Integrated Epidemiological Study of Dengue Virus Transmission in Java, Indonesia

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By

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Abstract

Dengue virus (DENV) is one of the most important arbovirus infections which continues to be spread to many parts of the world. The widespread distribution of the vector *Aedes sp*, DENV genetic evolution, emergence of a new serotype, global warming, environmental changes, population growth and human mobility are some of the factors affecting DENV transmission. From the many studies conducted on DENV, there is still a lack of integrated research that includes several aspects that affect DENV transmission at a local scale. The aims for this study was to conduct an integrated study of DENV tranmission, covering entomology, DENV, and socio-economic and environmental factors using Banyumas Regency, Java Indonesia, as a model area. The uniqueness of demography, socioeconomy and environment of each area emphasizes the importance of this research.

For the entomology factors, this study found that traditional larvae indices such as House Index (HI), Breteau Index (BI) and Container Index (CI), which have been applied for many decades in entomology surveys, are not relevant measurements for determing mosquito populations. These findings supported previous findings that larvae indices cannot predict the transmission risk level and is not correlated with DENV incidence. In this study, adult mosquito collections were found to be a better measurement of risk of DENV transmission. A high vertical transmission rate was also confirmed in an endemic area, which is possibly one explanation for DENV persistence in that area. From a knowledge, awareness and practice (KAP) survey, there is no correlation between knowledge, awareness and practice of DENV prevention and control, and there is also no association between KAP of people with the mosquito infestations in the area of study. This finding leads to the need for better strategies such as education campaigns about DENV prevention to ensure not only an increase in knowledge but also this knowledge translates into practices.

During collection of serum samples from DENV infected patients a higher number of adult age groups reported DENV cases, indicating an age group shift from children to adults. Most of the samples (89%) from positive result of IgG/IgM test had a secondary infection by serological test, which likely increases the possibility of developing severe clinical manisfestations. Many publications believe that secondary infection by different serotypes could cause severe DENV infection. Unfortunately, the serotyping and genotyping of the patient samples could not be completed due to time constraints, so the information of circulating serotypes and genotypes could not be obtained. It would be interesting to

further analyse the serotypes and then correlate them with the less or more severe clinical manifestations and also capture the spread of disease from pylogenetic trees from the genotyping results.

Based on spatio and spatio-temporal models, it can be concluded that socioeconomic factors, particularly the level of education and the employment structure were the most important risk factors of DENV infection. It was also revealed that enviromental factors had only a little influence on DENV infection, in contrast with many previous beliefs that global warming and environmental changes are the main factors of DENV infection. Human mobility was proposed to be the main explanation of this phenomenon since more educated people and people with good job type tend to have higher exposure to DENV infection due to their movement from home to work places or public areas. This also complements the fact that more adults reported DENV infection during the patient sample collection, suggesting that adult age groups possibly have a higher risk of DENV infection due to higher mobility, which means higher exposure to DENV infection. The possibility of having a secondary infection is also higher in adults since there has been more time to have the first infection and then the second infection.

In order to complete this integrated study, the influence of temperature on mosquito immunity, in particular the RNA interference (RNAi) response was tested. Based on RNAi activity in 24°C, 28°C and 32°C, RNAi activity was slightly more efficient following the increase of temperature. In addition, the infection of Aag2 cells with SFV showed that the increasing temperature will result in lower virus replication. We can assume that the lower or higher temperature only contributes a minor effect on RNAi machinery *in vitro*.

In conclusion, this integrated epidemiological study finds that current entomology surveys are not relevant, because they are not associated with the risk of transmission. In addition, socioeconomic factors rather than environmental factors are proposed to be the most significant factor for DENV infection. Findings such as age shift, secondary infection, human mobility and a high vertical transmission rate are important information which could help the public health sector in their planning and action on DENV prevention and control strategies.

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Author's declaration

I declare that the research work reported in this thesis is is my own work except where otherwise stated. Two chapters in this thesis have been produced as independent papers for publication :

Chapter 2. In revision stage in PLOS *Neglected Tropical Medicine*. Title : Dengue in Java, Indonesia: relevance of mosquito indices and vertical transmission as risk predictors. Siwi P. M. Wijayanti, Sunaryo Sunaryo, Suprihatin Suprihatin, Melanie McDonald, Stephanie M. Rainey, Esther Schnettler, Roman Biek, and Alain Kohl

Chapter 4. In preparation for submission to *Lancet Infectious Diseases*. Title : Spatial and spatio-temporal models reveal the importance of socio-economic versus environmental risk factors for reported dengue cases in the tropical environment of Java, Indonesia. Siwi P. M. Wijayanti*, Thibaud Porphyre*, Stephanie M. Rainey, Melanie McDonald, Esther Schnettler, Margo Chase-Topping, Alain Kohl

I further declare that no part of this research work has been submitted for the requirement of any other degree.

Siwi Pramatama Mars Wijayanti

Abbreviations

DENV	dengue virus
DENV-1	dengue virus serotype 1
DENV-2	dengue virus serotype 2
DENV-3	dengue virus serotype 3
DENV-4	dengue virus serotype 4
DENV-5	dengue virus serotype 5
ORF	open reading frame
NS	non structural
E	envelope
UTR	untranslated region
C	capsid
IFN	interferon
Mtase	methyltransferase
RdRp	RNA-dependent RNA polymerase
DHF	dengue Haemorrhagic Fever
DSS	dengue Shock Syndrome
DF	dengue fever
ADE	antibody-dependent enhancement
SEM	scanning electron microscopy
EIP	extrinsic incubation period
MIB	midgut infection barrier
MEB	midgut escape barier
SGIB	salivary gland infection barier
SGEB	salivary gland escape barier
RNAi	RNA interference
dsRNA	double-stranded RNA
viRNA	virus-derived small interfering RNA
Dcr-2	Dicer-2
RISC	RNA-induced silencing complex
Ago-2	argonaute
DC	dendritic cells
NK	natural killer
CFR	case fatality rate
HI	haemagglutination inhibition
INLA	integrated nested laplace approximation
HI	house index
BI	breteau index
CI	container index
CPI	container pupae index
HPI	house pupae index
FLI	free larvae index
OI	ovitrap index
F1	Filial 1
VIR	virus infection rate
SBPC	streptavidin-biotin-peroxidase-complex
IHC	immunocytochemistry
PBS	phosphate buffered saline
KAP	knowledge, awareness, practice
GIS	geography information system

GPS	global positioning system
PCR	polymerase chain reaction
ELISA	enzyme-linked immunosorbent assay
cDNA	complementary DNA
IgG	Immunoglobulin G
IgM	Immunoglobulim M
RNA	ribonucleic acid
PCA	principal component analysis
LST	land surface temperature
IRR	incidence risk ratio
DIC	deviance information criterion
MOI	multiplicity of infection

Chapter 1

1 General Introduction

Dengue virus (DENV) infection is the most common arthropod-borne virus (arbovirus) disease affecting many countries around the world, especially in the tropics (Bhatt et al., 2013; Rogers, D. J. et al., 2006). The increasingly widespread distribution of DENV vector, *Aedes sp*, increase the threat of this virus in the future (Higa, 2011). Currently there is no licensed vaccine against the disease, and efforts still focus on prevention. Extensive molecular, entomology, environmental, demographic, and mathematical models of dengue virus infection have been carried out, but there is an urgent need for integrated research to be carried out. Dengue virus infection can be affected by several complex variables each of which must be considered in its role on dengue transmission. The main aim of my project was to integrate several variables such as molecular, entomology, demography, socioeconomic and environment in one location, Banyumas Regency in Java (Indonesia) as study area in order to gain a better understanding of DENV transmission that can inform local preventive measures.

