a total of 300 mosquitoes kept in the cages labeled as A1, B1 and C1 were fed with 2 mL of DENV-1 infected blood meal in each cage. The virus titer in the blood meal was $1x10^7$pfu/mL. The other 300 mosquitoes kept in the cages labeled A2, B2 and C2 were fed with 2 mL of uninfected blood meal as control (Figure 3.4).

Twelve hours prior to the blood meal, the female *Aedes aegypti* mosquitoes were held without sucrose solution (Rohani et al., 2009). The feedings were repeated with the mosquitoes kept in the cages labeled as A1, B1 and C1 fed with DENV-3 and DENV-4 infected blood meal accordingly.

The feeding was accomplished using a Hemotek artificial feeding system (Figure 3.5) with fresh blood from healthy human volunteers who were screened for dengue infections by using the RT-PCR to ensure that volunteers were free of the disease. Consent was obtained from the volunteers preceding to blood donation. During the first 30 minutes of feeding, three human volunteers sat close to each cage such that she or he was breathing into the cage to boost the blood feeding.

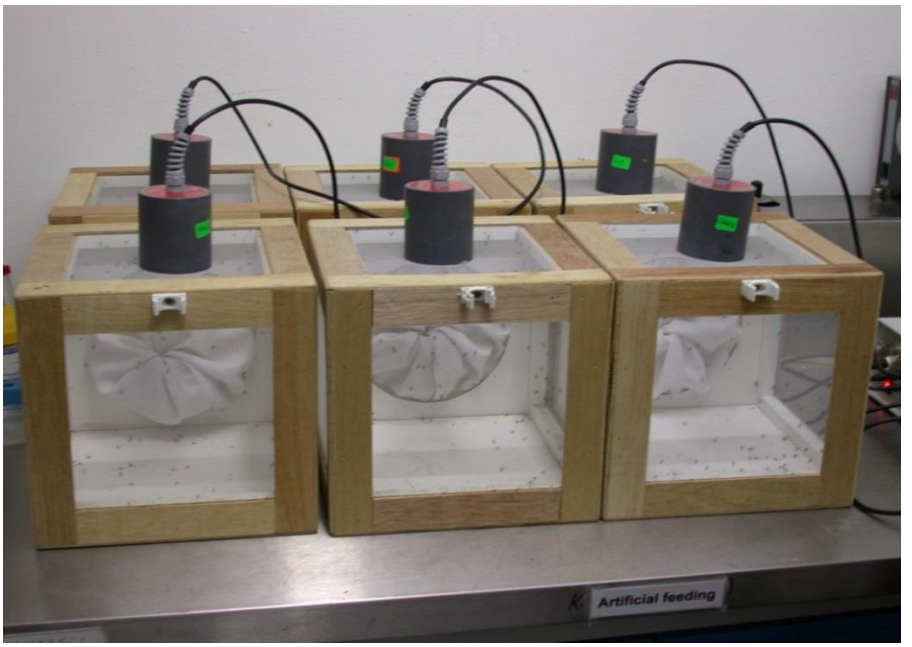

Figure 3.4: Female mosquitoes were subjected to artificial blood feeding

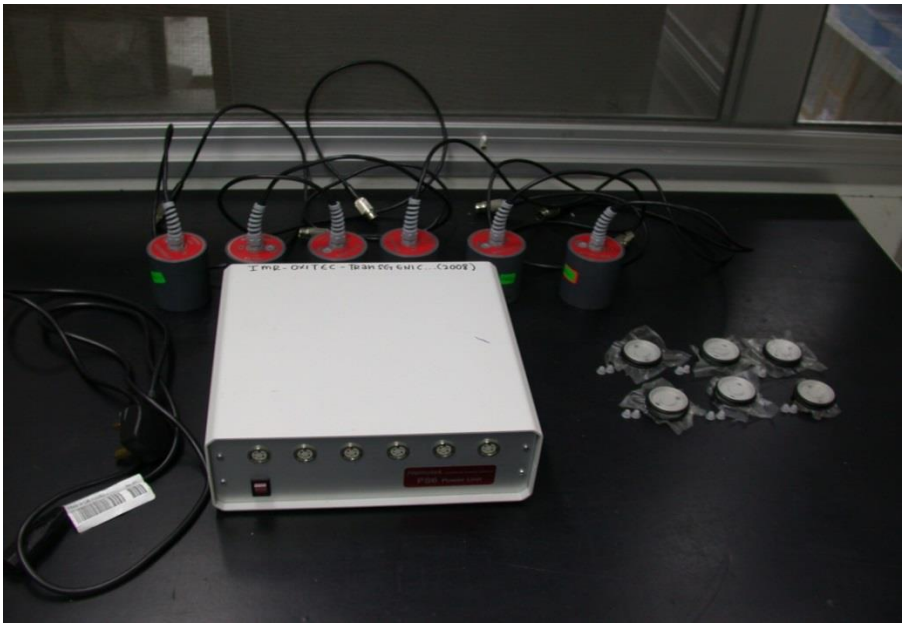

Figure 3.5: Hemotek artificial feeding system

3.4 Post-Infection

3.4.1 Experimental Transovarial Transmission

After 24 hours of blood meal, the female mosquitoes were individually separated into glass tubes (Figure 3.6). Each glass tube was supplied with wet cotton and filter paper for eggs laying. In this experiment, the occurrence of transovarial transmission was determined by using the eggs that were produced by infected and non-infected female mosquitoes. The eggs were collected from single glass tubes after the female mosquitoes were sacrificed every day from day 0 until day 14, and the number of eggs was recorded. The eggs were submerged in dechlorinated tap water for wild type strain while RIDL strain was submerged in

dechlorinated tap water containing tetracycline. The eggs from each isolated female were reared separately according to the standard protocol recommended by Nimmo (2007) until the third or fourth larval instar. They were then pooled (20 larvae per pool) in sterile 1.5 mL microcentrifuge tube for virus isolation as well as for RT-PCR assay.

Figure 3.6: Mosquitoes were individually separated in a glass tube

3.4.2 RNA Extraction

All adult females and larvae samples were homogenized using a sterile homogenizer (Kontes Pellet Pestle®) and spun for 15 minutes at 8,000 rpm at 10ºC. Viral RNA extraction kit (Bioneer, Daejeon, Republic of Korea) was used to

extract the viral RNA of larvae. The extraction was carried out according to the manufacturer's instructions. Briefly, 200 μL of supernatant was added in a 1.5 mL sterile microcentrifuge tube. Four hundred microliter of Binding Buffer was added in the tube and vortexed for 5 seconds to ensure the complete lysis of samples and incubated for 10 minutes at room temperature. After 100 μL of isopropanol was added, the solution was vortexed for 5 seconds and spun for 10 seconds at 8,000 rpm. The solution was transferred into a 2 mL collection tube which was fitted with the binding column. The solution was washed with five 500 μL of Washing 1 Buffer and centrifuged for 1 minute at 8,000 rpm. The binding column was transferred to a new 2 mL collection tube and the same procedure was repeated with Washing 2 Buffer. In order to remove the ethanol from the solution completely, all the samples were spun down at 13,000 rpm for 1 minute. Finally, the binding column was transferred to a 1.5 mL collection tube and was left to stand for 1 minute after 50 μL of Elution Buffer was added to allow the buffer to permeate the column. The RNA was eluted by spinning the column at 8,000 rpm for 1 minute. The eluted RNA was stored at -70ºC.

3.4.3 Dengue Virus Detection Using One-Step TaqMan Real-time RT-PCR

The one-step TaqMan real-time RT-PCR (Figure 3.7) was carried out on an iCycler real-time PCR machine (BioRad, Hercules, California, USA) using QuantiTect Probe RT-PCR Kit (Qiagen, Hilden, Germany) following the procedure of Kong et al. (2006). The fluorogenic TaqMan probes used were the

following: DENV-1, 5’ (FAM) CTC AGA GAC ATA TCA AAG ATT CCC GGG (BHQ1); DENV-3, 5’ (Cy5) ACA TTT CCA AGA TAC CCG GAG GAG (BHQ3); DENV-4, 5’ (HEX) CCT AGA GGA CAT AGA CAA AAA GGA AGG AGA CC (BHQ1). Samples were assessed in 25 µL solution containing 5 µL of extracted RNA, 0.25 µL of RNA transcriptase, 12.5 µL of RT-PCR master mix, 0.5 µL of TaqMan probe and 0.5 µL of each primer according to the recommendations of the kit’s manufacturer. The thermal cycling profile for the assay comprised 30 minutes of reverse transcription step at 50ºC, 15 minutes of Taq polymerase activation at 95ºC, followed by 40 cycles of amplification steps of denaturation at 95ºC for 30 seconds, then annealing and extension at 60ºC for 60 seconds.