1.1 Dengue Virus

The causative agent of dengue disease is DENV, which consists of five DENV serotypes 1, 2, 3, 4 and 5 (Bäck & Lundkvist, 2013; Halstead, 2008a; Mustafa et al., 2015; Reich et al., 2013). The origin of DENV is still unknown. It is hypothesized that human DENV evolved from a sylvatic DENV virus strain, transmitted among nonhuman primates in Africa or Asia between 100-1,500 years ago (Wang et al., 2000). This sylvatic cycle is proposed to be ancestral because it requires a minimum human population size of 10,000-1 million for interhuman transmission to occur, which did not exist or where unlikely to exist in Africa 4000 years ago (Gubler, D.J, 1998).

1.1.1 Classification of Dengue Virus

DENV are members of the genus *Flavivirus* in the Family *Flaviviridae* (Calisher et al., 1989). The determination of this taxonomy is based on antigenic cross-reactivity with other flavivirus, genomic organization and sequence homology (Rico-Hesse, 2003). Because of common morphology and genomic structures, it is difficult to identify members of the flavivirus family by classical serological techniques (Henchal & Putnak, 1990). Initially, DENV was classified using T1 RNase fingerprinting into topotypes (Repik et al., 1983). The clusters are defined as serotype and termed as dengue virus type 1 (DENV-1), dengue

virus type 2 (DENV-2), dengue virus type 3 (DENV-3), dengue virus type 4 (DENV-4) and recently dengue virus type 5 (DENV-5). Among the serotypes of dengue viruses, they are distinct but related antigenically (Rico-Hesse, 2003). Furthermore, the DENV classification was carried out using nucleic acid sequencing within each serotypes (Rico-Hesse, 1990). The "genotype" term defined as cluster of DENV having nucleotide divergence no more than 6% within the E/NS1 junction (Rico-Hesse, 1990).

1.1.2 Dengue Virus Structure and Genome Organization

DENV, similar to other flavivirus, morphologically is a spherical shape with a diameter of 40-50 nm. This virus contains a nucleocapsid with a diameter of about 30 nm, surrounded by a lipid envelope. The viral capsid and nucleocapsid contain an RNA genome,which encodes a single large polyprotein, then cleaved into several structural and non-structural mature peptides. Envelopes (E) contain a lipid bilayer consisting of a layer of lipids, around 51000-59000 Dalton (Perera & Kuhn, 2008). DENV genome is a single stranded positive sense RNA, of approximately 11,000 nucleotides, which has a 5' cap but no poly (A) tail (Lindenbach & Rice, 2003). DENV structure can be illustrated in Figure 1-1.

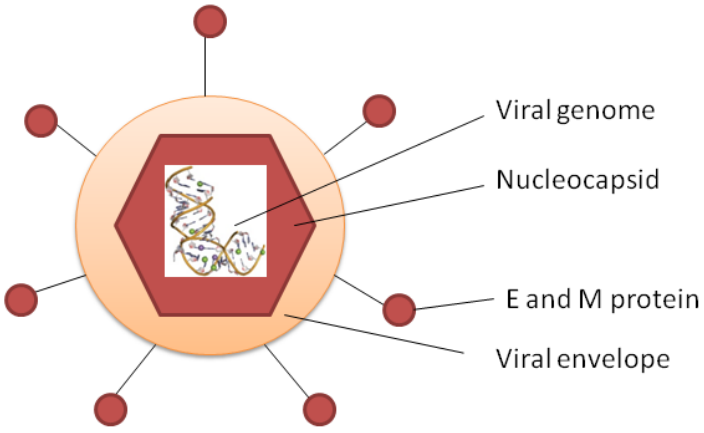


Figure 1-1 DENV structure.
DENV has spherical shape, contain nucleocapsid surrounded by viral envelope. Envelope (E) and Matrix (M) protein span through the lipid bilayer. Adapted from http://www.nature.com/scitable/topicpage/dengue-viruses-22400925

The genome encodes an open reading frame (ORF) of about 10, 200 nucleotides and encodes 10 proteins, with 3 structural proteins encoded from a quarter of the genome and 7 nonstructural proteins (NS) encoded by the coding region of the rest. The protein sequences are as follows : 5' C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-

3’(Halstead, 2008a). The structural proteins are the capsid protein (C) and membrane (M) or its precursor (prM) and Envelope (E) protein, while the nonstructural proteins consist of the NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 proteins (Chambers et al., 1990). The schematic diagram of DENV genome organization can be seen in Figure 1-2.

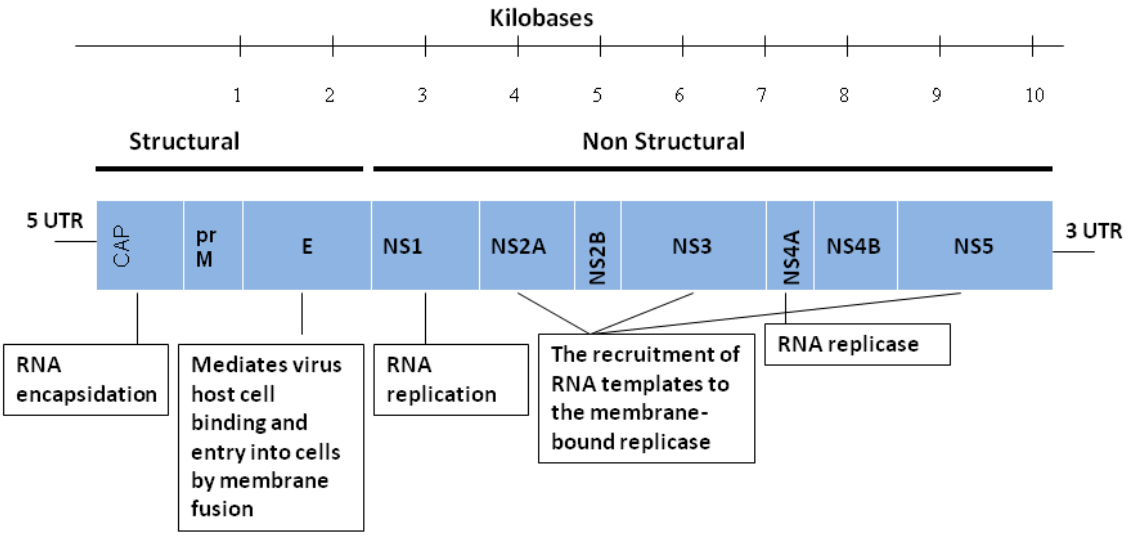


Figure 1-2 The schematic diagram of DENV genome organization.
The DENV genome consists of structural and non structural protein. It also showed the major function of some dengue protein. Adapted from (Weaver & Vasilakis, 2009).