A serial dilution of identified copy number of dengue viruses RNA based on the dilution factor ranging from 10^0 to 10^{10} was prepared as to construct a standard curve for the quantification using the real-time RT-PCR. A standard curve was generated from samples of known RNA quantity which subjected to target real-time RT-PCR unknown samples (Pfaffl, 2004). The unknown samples were amplified as the PCR product spotted during the cycle and thus had constructed amplification plot. An ample increment in fluorescence was detected regards to the higher starting copy number of RNA target (Pfaffl, 2004). In the other hand, the positive pools of larvae were detected by real time RT-PCR through the number copies of virus RNA.

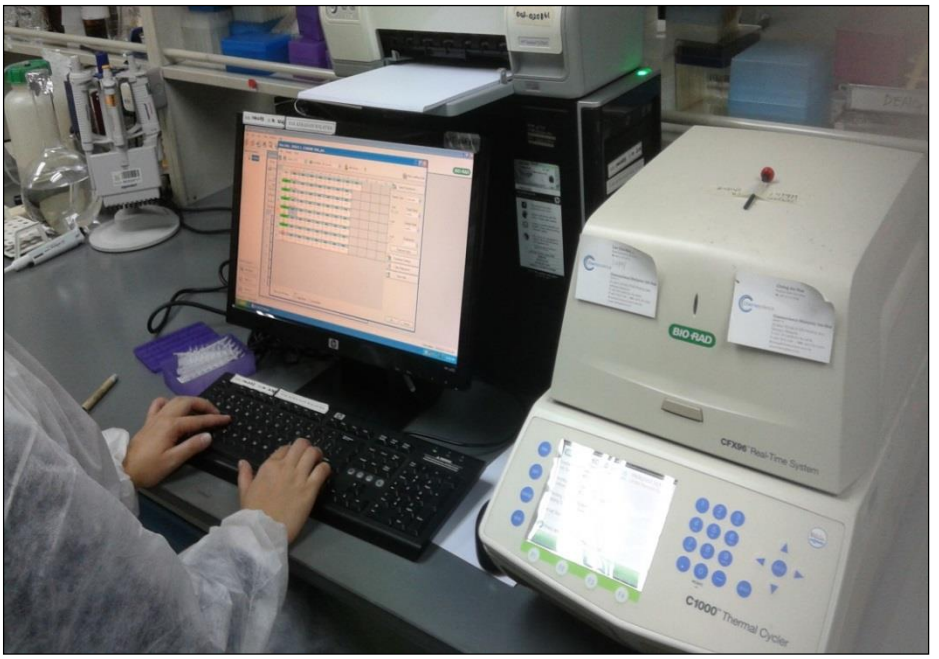

Figure 3.7: One-step TaqMan real-time RT-PCR was carried out using an iCycler real-time PCR machine

3.4.4 Virus Isolation

Isolation of virus from the larval homogenate samples that were used for real-time reverse transcriptase-polymerase chain reaction (RT-PCR) analysis was attempted using *Aedes albopictus* C6/36 cell line (Figure 3.8) (Chakravartiar et al., 2006). All the positive pools of larvae from real-time RT-PCR infected with DENV-1, DENV-3 and DENV-4 were inoculated into a C6/36 cell monolayer and incubated at 28°C. The cells were maintained in L-15 medium supplemented with 2% fetal calf serum and 10% (v/v) of tryptose phosphate broth (TPB). Once the prominent cytopathic effect (Figure 3.9) was observed within 4 to 5 days of post infection, it is indicated that the virus was viable and thus demonstrated of transovarial transmission.

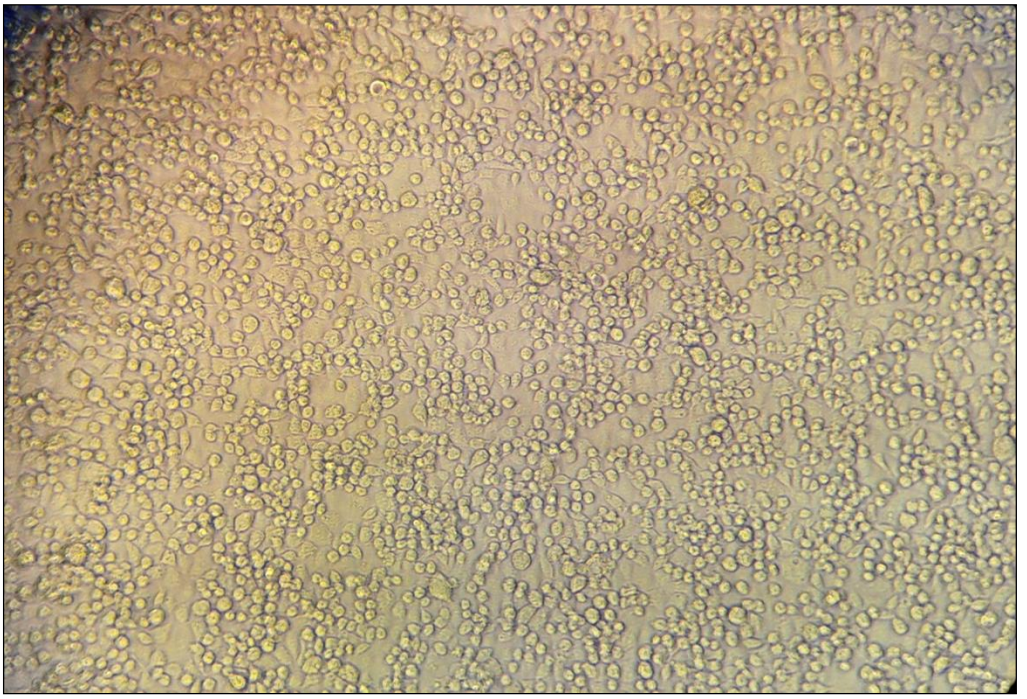

Figure 3.8: Uninfected *Aedes albopictus* cells (C6/36) (100x)

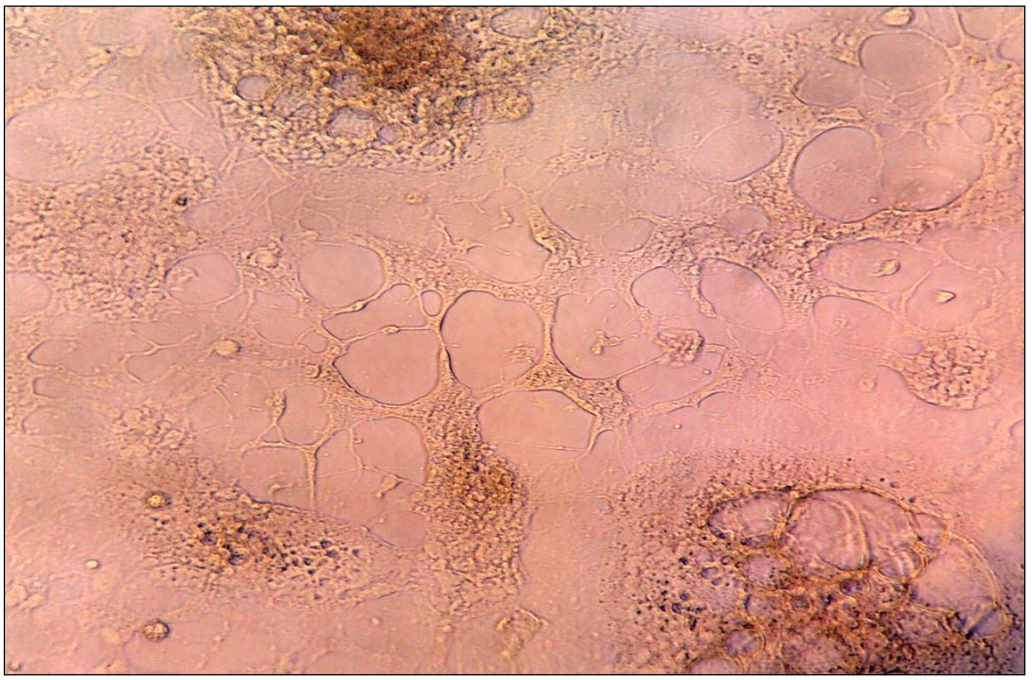

Figure 3.9: Cytopathic effects of DENV-1 on *Aedes albopictus* cells (C6/36) (100x)