The DENV genome consist of a 5 ’ (100 nt) and 3’(400-800 nt) untranslated region (UTR) (Figure 1-2). The 5’ UTR consist of 95 to 101 nucleotides long, contains two elements which important for viral replication (Lodeiro et al, 2009; Gebhard et al, 2011). The 3’UTR plays an important part in viral replication and translation (Brinton et al., 1986), while the capsid (C) protein, an 11 kDa homodimer protein, is important for RNA genome encapsidation (Chang et al., 2001). The membrane protein (prM /M) is a part of the virion, plays an essential role in release of mature virions during the late stages of virus assembly (Kuhn et al., 2002). Envelope (E) protein, 53 kDa class II N-glycosylated dimeric membrane fusion protein, is one of the antigenic determinants of the virus and important for entry and viral attachment (Johnson et al., 1994; Rey, 2003). Many key epitopes were observed in E glycoprotein which are recognized by neutralizing antibodies (Rouvinski et al., 2015). The 3’ end of DENV genome encoding the non-structural proteins is in the following order : NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5, as shown in Figure 1-2. The function of each non structural protein is listed in Table 1-1.

Table 1-1 Non structural proteins of DENV and their function

NS Protein	Functions	References
NS1	Plays a role in viral RNA replication complex ; soluble complement-fixing antigen	(Fan et al., 2014; Lindenbach & Rice, 1997)
NS2A	Involved in the coordination of RNA packaging and replication, possibly antagonism of interferon (IFN)	(Jones et al., 2005; Khromykh et al., 2001; Munoz-Jordan et al., 2003)
NS2B	Associates with NS3 to form the viral protease complex and serves as a cofactor in the structural activation of the DENV serine protease of NS3	(Erbel et al., 2006; Leung et al., 2001)
NS3	Involved in the processing of the viral polyprotein, and RNA replication	(Natarajan, 2010)
NS4A	Essential components of the endoplasmic reticulum membrane-associated replication complex.	(Zou et al., 2015)
NS4B	An interferon (IFN)-signaling inhibitor	(Zou et al., 2015)
NS5	Methyltransferase (Mtase) and RNA-dependent RNA polymerase (RdRp)	(Ackermann & Padmanabhan, 2001; Dong et al., 2012; Egloff et al., 2002)

1.1.3 Dengue Disease

DENV infections cause wide ranging clinical symptoms from asymtomatic to severe manifestations such as dengue Haemorrhagic Fever (DHF) and dengue Shock Syndrome (DSS) (Yacoub et al., 2013). Infection with one of five dengue serotypes could induce protective immunity to that serotype, but also showed short term immunity to other serotypes (Reich et al., 2013). Most of DENV infections are asymptomatic, however some lead to more severe infections. Clinical manifestations in children are usually different from the infection in adults, with the mortality rate also higher than in adults (Chastel, 2012).

In the early stage of DENV infection, the symptoms are similar to other Flavivirus infections, meaning of the likelihood of developing severe infection can be challenging. Dengue infected patients can develop either asymptomatic infections or one of three clinical manifestations, fever, dengue Fever (DF) with or without hemorrhage or dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS) (WHO, 2009). The clinical classification of DENV infection is summarized in the schematic diagram in Figure 1-3.

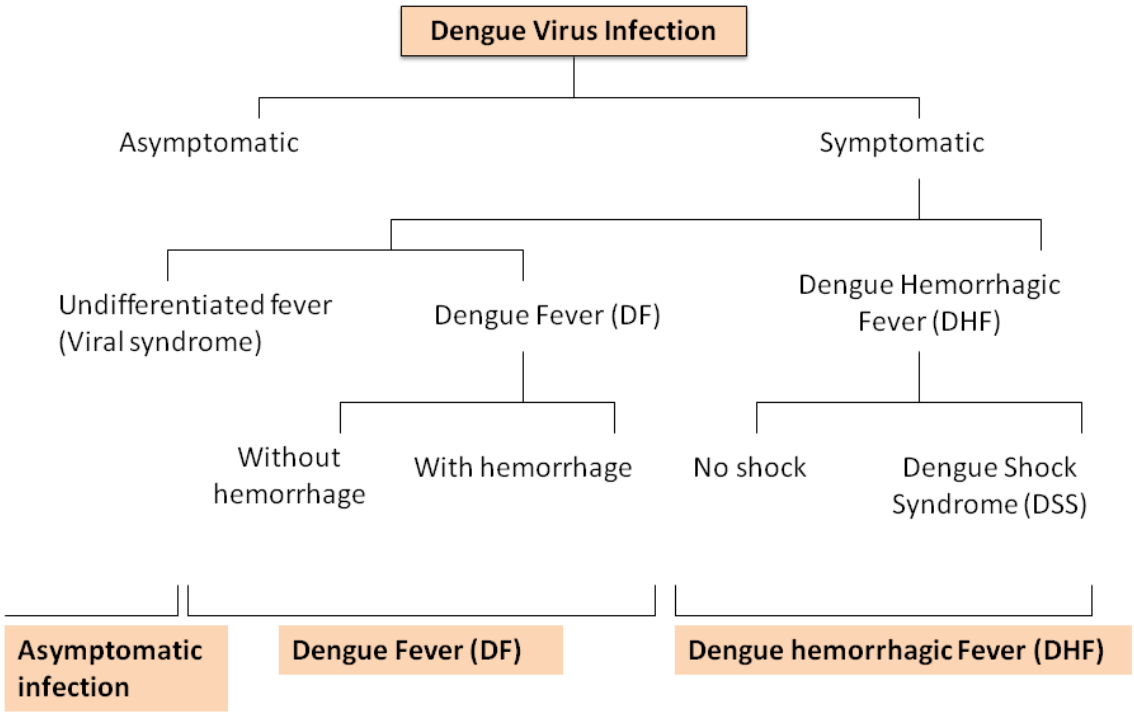


Figure 1-3 Schematic diagram of the DENV infection clinical classification.
The diagram shows the clinical classification and manifestations of DENV infection, from asymptomatic infection to dengue fever and dengue hemorrhagic fever. Adapted from (WHO, 2009).

Dengue fever usually occurs after an incubation period of 4-7 days, with common symptoms such as coughing, vomiting and pain in the abdominal area. For symptomatic DENV, after the incubation period the illness will go through three phases which are febrile illness, critical illness and recovery (WHO, 2009). The course of dengue illness is illustrated in the Figure 1-4.

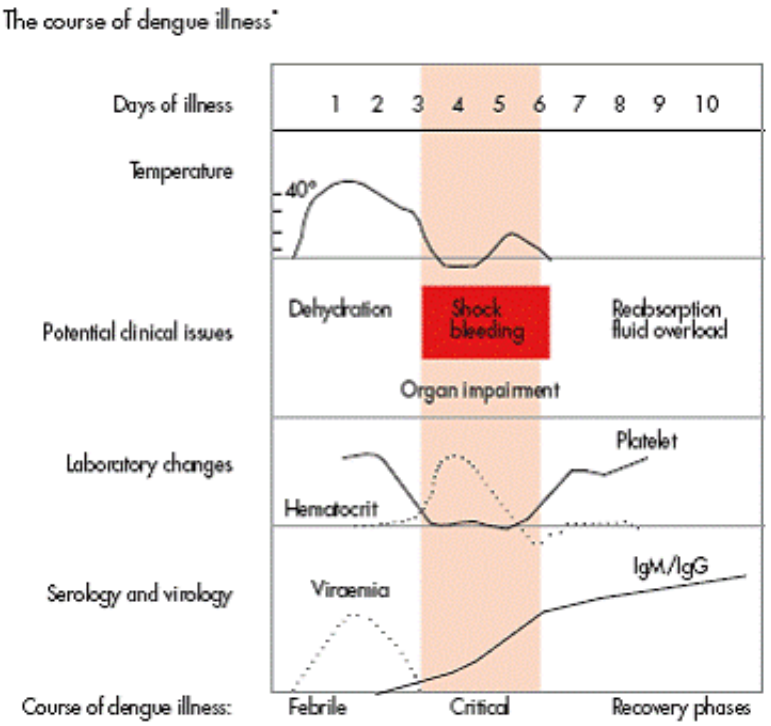


Figure 1-4 The course of dengue illness.
The three phases of dengue disease: febrile, critical and recovery phases. The figure illustrates the temperature, potential clinical issues, laboratory-detected changes in blood and serology and virology of each phases (WHO, 2009).

In the febrile phase, the typical feature is that patients experience high fever, which lowers for a while and then become very high again. This acute febrile phase usually lasts 2–7 days, and is often accompanied by facial flushing, skin erythema, generalized body ache, myalgia, arthralgia and headache. A positive tourniquet (capillary fragility) test in this phase increases the probability of dengue disease (Chakraborty, 2008; WHO, 2009). Afterwards, patients enter the critical phase, when the body temperature declines to 37.5-38 °C or less. This phase usually happens between days 3-7 of the illness, and the patient can develop severe infection symptoms, such as DHF and DSS with marks of bleeding and plasma leakage. The hallmarks of the critical phase is an increase of capillary permeability and haematocrit level. Bleeding can present in a wide range of manifestations from petechiae (small skin hemorrhages) to severe gastrointestinal bleeding, caused by capillary fragility and thrombocytopenia (a low number of blood platelets)(Chakraborty, 2008). Recovery phase occurs when the patient survives the 24–48 hour critical phase, then the extravascular fluid reabsorption will occur gradually over the next 48-72 hours. In this phase, they enter the recovery phase with improvement of well being, recovery of appetite and the stability of haemodynamic status. The recovery of the platelet count and the

increase of number of white blood happened after defervescence phase (Lum et al., 2014; WHO, 2009).

Several studies suggested that most of the patients who develop severe illness (DHF and DSS) had prior infection with a different DENV serotype (secondary infection) (Graham et al., 1999; Suwandono et al., 2006; Yamanaka et al., 2011). The accepted hypothesis of this phenomenon is called antibody-dependent enhancement (ADE) of DENV infection. The mechanism of ADE is proposed when circulating antibodies bind to newly infecting dengue virus particles but do not clear the infection and neutralisation does not occur (Halstead, S B., 2014). This inefficient antibody binding triggers infection of target cells such as macrophages and virus production, leading to severe disease development (Flipse et al., 2013). Several studies also proposed that some serotypes or genotypes within serotypes are more virulent than others, and cause more severe infections (Chakraborty, 2008; Fried et al., 2010).

1.1.4 The Global Distribution of Dengue Disease

The first well-characterised clinical description of dengue disease was noted by Benjamin Rush in 1780 during an outbreak in Philadelphia (Rush, 1951). Subsequently, DENV epidemics happened during the 18th and 19th centuries in newly colonised lands in areas of Asia and Africa (Halstead, S., 2008). World war II played an important role in the spread of DENV by movement of troops, war material and population mobility which transported the viruses and mosquito vectors. After World war II, many Asian countries became hyperendemic with DENV infections, and this is getting worse by unprecedented urban growth since 1950s (Gubler, D., 2011; Sabin, 1952). Before 1970, only 9 countries had suffered under severe dengue epidemics. Currently, more than 100 countries, in the World Health Organization (WHO) regions of Africa, South-East Asia, the Americas, the Eastern Mediterranean and the Western Pacific are endemic with DENV. The tropical areas of the Americas, South-East Asia and Western Pacific have experienced the most devastating dengue outbreaks (WHO, 2015a). In another study, 128 countries were identified as dengue-present and 3.9 billion people at risk of infection (Brady et al., 2012). The map below illustrated the DENV risk area worldwide.

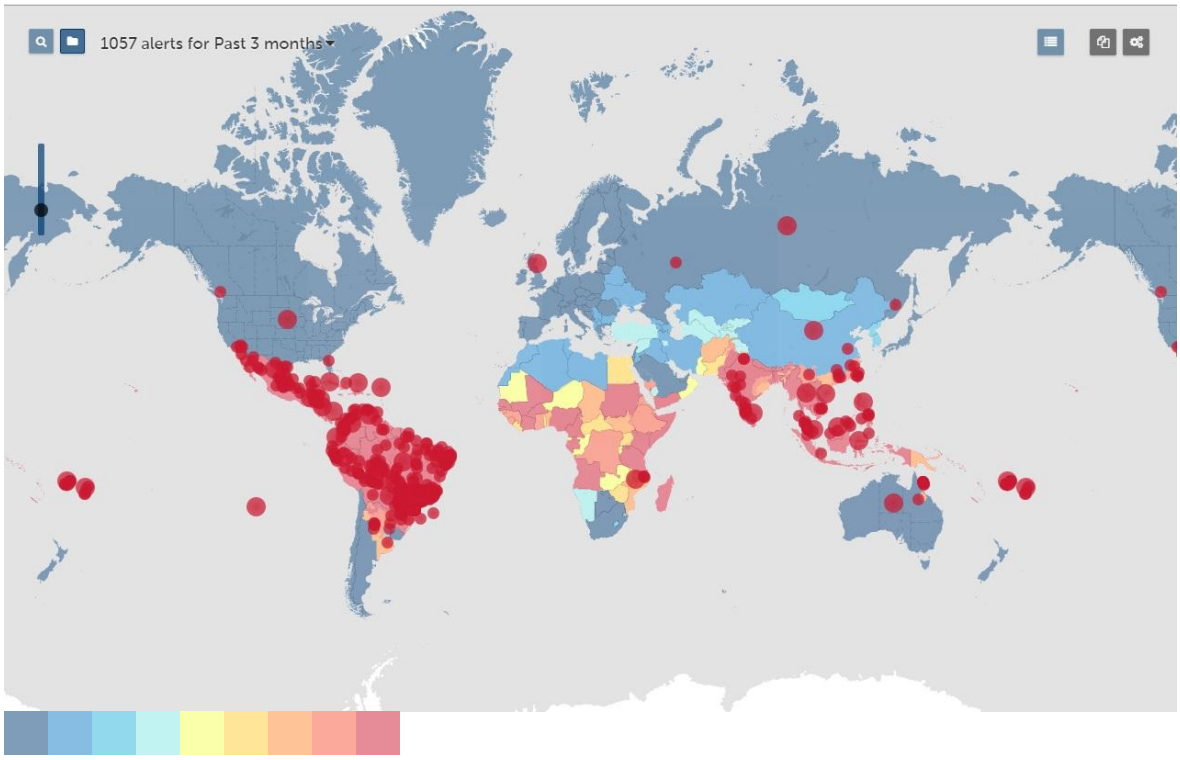


Figure 1-5 DENV risks area worldwide.
The circle with each color illustrated the degree of endemicity, from dark blue (absent) to dark red (present). Big red dots are imported dengue cases in country level; small red dots are imported dengue cases in local level. Risk areas are determined by consensus between sources including: national surveillance systems, published literature, questionnaires and formal and informal news reports. Taken from the CDC Health Map Report (http://www.healthmap.org/dengue/en/) (CDC, 2015).