3.5 Statistical Analysis

The Statistical Package for the Social Sciences Software (SPSS, version 15.0) was used to analyze all the data. The data were first analyzed by using the Kolmogorov-Smirnov (Coakes et al., 2008) to test for the normality of the data distribution. The number of eggs that were laid from each single female mosquito from both cohorts of infected and non-infected blood meal was recorded as well as the number of larvae that hatched from the respective eggs. From the data recorded, Mann-Whitney U test was used to determine the differences in number of eggs and larvae hatched from the single females blood-fed with DENV-1, DENV-3 and DENV-4 accordingly and non-infected blood meals for both RIDL and WT *Aedes aegypti*.

The Mann–Whitney U test is a non-parametric test analog to the independent samples t-test and can be used when one does not assume that the independent variable is a normally distributed interval variable. It is one of the best-known non-parametric significance tests. The null hypothesis for this test is H_0: The population medians are equal (p=0.05). The non-directional alternative hypothesis is H_1: The population medians are not equal (if p<0.05 or p>0.05). In this test, the result is considered as significantly different between the two strains (RIDL and WT *Aedes aegypti*) if the significance value (p-value) is less than 0.05.

CHAPTER 4

RESULTS

4.1 Fecundity Rate of Infected and Non-Infected WT versus RIDL Female *Aedes aegypti* Mosquitoes

The fecundity rates of both infected and non-infected mosquitoes for the WT and RIDL strains were measured by the number of eggs and the number of larvae hatched. The data of number of eggs and number of larvae were found not to be normally distributed. The Mann-Whitney U test was applied to the data analysis.

4.1.1 Dengue Virus Serotype 1

There were 102 and 90 from 150 of RIDL and WT females respectively that found to be positive of real-time RT-PCR. Table 4.1 showed that the infection rate detected using real-time RT-PCR for RIDL females were 68% and 60% for WT females. From a total of 150 RIDL females that were blood-fed with DENV-1,

only 90 females laid eggs with a hatching rate of 36% (Table 4.1). As for WT, there were 89 females laid eggs and the hatching rate was 33%. There was no significant difference in the number of eggs laid by both infected WT and RIDL females (Mann Whitney: $p>0.05$). The mean number of eggs laid by DENV-1 infected RIDL females was 28 ± 35 eggs while the DENV-1 infected WT females laid 25 ± 28 eggs (Table 4.2). The mean number of larvae hatched from eggs laid by infected RIDL females was 10 ± 22 larvae, while for infected WT females was 8 ± 12 larvae. Statistical analysis showed that there was no significant difference between the number of larvae hatched from the eggs laid by infected RIDL and infected WT females ($p>0.05$) (Table 4.2).

The number of eggs laid by infected and non-infected RIDL females was found significantly different ($p=0.001$) (Table 4.2). The mean number of eggs laid by infected RIDL females was 28 ± 35 eggs, while the number of eggs laid by non-infected RIDL females was 12 ± 24 eggs. The statistical analysis showed that infected RIDL females laid higher number of eggs than the non-infected RIDL females (Table 4.2). However, there was no significant difference ($p>0.05$) in the number of larvae hatched from the eggs laid for both infected and non-infected RIDL females. The mean number of larvae hatched from the eggs laid by infected RIDL females was 10 ± 22 larvae, while for non-infected RIDL females, the mean was 3 ± 10 larvae.

Statistically, there was significant difference (p=0.009) of number of eggs laid by infected and non-infected WT females (Table 4.2). The mean number of eggs laid by infected and non-infected WT females was 25 ± 28 and 33 ± 27 respectively. The number of eggs laid by the infected females was lower than the non-infected ones.

The mean number of larvae of infected WT females was 8 ± 12 larvae, while for non-infected WT females was 14 ± 20 larvae. As shown in Table 4.2, there was significant difference (p=0.038) for larvae count for both infected and non-infected WT females. The number of larvae hatched of infected WT females was lower compared to the non-infected WT females.

The results in Table 4.2 show that there is significant difference (p<0.05) of number of eggs laid by both non-infected RIDL and WT females. The mean number of eggs laid by non-infected RIDL females was 12 ± 24 eggs, while for non-infected WT females was 33 ± 27 eggs. The non-infected WT females laid more eggs compared to the non-infected RIDL females. Similarly, the number of larvae was also found to be significantly different (p<0.05) between both non-infected strains (Table 4.2). The number of larvae for non-infected WT females was 14 ± 20 while the number of larvae for non-infected RIDL females was 3 ± 10.

Table 4.1: Number of females laid eggs and hatching rate by DENV-1 infected and non-infected WT and RIDL female *Aedes aegypti*

Type of blood meal	Strain	Number of females	*Number of infective females	Infection rate (%)	Number of females laid eggs	Hatching rate (%)
Infective blood meal (DENV-1)	RIDL	150	102	68	90	36
	WT	150	90	60	89	33
Non-infective blood meal (No virus)	RIDL	150	0	0	72	29
	WT	150	0	0	107	44

*****Positive confirmation by real-time RT-PCR

Table 4.2: Number of eggs laid and number of larvae hatched for WT and RIDL female *Aedes aegypti* that were blood-fed with infective blood meal (DENV-1) and non-infective blood meal (blood without virus).

Parameter	**Strain**	**Mean±SD**	***p*-value***
Number of eggs laid	RIDL (infected)	28 ± 35	0.001
	RIDL (non-infected)	12 ± 24	
	WT (infected)	25 ± 28	0.009
	WT (non-infected)	33 ± 27	
	RIDL (non-infected)	12 ± 24	0.000
	WT (non-infected)	33 ± 27	
	RIDL (infected)	**28 ± 35**	**0.653**
	WT (infected)	**25 ± 28**	
Number of larvae hatched	RIDL (infected)	10 ± 22	0.093
	RIDL (non-infected)	3 ± 10	
	WT (infected)	8 ± 12	0.038
	WT (non-infected)	14 ± 20	
	RIDL (non-infected)	3 ± 10	0.000
	WT (non-infected)	14 ± 20	
	RIDL (infected)	**10 ± 22**	**0.233**
	WT (infected)	**8 ± 12**	

*****Statistically analyzed using Mann-Whitney U test with significance level set as $p < 0.05$

4.1.2 Dengue Virus Serotype 3

For DENV-3, the number of infective RIDL females was 60 as compared to WT females which was 66 from the total of 150 females for each strain (Table 4.3). The infection rate based on the positive confirmation by real-time RT-PCR for both strain was 40% for RIDL and 44% for WT. A total number of 99 RIDL females that were fed with DENV-3 infected blood meal had laid eggs and the hatching rate was 47%. As for the DENV-3 fed WT females, there were 87 females that laid eggs and of these 49% of the eggs had hatched (Table 4.3). As shown in Table 4.4, there was no significant difference (p>0.05) between the numbers of eggs laid by both infected RIDL and WT females. The mean number of eggs laid by RIDL females was higher compared to WT females which was 25 ± 26 and 22 ± 22 respectively. There was also no significant difference (p>0.05) between the numbers of larvae hatched for infected RIDL and infected WT females. The mean number of larvae hatched for infected RIDL females was 12 ± 17 while for infected WT females was 11 ± 14 (Table 4.4).

Comparing infected RIDL and non-infected RIDL females, there was significant different (p=0.001) in the number of eggs laid by both groups (Table 4.4). The mean number of eggs laid by DENV-3 infected RIDL females was 25 ± 26 while the mean number of eggs for non-infected RIDL females was 37 ± 30. This shows that the non-infected RIDL females laid more eggs compared to the infected ones. In contrast, there was no significant difference (p>0.05) between the number of larvae hatched from the eggs laid by infected RIDL and non-infected

RIDL females (Table 4.4). The mean number of larvae hatched from the eggs laid by infected RIDL females was 12 ± 17, while for non-infected RIDL females was 17 ± 21.