Quite clearly, DENV infection is threatening the lives of billions of people around the world. The high burden of disease continues in the Asian continent, particularly in South East Asia. Dengue fever cases also continue to be established and reported in Africa and Australia. In recent decades, the dengue incidence in South America and the Carribean has increased significantly. In addition, the high movement of people through travel provides continuing exposure to DENV infection in North America and Europe (Guzman, A. & Isturiz, 2010).

1.2 Mosquito Vectors of DENV

1.2.1 Vector of dengue virus transmission

Aedes aegypti is considered to be the primary vector of DENV transmission, while *Aedes albopictus* acts as a secondary vector (Halstead, 2008b; Higa, 2011). *Aedes sp* acquire DENV when they bite a human or primates infected with DENV. These species can transmit DENV though biting another host, and the multiple host biting preferences of *Aedes sp* facilitate DENV to be spread in communities (Scott & Takken, 2012).

Mosquitoes have the potential to transmit DENV throughout their life span (15-65 days) (Chakraborty, 2008). Only female mosquites take human blood meals in order to obtain proteins required for egg maturation. The low concentration of the amino acid isoleucine in human blood is the reason of the exclusivity of female *Aedes aegypti* feeding on human blood, instead of plant carbohydrates (Harrington et al., 2001; Scott & Takken, 2012). The unique concentration of isoleucine in human blood is meet the requirement for energy, fitness and reproduction of female mosquito, compared to the non human blood. . (Harrington et al., 2001; Scott & Takken, 2012).

1.2.1.1 *Aedes aegypti*: a key vector of DENV.

The origin of *Aedes aegypti* (Linn.) is recognized to be in Sub Saharan Africa (Powell 2013). *Aedes aegypti* L belongs to the Order *Diptera* and Family *Culicidae* (Halstead, 2008a). *Aedes aegypti* is known as the principle vector of DENV, although the official common name for *Aedes aegypti* is "yellow fever mosquito" as this species was previously known as the vector of yellow fever virus (YFV) (Family: *Flaviviridae*; Genus : *Flavivirus*). Besides DENV and YFV, this species can also transmit chikungunya virus (CHIKV) (Family : Togaviridae; Genus : *Alphavirus*) (Powell 2013). Distribution of *Aedes aegypti* is principally well adapted to tropical and sub-tropical areas in South-East Asia, between latitude 40°N and 40°S (WHO, 2011).

Generally, *Aedes aegypti* are well adapted to live close to human dwellings in urban areas (anthrophophilic) and breed primarily in man-made containers (Christophers, 1960). However they can develop in natural containers such as coconut shells, snail shells, leaf axils and tree holes (Tandon & Ray, 2000). The type of container can vary depending on the behaviour of people in certain areas (Kumar et al., 2002; Seng & Jute, 1994; Tandon & Ray, 2000). This species is a day-time feeder, with the peak time of biting during early morning. Multiple host feeding is also demonstrated by *Aedes aegypti*, supporting the spread of DENV (Farjana & Tuno, 2013; Scott & Takken, 2012; Takken & Verhulst, 2013).

1.2.1.2 Aedes albopictus: the secondary vector.

Aedes albopictus (Skuse), also known as the "Asian tiger mosquito", is considered as a secondary vector of DENV transmission. In 1931, *Ae. albopictus* was identified as a vector of DENV (Gratz, 2004). *Aedes albopictus* has also been identified as a vector for West Nile Virus (WNV) (Family : *Flaviviridae*; Genus *Flavivirus*), eastern equine encephalitis

virus (EEEV) (Family *Togaviridae*; Genus *Alphavirus*), Japanese encephalitis virus (JEV) (Family *Flaviviridae*; Genus *Flavivirus*) and chikungunya virus (Family *Togaviridae*; Genus *Alphavirus*) (Mitchell et al., 1992). The global distribution of *Aedes albopictus* has changed significantly through intercontinental shipment of tyres and has introduced this species to America, Europe and Africa (Hawley, 1988; Reiter & Sprenger, 1987). Currently, *Aedes albopictus* can be found in temperate regions and its area of origin (Asia), Europe, America, Africa and a number of locations in the Pacific and Indian Oceans (Carvalho et al., 2014; Kraemer et al., 2015; Paupy et al., 2009; Roche et al., 2015). The wide spread distribution of *Aedes albopictus* is likely because this species has better adaptation to cold temperatures, unlike *Aedes aegypti* (Paupy et al., 2009). Other factors such as uncontrolled urbanisation and global warming also affect the density, larval development rate, and survival of adult *Aedes albopictus*, increasing the vector capacity and DENV transmission (Li, Y. et al., 2014). The timeline of *Aedes albopictus* invasion into many countries around the world is shown in Figure 1-6.

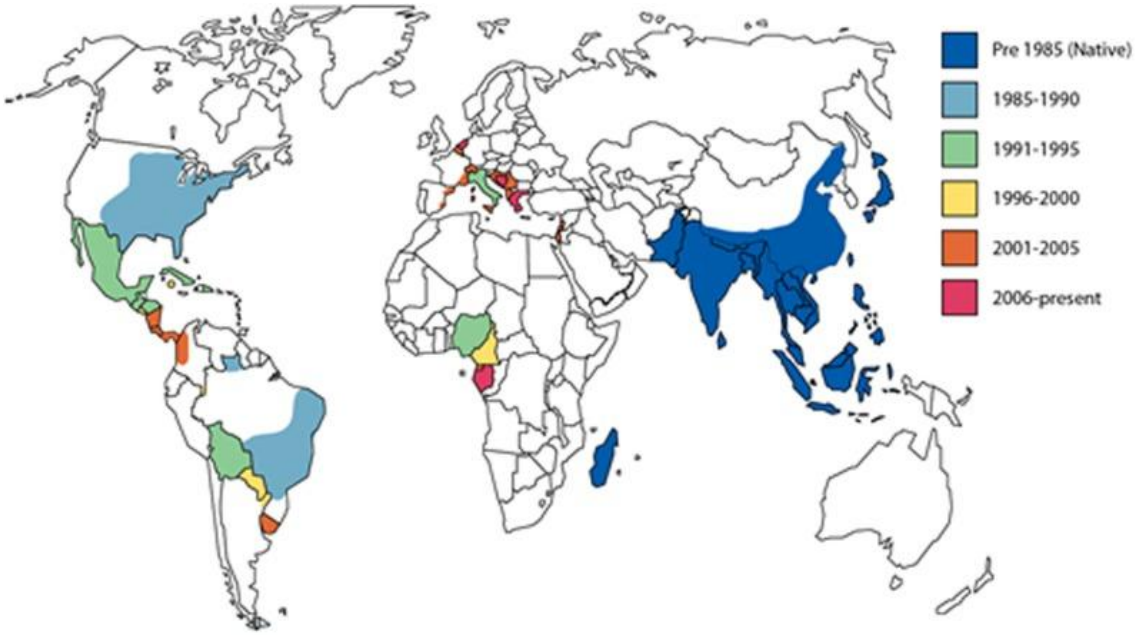


Figure 1-6 Timeline of *Aedes albopictus* global invasion.
Aedes albopictus has spread around the world, mainly because of its physiological and ecological plasticity. The colours in the map represent the time of *Aedes albopictus* invasion into many countries in world. The native habitat of *Aedes albopictus* is shown in dark blue, then subsequent invasion to North America, South America, Europe and Africa in light blue (1985-1990), green (1991–1995), yellow (1996–2000), orange (2001–2005), and red (2006–present) (Waldock et al., 2013).