Statistically, there was significantly different ($p<0.05$) between the number of eggs laid by infected and non-infected WT females (Table 4.4). The mean numbers of eggs for infected and non-infected WT females were 22 ± 22 and 34 ± 26 respectively. This shows that the number of eggs of infected WT females was lower than the number of eggs of non-infected WT females. Similarly, there was also significantly different ($p<0.05$) of larvae count for both infected and non-infected WT females. The mean number of larvae was lower in WT infected females (11 ± 14 larvae) compared to non-infected WT females (19 ± 18 larvae).

As shown in Table 4.4, there was no significant difference ($p>0.05$) of the number of eggs laid and the number of larvae hatched by both non-infected RIDL and WT females. The mean numbers of eggs for non-infected RIDL and WT females were 37 ± 30 and 34 ± 26 respectively. The numbers of larvae for both non-infected RIDL and WT females were 17 ± 21 and 19 ± 18 respectively.

Table 4.3: Number of females laid eggs and hatching rate by DENV-3 infected and non-infected WT and RIDL female *Aedes aegypti*.

Type of blood meal	Strain	Number of females	*Number of infective females	Infection rate (%)	Number of females laid eggs	Hatching rate (%)
Infective blood meal (DENV-3)	RIDL	150	60	40	99	47
	WT	150	66	44	87	49
Non-infective blood meal (No virus)	RIDL	150	0	0	111	46
	WT	150	0	0	109	55

*****Positive confirmation by real-time RT-PCR

Table 4.4: Number of eggs laid and number of larvae hatched for WT and RIDL female *Aedes aegypti* that were blood-fed with infective blood meal (DENV-3) and non-infective blood meal (blood without virus).

Parameter	Strain	Mean±SD	*p*-value*
Number of eggs laid	RIDL (infected)	25 ± 26	0.001
	RIDL (non-infected)	37 ± 30	
	WT (infected)	22 ± 22	0.000
	WT (non-infected)	34 ± 26	
	RIDL (non-infected)	37 ± 30	0.214
	WT (non-infected)	34 ± 26	
	RIDL (infected)	**25 ± 26**	**0.113**
	WT (infected)	**22 ± 22**	
Number of larvae hatched	RIDL (infected)	12 ± 17	0.116
	RIDL (non-infected)	17 ± 21	
	WT (infected)	11± 14	0.000
	WT (non-infected)	19 ± 18	
	RIDL (non-infected)	17 ± 21	0.262
	WT (non-infected)	19 ± 18	
	RIDL (infected)	**12 ± 17**	**0.684**
	WT (infected)	**11± 14**	

*****Statistically analyzed using Mann-Whitney U test with significance level set as $p < 0.05$

4.1.3 Dengue Virus Serotype 4

In Table 4.5, the numbers of infective RIDL females were recorded as 66 compared to 93 infected WT females prior to positive result of real-time RT-PCR. The infection rate of females RIDL was 44% and 62% for WT females respectively. There were 66 out of 150 RIDL females that were fed with DENV-4 infective blood meal, had laid eggs with a hatching rate of 54% (Table 4.5). As for the positive WT females, there were 93 females that laid eggs and of these 28% of the eggs hatched. As shown in Table 4.6, there was no significant difference (p>0.05) of the numbers of eggs laid between RIDL and WT females fed with DENV-4 infective blood (30 ± 28 vs. 22 ± 23, respectively). In contrast, there was a significant difference (p=0.016) between the numbers of larvae. The mean number of larvae for infected RIDL females was 17 ± 21 which was higher compared to infected WT females; 6 ± 7 (Table 4.6).

The number of eggs laid by both infected RIDL and non-infected RIDL females was found significantly different (p=0.003) as shown in Table 4.6. The mean number of eggs laid by infected RIDL females was 30 ± 28, while the mean number of eggs laid by non-infected RIDL females was 41 ± 32. This indicated that the number of eggs laid by infected RIDL females was lower than the non-infected ones.

The mean number of larvae hatched from the eggs laid by infected RIDL females was 17 ± 21, while for non-infected RIDL females; the mean was 9 ± 12. The statistical analysis showed that there was a significant difference (p=0.047) between the numbers of larvae for infected and non-infected RIDL females (Table 4.6). It showed that the number of larvae hatched for infected RIDL females was higher than for the non-infected RIDL females.

As shown in Table 4.6, there was a significance different (p<0.05) of number of eggs laid by infected and non-infected WT females. The mean numbers of eggs laid for infected and non-infected WT females were 22 ± 23 and 8 ± 10 respectively. This indicated that DENV-4 infected WT females laid more eggs than the non-infected WT females. Similarly, there was a significant difference (p<0.05) for larvae count for both infected and non-infected WT females. The mean number of larvae hatched for infected WT females was higher than that of the non-infected WT females (6± 7 versus 3± 5 larvae).

The number of eggs laid by both non-infected RIDL and WT females was found significantly different (p<0.05) (Table 4.6). The mean number of eggs laid by non-infected RIDL females was 41 ± 32, while the corresponding number for non-infected WT females was 8 ± 10. The statistical analysis showed that non-infected RIDL females laid higher number of eggs than the non-infected WT females.

As shown in Table 4.6, there was a significant difference ($p<0.05$) of number of larvae for both non-infected RIDL and WT females. The mean number of larvae for non-infected RIDL and WT females was 9 ±12 and 3 ± 5 respectively.

Table 4.5: Number of females laid eggs and hatching rate by DENV-4 infected and non-infected WT and RIDL female *Aedes aegypti*.

Type of blood meal	Strain	Number of females	*Number of infective females	Infection rate (%)	Number of females laid eggs	Hatching rate (%)
Infective blood meal (DENV-4)	RIDL	150	66	44	103	54
	WT	150	93	62	115	28
Non-infective blood meal (No virus)	RIDL	150	0	0	115	22
	WT	150	0	0	94	35

*****Positive confirmation by real-time RT-PCR

Table 4.6: Number of eggs laid and number of larvae hatched for WT and RIDL female *Aedes aegypti* that were blood-fed with infective blood meal (DENV-4) and non-infective blood meal (blood without virus).

Parameter	Strain	Mean±SD	*p*-value*
Number of eggs laid	RIDL (infected)	30 ± 28	0.003
	RIDL (non-infected)	41 ± 32	
	WT (infected)	22 ± 23	0.000
	WT (non-infected)	8 ± 10	
	RIDL (non-infected)	41 ± 32	0.00
	WT (non-infected)	8 ± 10	
	RIDL (infected)	**30 ± 28**	**0.069**
	WT (infected)	**22 ± 23**	
Number of larvae hatched	RIDL (infected)	17 ± 21	0.047
	RIDL (non-infected)	9 ± 12	
	WT (infected)	6 ± 7	0.000
	WT (non-infected)	3 ± 5	
	RIDL (non-infected)	9 ± 12	0.000
	WT (non-infected)	3 ± 5	
	RIDL (infected)	**17 ± 21**	**0.016**
	WT (infected)	**6 ± 7**	

*****Statistically analyzed using Mann-Whitney U test with significance level set as $p < 0.05$

4.2 Quantification of Viruses in Larvae by Real-Time Reverse Transcriptase–Polymerase Chain Reaction and Virus Isolation

Mosquitoes of both strain RIDL and wild type (WT) received an infective blood meal of DENV with a titer of $1x10^7$ pfu/mL. The observation of the blood-fed mosquitoes was done from day 0 until day 14 post infection. In each of post infection days, 10 female mosquitoes were sacrificed and the eggs laid were counted before submerged in the tray for larvae hatching. All the larvae were pooled into groups of 20 larvae and were subjected to real-time RT-PCR for virus detection. Isolation of virus from positive pools was carried out in *Aedes albopictus* C6/36 cell line (Lee and Rohani, 2005; Rohani et al., 2007) to observe the infectious virus particles. As shown in Figure 4.1, the highest transovarial transmission rate was demonstrated in DENV-1 (14% for RIDL, 17% for WT), followed by DENV-3 (10% for RIDL, 6% for WT) and the lowest transovarial transmission was observed in DENV-4 (3% and 2% respectively for RIDL and WT), statistically indicated that there is significant different of transovarial rate between the three DENV serotypes (Chi square: $p<0.05$).

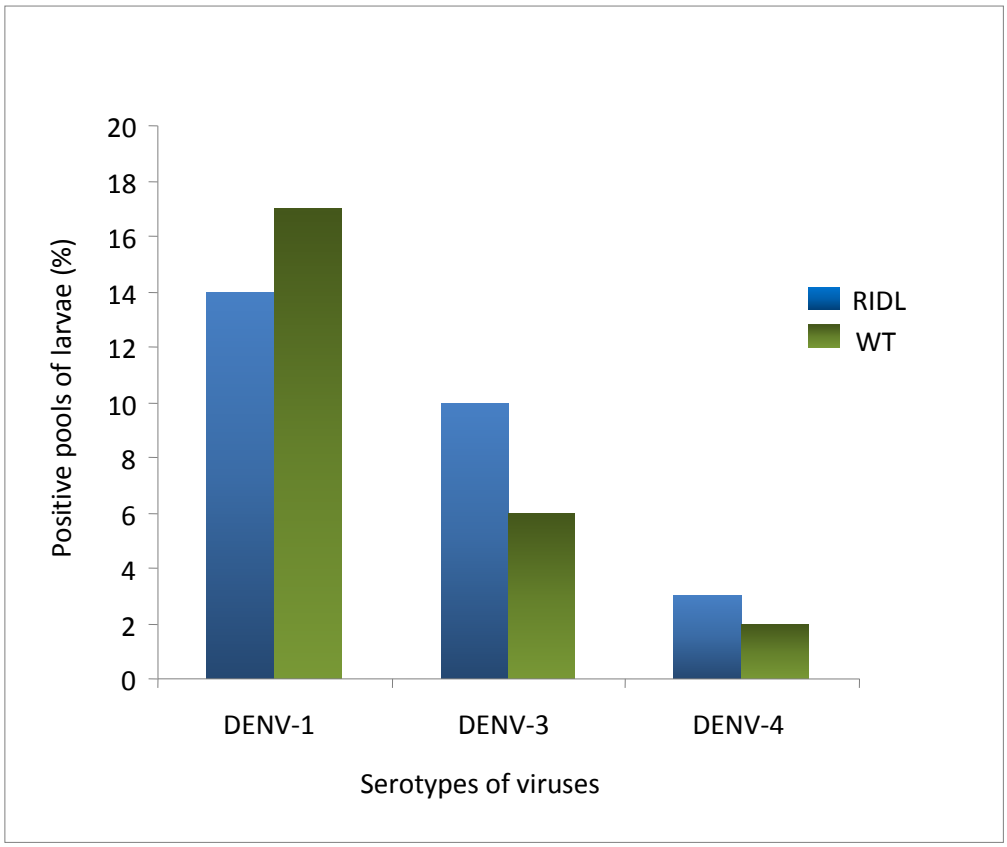

Figure 4.1: Transovarial transmission of RIDL and WT *Aedes aegypti* females infected by DENV-1, DENV-3 and DENV-4 respectively

4.2.1 Dengue Virus Serotype 1

From the total of 102 infective RIDL females (Table 4.1), only 20 of them produced larvae and those larvae were tested for the presence of DENV-1. Only 22 pools of larvae showed positive result by real-time RT-PCR and virus was isolated from eight pools (Table 4.7). As for WT females, 90 (Table 4.1) was found to be infective and only 39 of them produced larvae that were screened for DENV-1. For WT, there were 24 pools from 45 pools of larvae were found positive by real-time RT-PCR (Table 4.7). There were eight pools

of larvae from both mosquito strains that showed the cytopathic effects (CPE) on the C6/36 cell line indicating the virus is alive. The highest copy numbers of DENV-1 of the pools of larvae were $2.22x10^9$ copies/pool on the fifth day of post-infection and $3.64x10^9$ copies/pool on the ninth day of post-infection for RIDL and WT respectively (Figure 4.2).

Table 4.7: Proportion of transovarial transmission from the total number of pools of larvae infected with DENV-1 of WT and RIDL *Aedes aegypti*.

Strain	**Number of infective females whose progeny were tested**	**Total pools of larvae**	**Number of positive pools by real-time RT-PCR**	**Number of positive pools by cell culture**
RIDL	20	53	22	8
WT	39	45	24	8

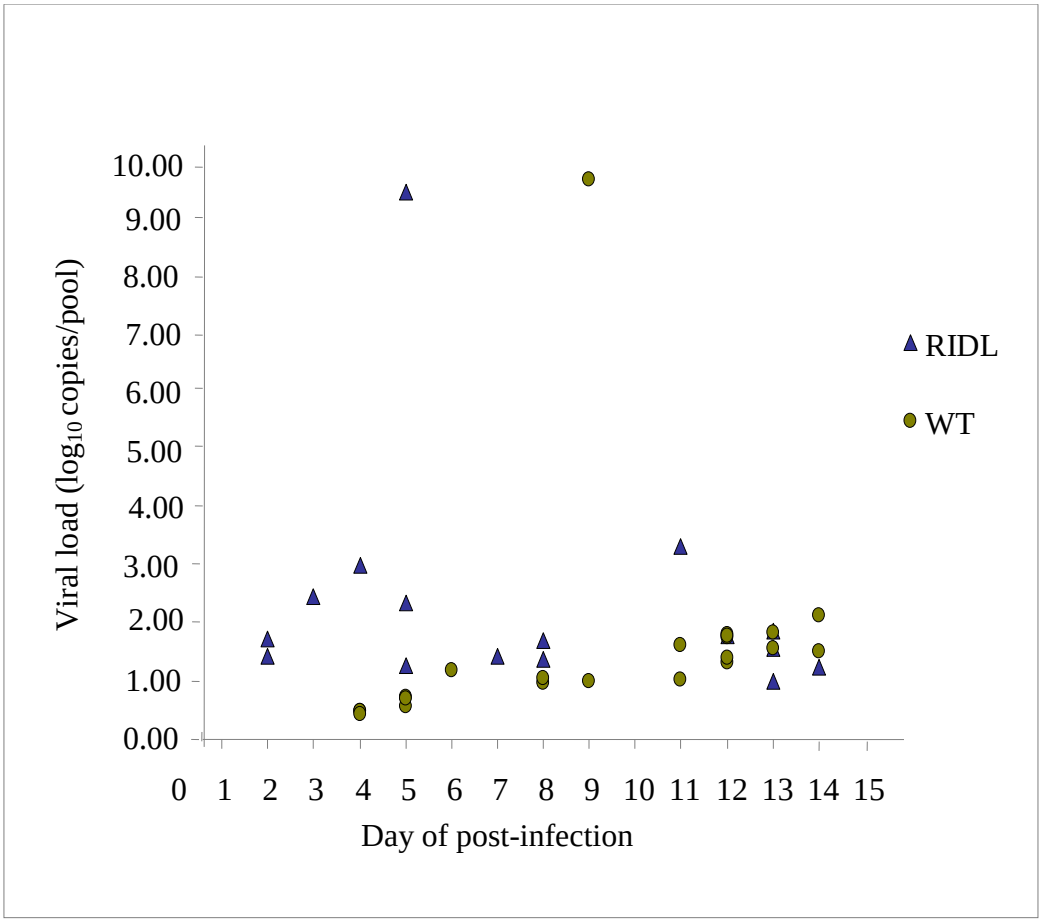

Figure 4.2: Viral load of pools of larvae of WT and RIDL *Aedes aegypti* infected with DENV-1 and quantified by real-time RT-PCR

4.2.2 Dengue Virus Serotype 3

There were 29 from 60 RIDL females that showed positive results of real-time RT-PCR (Table 4.3) which had produced fertilized eggs. As for WT strain, from 66 infective females (Table 4.3), 33 WT females did produce larvae. Only five pools of RIDL larvae and three pools of WT larvae were found positive by real-time RT-PCR. The viruses were isolated from three and two pools of larvae of RIDL and WT respectively. The highest copy number

of DENV-3 for RIDL larvae was $3.60x10^1$ copies/pool on the eighth day of post-infection and for WT was $5.94x10^4$ copies/pool on the second day of post-infection (Figure 4.3).