The breeding sites of *Aedes albopictus* vary widely in variety with both artificial habitats such as water containers, buckets, discarded tires and flower vases and natural habitats

such as tree holes being used (Hawley, 1988; Higa, 2011). However, this species prefers natural habitats with an abundance of vegetation, feed and outdoor resting areas. Populations of *Aedes albopictus* are usually high in rural and suburban areas because of this preference (Braks et al., 2003; Scott & Morrison, 2010). Biting time of this mosquito is primarily in the early morning and late afternoon, with exceptions such as night time biting sometimes observed depending on the season, region, and the nature of the human habitat. This species not only bites humans, but also a wide variety of amphibians, reptiles and vertebrates from cold to warm blooded animals (Niebylski et al., 1994; Ponlawat & Harrington, 2005; Savage et al., 1993; Sawabe et al., 2010).

1.2.2 *Aedes sp* Life Cycle

Aedes sp are holometabolous insect which go through a complete metamorphosis process consists of egg, larvae, pupae and adult stages. The entire life cycle of *Aedes sp* can be completed within one and a half to three weeks depending on environmental conditions. Female mosquitoes lay their eggs on the inside of the wet walls in the water container. Larvae hatch after eggs come into contact with water. Larvae live by eating microorganisms and organic materials, then go through four stages of larval development from first instar to fourth instar. Subsequently, larvae transform to pupae and then adult mosquitoes (Christophers, 1960; Harker et al., 2013). The schematic metamorphosis process is illustrated in Figure 1-7.

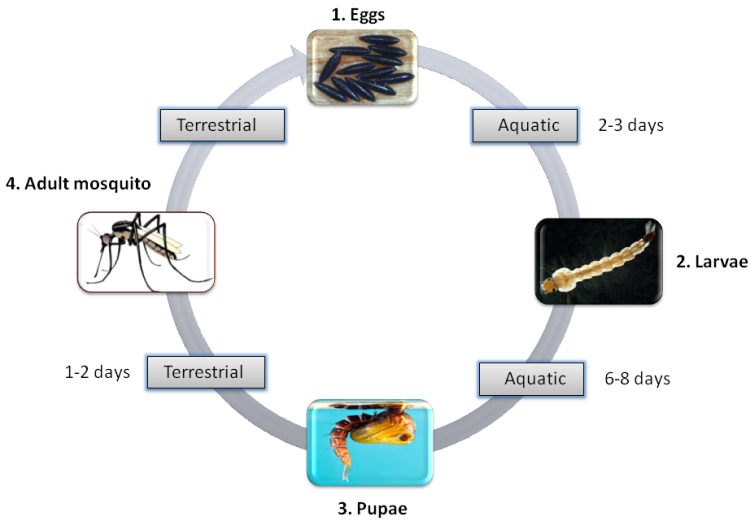


Figure 1-7 The scheme of *Aedes sp* life cycle.
The cycle consist of eggs, larvae, pupae and adult mosquito. *Aedes sp* through complete metamorphosis, has terrestrial and aquatic stages. The process of metamorphosis follows the direction of arrows, from eggs to adult mosquito.

Female mosquitoes lay their eggs (around 50-500) individually on the walls of water containers 2-4 days after a blood meal. The eggs can survive for weeks to one month, and then hatch after being submerged in water (Becker, 2010). Morphologically, the eggs are small, black in colour, in elongated oval shapes, approximately under a milimeter in length (Christophers, 1960). Using Scanning Electron Microscopy (SEM), the surface of *Aedes aegypti* eggs are rough, some smaller tubercles are observed on each side of the ridge (Matsuo, 1972). *Aedes albopictus* eggs are more cold resistant than *Aedes aegypti*, and they can survive in cold temperatures (Waldock et al., 2013).

After completion of embryonic development, the eggs enter a dormant stage and then hatch after being submerged in water. There are two stages involved in hatching of *Aedes aegypti* eggs, which are breakdown of the water-impermiable barrier due to a decreasing oxygen concentration and influx of water to the larvae, then swelling-out of larvae from the shells (Strum, 1963). The entire process of hatching happens in a few minutes (Becker, 2010). Larvae moult four times during development, with the stages called larval instar 1 to larval instar 4, which takes about 5 days to complete. Each moult, is characterised by increase of the head capsule of larvae as the body grows. This process is coordinated by juvenile hormone and the moulting hormone, ecdysone (Becker, 2010).

Pupae of *Aedes aegypti* are shaped like a comma, large but more slender compared to other mosquito species pupae. These pupae are inactive, they do not need to eat to survive, but still need oxygen to breathe. For the purposes of breathing, pupae are located near the surface of the water. The duration of the pupal stage is usually around 2 days, however this can be reduced or extended depending on the water temperature (Christophers, 1960). In the pupal stage, some larval organs are then histolysed and the body of the adult is formed. The fat body of the larvae is also transfered to the adult stage, as a source of vitellogenin. In the resting position, pupae passively float motionless on the surface of the water (Becker, 2010).

The final stage of mosquito metamorphosis is the emergence of the adult. It occurs when gas is forced between the pupal and the pharate adult cuticle. After emergence, adult mosquitoes increase the volume pressure of haemolymph (fluid-analogous to the blood in vertebrates) which enables the legs and wings to stretch. Within a few minutes, adult mosquitoes can fly, however they need 1-1.5 days to adjust their metabolism to adult stage.

Subsequently, the adults are ready to start their life cycle again by mating, feeding and oviposition (Becker, 2010).

1.2.3 DENV Infection

The mosquito becomes infected after blood feeding on a DENV infected human during the period of viremia (Guzman, M. G. et al., 2010). Afterwards, DENV amplification begins in the midgut epithelial cells, then the virus escapes and spreads to secondary tissues such as salivary glands. The time during the process of infection of DENV from the midgut until the virus reaches the salivary glands is called the extrinsic incubation period (EIP) (Hardy et al., 1983). Generally, the duration of EIP in competent *Aedes sp* is around 8-12 days (WHO, 2009). Then, virus in salivary glands can be tranmitted to humans by mosquito blood feeding following saliva injection (Sim et al., 2012). The cycle of DENV infection through mosquito and human hosts is illustrated in Figure 1-8.