Table 4.8: Proportion of transovarial transmission from the total number of pools of larvae infected with DENV-3 of WT and RIDL *Aedes aegypti*.

Strain	**Number of infective females whose progeny were tested**	**Total pools of larvae**	**Number of positive pools by real-time RT-PCR**	**Number of positive pools by cell culture**
RIDL	29	53	5	3
WT	33	43	3	2

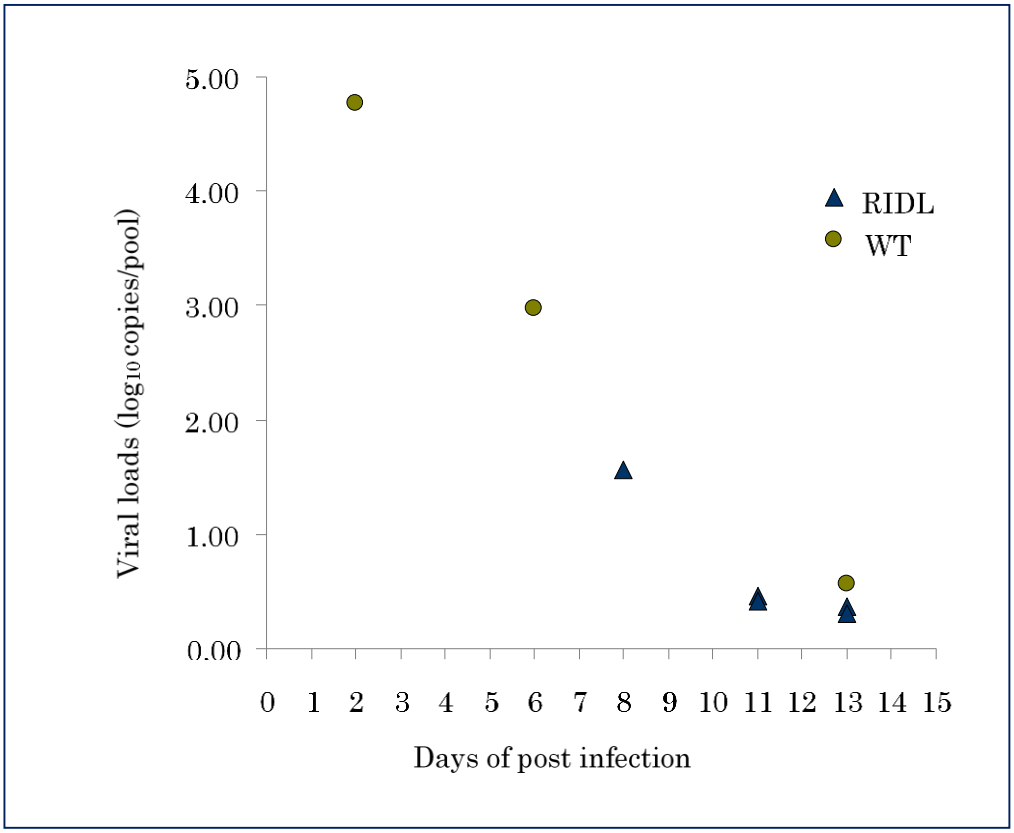

Figure 4.3: Viral load of pools of larvae of WT and RIDL *Aedes aegypti* infected with DENV-3 and quantified by real-time RT-PCR

4.2.3 Dengue Virus Serotype 4

From the total number of 66 infective RIDL females of real-time RT-PCR (Table 4.5), 39 of them had produced larvae (Table 4.9). For infective WT females, there were 93 females that showed positive result of real-time RT-PCR (Table 4.5) but only 63 females were able to produce larvae (Table 4.9). Only one pool from both strains was detected as positive by real-time RT-PCR (Table 4.9) and virus was successfully isolated from the pool of larvae for each of the mosquito strains. The copy numbers of virus of the pool

for RIDL on the fifth day of post-infection was $4.82x10^6$ copies/pool and for WT was $1.32x10^1$ copies/pool on the tenth day of post-infection (Figure 4.4).

Table 4.9: Proportion of transovarial transmission from the total number of pools of larvae infected with DENV-4 of WT and RIDL *Aedes aegypti*.

Strain	**Number of infective females whose progeny were tested**	**Total pools of larvae**	**Number of positive pools by real-time RT-PCR**	**Number of positive pools by cell culture**
RIDL	39	68	1	1
WT	63	62	1	1

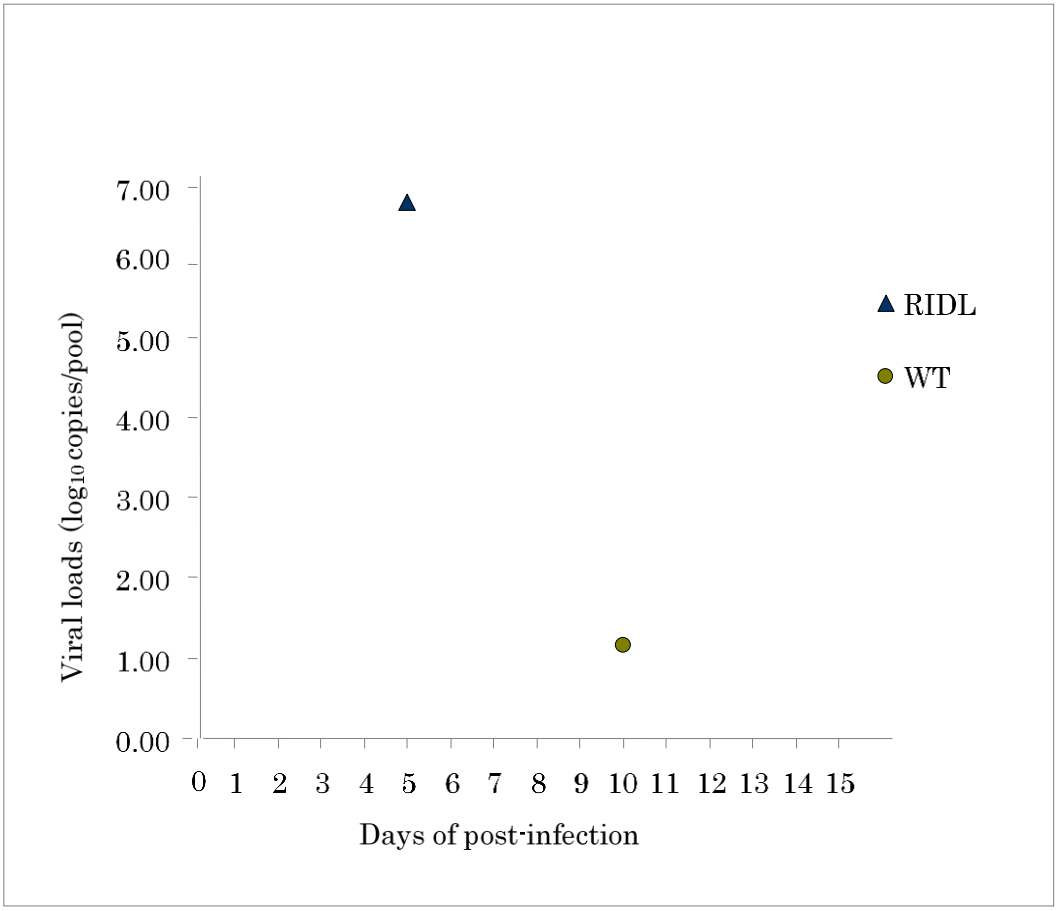

Figure 4.4: Viral load of pools of larvae of WT and RIDL *Aedes aegypti* infected with DENV-4 and quantified by real-time RT-PCR

CHAPTER 5

DISCUSSION

5.1 Transovarial Transmission

Transovarial transmission of dengue viruses (DENV) in *Aedes aegypti* mosquitoes, its major vector, may play a vital role in the preservation of DENV in environment (Lee et al., 1997). In this study, molecular surveillance of DENV is based on the screening of DENV genes in *Aedes aegypti* larvae. An infected mosquito can pass the DENV onto her offspring transovarially and this has been demonstrated to occur in nature, hence triggering a mechanism to permit the virus to live during the dry or cold seasons or the interim absence of non-immune vertebrate hosts (Rosen et al., 1983; Mourya et al., 2001a). Rohani et al. (2007) reported that field caught male *Aedes* mosquitoes were found to be the infected hosts which could pass the virus to females during mating and latter in turn transfer the virus to their progeny transovarially.

Several studies on *Aedes* mosquitoes have shown the presence of viruses in the ovaries indicating the transovarial transmission of the mosquitoes. In this study, both infected RIDL and WT females had transovarially transferred the DENV-1, DENV-3 and DENV-4 to its progeny. Cecilio et al. (2004) reported that DENV-1 and DENV-2 were detected in only one part of ovaries (oocytes), suggesting that the infection was located in the follicular epithelium and that *Aedes aegypti* are capable of transmitting the virus to its progeny. In Brazilian colonized *Aedes aegypti* females fed on a blood meal containing DENV-2, it has been shown that the virus was present in the ovary starting from day 3 until day 15 post infection (Campanelli et al., 2006).

DENV-2 can replicate in a special cell type or special part of mosquito reproductive system (Zhang et al., 2010). The access of dengue virus into the embryonic eggs took place in the genital chamber of the females as the established eggs were fertilized during oviposition (Frier and Rosen, 1987). Romoser et al. (2011) discovered that ovaries of *Aedes mcintoshi* were found positive with Rift Valley fever virus, indicating that ovarian infection from the hemocoel is an important step in initiating a vertically transmitting mosquito line.

Several studies in virologic surveillance have revealed that transovarial transmission was identified through all four serotypes of dengue virus in both

larvae and field-caught male adult *Aedes aegypti* (Hull et al., 1984; Lee and Rohani, 2005; Angel and Joshi, 2008; Guedes et al., 2010; Thongrungkiat et al., 2011). The association of dengue virus detected in nature with documented dengue cases was explored as *Aedes aegypti* field mosquitoes infected with dengue virus were captured approximately from one to two months prior to dengue outbreak and the infection rate of transovarial transmission was high during dry season once dengue cases started to upsurge (Chow et al., 1998; Urdaneta et al., 2005).

Numerous studies conducted under the laboratory condition, found that infected *Aedes aegypti* are capable of transferring the dengue viruses (DENV-1, 2, 3 and 4) transovarially to its progeny (Rosen et al., 1983; Lee et al., 1997; Mourya et al., 2001b; Joshi et al., 2002; Wasinpiyamongkol et al., 2003; Cruz et al., 2015).

5.2 Comparative Life Parameters of RIDL and WT Mosquitoes

A bionomics study conducted by Lee et al. (2009) showed that there was no significant difference of adult fecundity and offspring sex ratio between RIDL and WT *Aedes aegypti* except higher mortality rate was recorded for RIDL compared to WT mosquitoes. RIDL mosquitoes showed an overall 5% lower survival rate as well as reduced adult longevity compared to

WT mosquitoes in the mass rearing study (Bargielowski et al., 2011). Yet, the basic life history and growth rate of the genetically-modified *Aedes aegypti* was not affected by the genetic manipulation and its mating competitiveness was expected to remain intact. In a competitive mating experiment under contained semi-field conditions, RIDL mosquitoes were found to be as competitive as WT mosquitoes (Lee et al., 2012). However, no study regarding the dengue virus infection of RIDL mosquitoes was done before.

From this study, although all the three dengue virus serotypes exhibited different rates of transovarial transmission, there is no significant different of infection rate percentage when compared between the RIDL and WT strain (Chi square: $p>0.05$).

5.3 Susceptibility of Dengue Virus Serotypes

In the present study, DENV-1 was observed to exhibit the highest transovarial transmission rate (> 10%) compared to DENV-3 and DENV-4 which were below 10%. The infection rate of females infected with DENV-1 was the highest among the three serotypes of viruses. It has been revealed that susceptibility of *Aedes aegypti* to dengue viruses is related with transovarial transmission rates, since greater transovarial transmission in a strain could be due to the occurrence of a higher number of susceptible, infected female

mosquitoes (Mourya et al., 2001b; Joshi et al., 2002). Throughout transovarial transmission, predominantly when DENV-3 replicated in the dormant embryo, the organ intricated in the virus propagation may be a significant factor in the durability and survival of the mosquitoes (Joshi et al., 2002). This present study showed that the female mosquitoes infected with DENV-1 have higher serotype infectivity than the female mosquitoes infected with the other two serotypes. The percentage of transovarial transmission of a virus may rely on the species and serotypes of the virus or the geographic strain of the mosquito (Mourya et al., 2001b).

According to Lambrechts et al. (2009), the vector competence of *Aedes aegypti* for DENV-1 is determined by specific vector genotype with virus genotype (GxG) interactions in which potential for mosquito infection and virus transmission depends on the specific combination of vector and virus genotypes. The immunity response of *Aedes aegypti* to dengue infection is constructed on genotype to genotype relations, indicating that the consequence of the infection will depend on the specific interaction between both genotypes (Armstrong and Rico-Hesse, 2001; Lambrechts et al., 2009). There is evidence that RNA interference, a major component of the *Aedes aegypti* intrinsic immune response, may modulate infection by producing molecules to inhibit DENV replication (Black IV, 2002).

5.4 Fecundity of Infected and Uninfected Mosquitoes

From the results, the number of infected females that laid infected eggs with DENV-1 was the highest compared to DENV-3 and DENV-4. In this study, due to logistics reason, the eggs from DENV-1 infected females were kept at room temperature for five weeks before hatching compared to one to two weeks of storage time for eggs of infected females for both DENV-3 and DENV-4. This could be the explanation of why the transovarial transmission rate was highest in progeny infected with DENV-1. In addition, Mourya et al. (2001b) showed that the rate of transovarial transmission significantly increases when eggs laid from *Aedes aegypti* females infected with DENV-2 were hatched after numerous weeks of incubation at room temperature. The possible reason accounting for this observation was that the virus was able replicate in the dormant embryos. In comparison with the present study, the incubation period of infected eggs did affect the infectivity rate of the dengue viruses as demonstrated in DENV-1. Therefore, further study on the infectivity rate of dengue viruses prior to eggs incubation period are essential as the finding is very crucial to comprehend further on the mode of dengue transmission.

Tesh and Shroyer (1980) showed that San Angelo virus survived for three months in desiccated *Aedes albopictus* eggs stored at 28°C. Studies also reported the survival of Japanese encephalitis virus (Rosen et al., 1978) and

yellow fever virus (Beaty et al., 1980) in desiccated infected mosquito eggs was two and four months, respectively. These observations have great epidemiological importance acting as a potential mechanism for the viruses to survive during the inter-epidemic periods whereby the vector population is low or absent (i.e. during winter or dry season). It is presumably the virus must also become latent in the mosquitoes until hatching occurs. The mechanism of virus survival in the egg warrants further study.

From the present study, it was found that there is a significant difference in the number of eggs laid by both strain of RIDL and WT of infected and uninfected females mosquitoes for all the three serotypes of viruses. The number of eggs laid by infected females was lower compared to uninfected ones except for infected RIDL females of DENV-1 and infected WT females of DENV-4. There is no clue yet has been discovered to describe these results since the experiments were conducted at the same condition but nevertheless only the fully engorged uninfected RIDL females of DENV-1and DENV-4 cohort after blood feeding were selected for the eggs laying phase. Perhaps these uninfected females did not mate during the introduction of sex starved male mosquitoes into the cage of virgin females for the mating purposes. Therefore these uninfected females would only produce unfertilized eggs which were not able to be laid.

These results also showed that the infection of dengue viruses did affect the fecundity of the female mosquitoes. Fecundity of West Nile virus (WNV) in infected *Culex tarsalis* females was significantly lower than that of uninfected *Culex tarsalis* (Styer et al., 2007). This is consistent with the finding in the present study that the fecundity in transovarially infected *Aedes aegypti* was discovered to be lower when matched with the control mosquitoes.