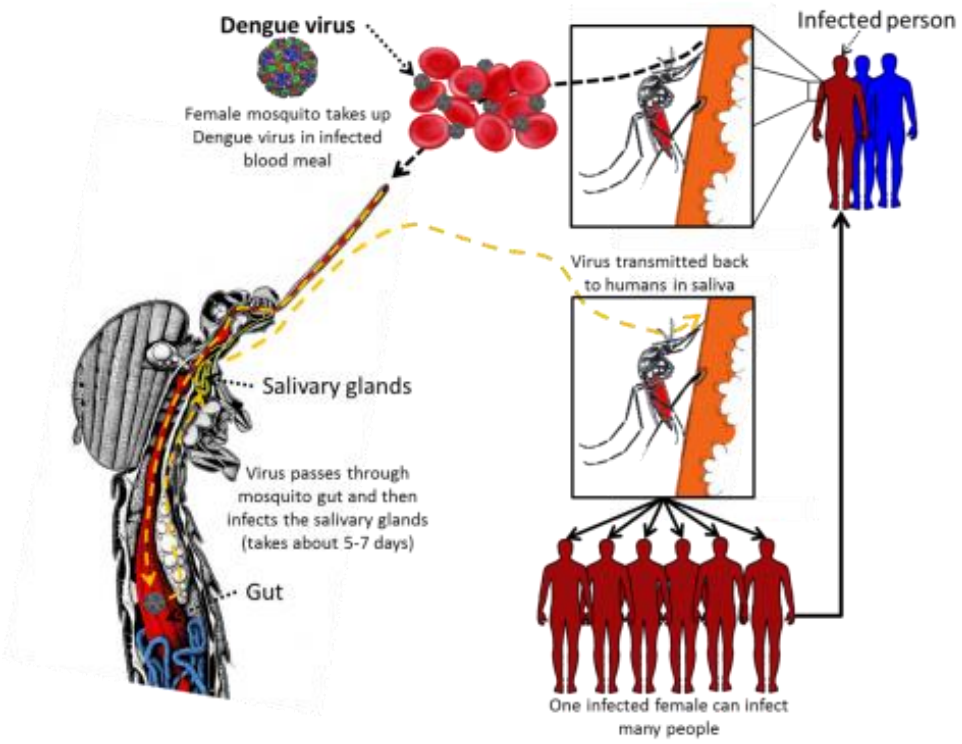


Figure 1-8 The schematic infection cycle of DENV in mosquitoes and humans.
DENV enters the mosquito by blood meal, amplifies in the midgut and then escapes to other tissues and the salivary glands. Then DENV in the mosquito salivary glands is transmitted to humans by mosquito bite and saliva injection. Taken from (http://www.oxitec.com/oxitec-video/introducing-haedes-and-aegypta-all-about-the-aedes-aegypti-mosquito/).

Before establishing a full infection in the mosquito, DENV has to overcome innate immune responses and several tissue barrier in the midgut and salivary glands (Franz et al.,

2015). There are several tissue barriers in mosquitoes which prevent DENV infection and dissemination: the midgut infection barrier (MIB), Midgut Escape Barrier (MEB), Salivary Gland Infection Barrier (SGIB) and Salivary Gland Escape Barrier (SGEB) (Black et al., 2002; Franz et al., 2015). MIB plays a role in the early stages of virus infection (receptor binding, uncoating, transcription or translation, immune responses), while MEB prevents the dissemination of infectious virions to secondary target organs (Black et al., 2002). The schematic diagram of several barriers can be seen in the Figure 1-9.

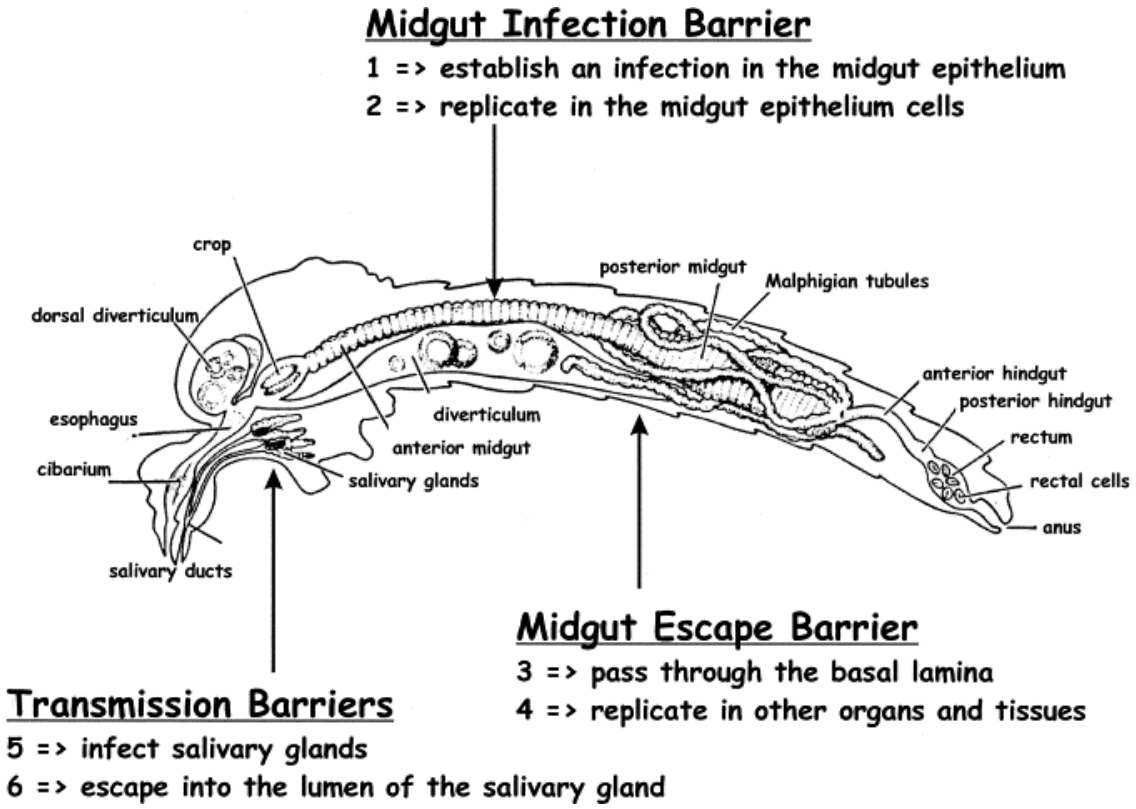


Figure 1-9 Schematic diagram of the several barriers for virus infection and spread in mosquitoes.
The barriers such as MIB, MEB and transmission barier (SGIB and SGEB) prevent virus infection amplification and/or stage in the various stages of virus infection in the mosquito (Black et al., 2002).

All of these barriers can be virus dose-dependent or independent, and this dose can be affected by innate immune response of the mosquito, particularly RNA interference (RNAi) (Khoo et al., 2010). RNAi is presumed to be the most important antiviral innate immune response of the mosquito, in addition to the Toll, IMD (Immune deficiency), JAK/STAT signalling pathways (Ding, 2010; Donald, C. et al., 2012; Siu et al., 2011). The principle mechanims of RNAi is to suppress gene expression by causing the destruction of certain mRNA molecules. RNAi will degrade long double-stranded RNA (dsRNA), which usually associated with virus infection, into smaller siRNAs (small

interfering RNA) or virus-derived small interfering RNAs (viRNAs) if derived from virus by Dicer-2 (Dcr-2) (Ding & Voinnet, 2007; Donald, C. et al., 2012). Subsequently, these small interfering RNAs are transferred to the multiprotein RNA-induced silencing complex (RISC), then degrade mRNA target by the function of the Argonaute protein (Ago-2) and caused the silencing of RNA target or virus (Donald, C. et al., 2012). The schematic diagram of RNAi pathway described in Figure 1-10.