From the results of the present study, it was found that the overall hatching rate was below 55% which could be due to the infection associated with development of the embryo inside the dormant eggs especially for DENV-1. Beaty et al. (1980) reported that *Aedes aegypti* larvae transovarially infected with yellow fever virus were retarded in their development. Similar results were also obtained with Japanese encephalitis virus (Tesh and Rosen, unpublished data). These observations suggest that flavivirus infection actually may have a lethal effect on the developing larvae. However, Shroyer and Craig (1980) stated that embryonic development of mosquitoes initiates immediately upon oviposition and proceeded without interruption until a fully formed embryo was produced. Therefore, this study could sustain the reason of low hatching rate observed in some of the uninfected females in the present study.

In the study, a high number of females laid eggs for both RIDL and WT strain for all three serotypes of viruses were recorded indicating a high

rate of fecundity. Prior to the human blood meal, all females were starved overnight. This is to ensure that the females' midgut is empty from the sugar solution as to promote to the blood meal for the egg laying purpose. Female mosquitoes that feed more regularly on human blood have higher fecundity whereas those that feed more regularly on sugar have higher longevity (Day et al., 1994).

Naksathit and Scott (1998) found that *Aedes aegypti* fed on human blood laid higher total numbers of eggs during their life time than did similar mosquitoes fed on the human blood plus the sugar. The lower number of eggs laid by mosquitoes may also be due to the physical interference of sugar meals with blood intake. Both infected and uninfected *Aedes aegypti* females were provided with sugar *ad libitium* which might have influenced severance of energy and thus mosquito survival and reproduction (Naksathit and Scott, 1998). Sugar feeding is known to decrease host-seeking behavior, reduce preoviposition behavior and delay oviposition (Foster, 1995).

Lee et al. (2009) showed that there was a significant difference in the hatching rate between both uninfected strains whereby the rate was higher for RIDL compared to WT mosquitoes. Studies on the life history traits have demonstrated that fitness cost in terms of both survival and fecundity are often associated with arboviruses infection in the mosquitoes (Lee et al., 2009).

However, from this study, there is no apparent indication that infection is associated with a decreased of egg hatching rate.

The number of females that laid eggs is also affected by temperature and moisture. In the present study, both WT and RIDL females were reared constantly at 26°C and relative humidity of 80%. The higher production of eggs rates was perceived at 25°C with relative humidity of 80% compared to 35°C with relative humidity of 80% (Costa et al., 2010). In *Anopheles krombeini*, the fecundity and longevity of females were severely reduced between 30 to 33.5°C when compared to individuals kept at 26°C (Huang, 1975; Joshi, 1996).

During the study, it was found that some females laid fewer eggs and some even did not oviposit at all. It is possibly related to the environmental stress whereby nutrients are redeployed to ensure the survival of mosquitoes which are else used to reproduce eggs (Chadee, 1997). Thus, if blood is used for the survival of the females instead of egg production (Nayar and Sauerman, 1975), the fecundity will be reduced during the unfavourable times.

5.5 Molecular Technique

5.5.1 Real-time RT-PCR Assay

In this study, all the samples were subjected to a real-time RT-PCR assay based on TaqMan technology (Chakravartiar et al., 2006) for the detection, serotyping and quantification of the viral load of the positive samples. Real-time RT-PCR assay is a rapid, sensitive and reproducible tool for large cohort studies. The sensitivity of detection was such that a 40^{th} fraction of the infected mosquito body lysate was sufficient to give a positive signal in the real-time RT-PCR (Gurukumar et al., 2009), thus even low levels of viral RNA in infected larvae could be detected. In this study, the results of real-time RT-PCR assay need to be supported with virus isolation. This is to ensure that the virus titer measurements quantified by real-time RT-PCR assay are not the fragments of dead virus that remained in the larvae.

In the present study, all the larvae samples that have the highest viral load of the DENV-1, 3 and 4 exhibited from day five onwards of post-infection except for the WT larvae infected with DENV-3 which the highest viral load was detected on day 2 of post-infection. The highest viral load detected was from the RIDL larvae samples infected with DENV-1 which is $2.22x10^9$ copies/pool. Tan et al. (2011) reported that *Aedes aegypti* adult

mosquitoes were found to have higher DENV-2 viral load on the fifth day onwards of post-infection (>1x10^7 copies viral load).

5.5.2 Virus Isolation

Virus isolation in cell culture remains a useful approach, especially when a viable isolate is needed, if viable and nonviable viruses are to be differentiated, and when infection is not of characteristics of any single virus. The high degree of susceptibility of the C6/36 cell line to dengue viruses has been documented (Igarashi, 1978; Kuno et al., 1985). White (1987) showed the susceptibility of C6/36 cells to infection by dengue viruses which might occur in low concentration in viremic sera.

In the present study, the larvae samples that were found positive for real-time RT-PCR were also subjected to virus isolation by using C6/36 cell line according to the protocol established by Gubler et al. (1984). The propagation of virus detected may require more than one passage to enable sufficient quantity of virus to be detected through virus isolation method. In the study, it was found that C6/36 cells infected with DENV-1 showed cytopathic effects as early as day four since it has the highest viral load compared to the DENV-3 and DENV-4.

The most sensitive virus isolation method is *in vivo* amplification through mosquito-derived cell cultures, such as C6/36 cell line (Vorndam and Kuno, 1997). However, the low competency of the infected cells and slow growth of virus are the major obstacles (Gubler et al., 1984; Rohani et al., 2007). Therefore, in the present study, samples that were found positive were subcultured up to four passages. In the study, progeny positive with DENV-1 had the highest number in transovarial transmission. Jarman et al. (2011) showed that serologically DENV-1 was the most isolated virus compared to other three serotypes of viruses from the C6/36 cell cultures. In addition, dengue serotypes also played an important role in the success of virus isolation (Jarman et al., 2011).

5.6 Future Study

In the present study, it was found that the incubation period of eggs before hatching associated with the susceptible of dengue virus to both *Aedes aegypti* female mosquitoes. Therefore, it is a significant finding to further study as to observe the perseverance of viruses in the environment through the transovarial transmission. This study might be extended to other species of arthropod vector-borne diseases. Future studies may also focus on evaluating inoculations of DENV-1, 3 and 4 serotypes in the *Aedes albopictus,* which is the secondary vector of DENV infection.

CHAPTER 6

CONCLUSION

Aedes aegypti is the main vector of the virus that causes dengue fever and its more severe form, dengue hemorrhagic fever. To date, a safe tetravalent vaccine is still in development and at least decades away from being available for widespread used and no specific medication for dengue. *Aedes* is a day-biting mosquito, bed nets are therefore not very effective against *Aedes*-borne diseases like dengue or chikungunya. As conventional control methods have been unable to prevent its global re-emergence as a significant threat to human health, the development of new control methods is gaining importance. One possibility is a genetics-based control strategy modelled on the traditional sterile insect technique (SIT). The RIDL system is such an approach, and has been engineered in *Aedes aegypti* with tetracycline-dependent repression of a dominant lethal gene construct.

In the present study, there were no significant differences of transovarial transmission between RIDL and WT female mosquitoes for DENV-1, DENV-3 and DENV-4. Except for DENV-1, the transovarial transmission rate was low (< 10%) and similar for both strains of females. The

study also found that RIDL and WT female mosquitoes were most susceptible to DENV-1. The infection rate of females infected with DENV 1 was the highest among the three serotypes of viruses.

As expected, the transgene did not affect the fecundity of the RIDL females after feeding on infected blood. The number of eggs laid per female *Aedes aegypti* usually showed high variability. The present study highlighted that transovarial transmission may provide a mechanism for the virus to maintain itself in an area through the low mosquito season; this is further suggested by studies demonstrating the possibility of maintenance of the virus through several generations of *Aedes aegypti* (Joshi et al., 2002) and *Aedes albopictus* (Shroyer, 1990) without further exposure to the virus. As the majority of RIDL female progeny will die before emerging as adults and being able to reproduce or bite; it is very unlikely that the strain would play any role in the maintenance of a virus in the field. Consequently, the lethal gene insertion does not affect the transovarial transmission when it occurs.

This study has also emphasized the importance of larval control since the immature stages may become the reservoir of the virus during the inter-epidemic period. It is clearly revealed that the suppression of the early phase of *Aedes* mosquito as well as the eradication of breeding sources should be emphasized.

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