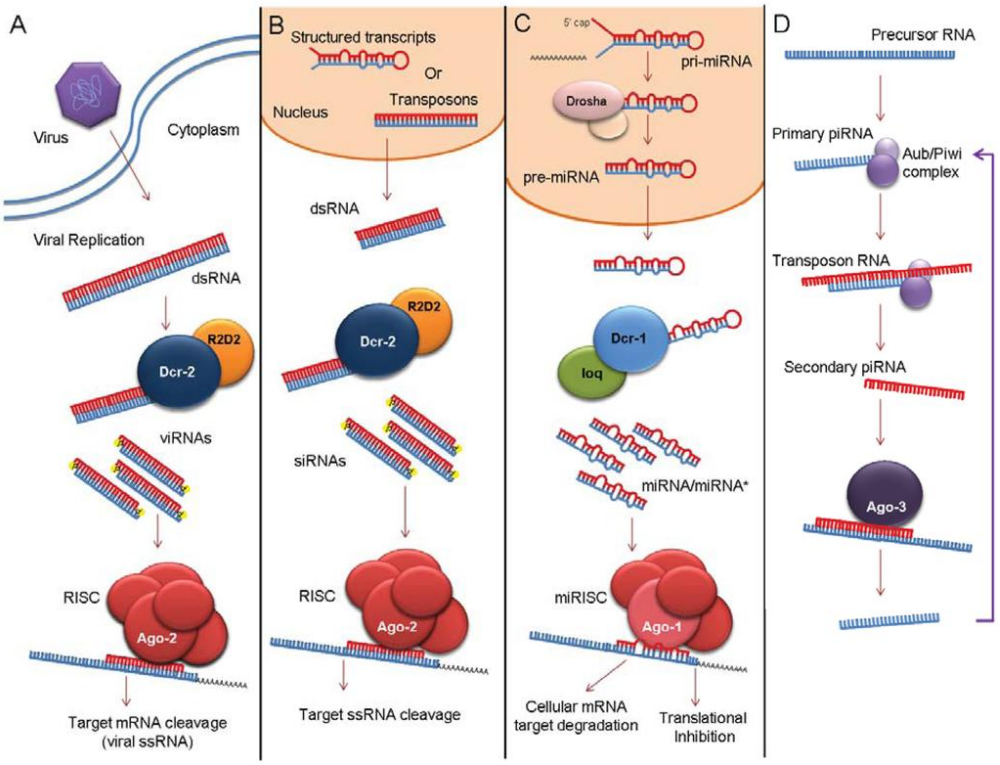


Figure 1-10. Schematic diagram of RNAi.

(A). Exogenous siRNA pathway ; (B) Endogenous siRNA pathway ; (C) microRNA pathway (miRNA); (D) PIWI-interacting RNA (piRNA). dsRNA = double stranded RNA; Dcr = Dicer; RISC= RNA-induced silencing complex; Ago= Argonaut; viRNA= viral specific small interfering RNA; siRNA= small interfering RNA; loq= loquacious; ssRNA =single stranded RNA (Donald et al, 2012).

Sanchez-Vargas et al (2009) reported that a failure in the RNAi system resulted in a significant increase in virus titers in the midgut, accelerating the spread to the salivary glands (Sanchez-Vargas et al., 2009). However, RNAi cannot be inhibited the infection completely once the arbovirus has established their replication in midgut (Adelman et al., 2013). Once DENV is transmitted to the human host by mosquito feeding, the virus then presumably infects immature Langerhands cells (epidermal dendritic cells) (DC) (Wu et al., 2000). It is believed that DC/NK (Natural Killer) cell interactions play the first line innate immune response against DENV (Navarro-Sanchez et al., 2005). Infected dentritic

cells then migrate to local lymph nodes, triggering cellular and humoral immune response by T cells (Guabiraba & Ryffel, 2014; Jessie et al., 2004). The process of how DENV infects the human body is illustrated in Figure 1-11.

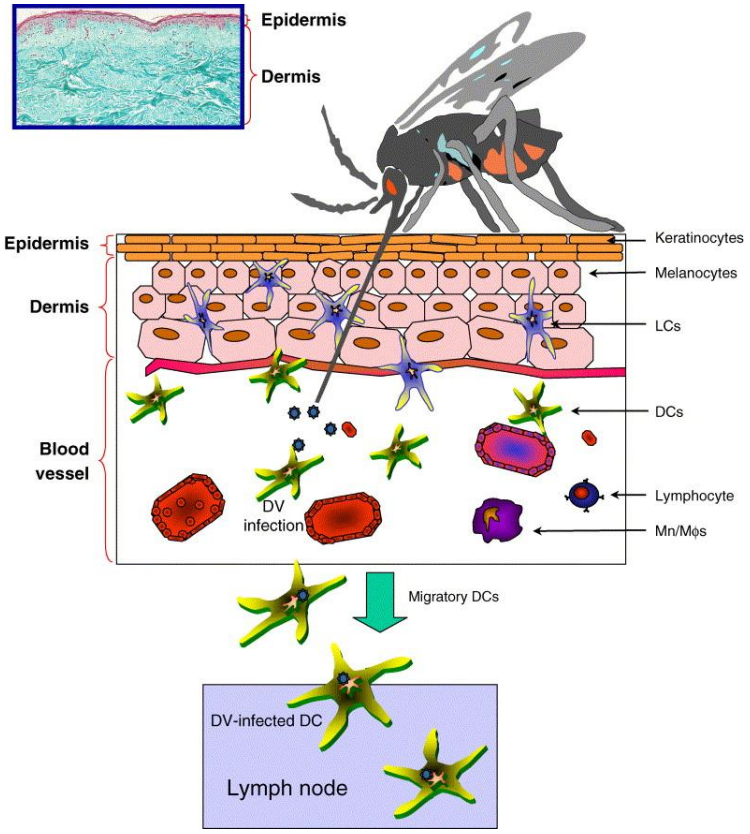


Figure 1-11 The process how DENV enters the human body.
DENV enters the mosquito through blood feeding, the virus then infects dendritic cells (DCs), and then migrates to regional lymph nodes (Navarro-Sanchez et al., 2005).

DENV migration to lymph nodes activates monocytes and macrophages to fight virus infection (Martina et al., 2009). Instead of eliminating the virus, these two white blood cell types can become infected by the virus as DENV evade the immune system by inhibiting type I Interferon signaling (Pagni & Fernandez-Sesma, 2012). Afterwards, infected monocytes and macrophages spread through the lymphatic system, and infect other cells including blood-derived monocytes, myeloid DCs, splenic and liver macrophages (Martina et al., 2009). The spread of virus throughout the body results in viremia, with DENV titres are high in bloodstream (Halstead, S. B., 1988).

1.2.4 DENV cycles and vertical transmission

DENV can also be transmitted from mosquito to their offspring during embryogenesis, a process called vertical transmission (Beaty, 1996; Halstead, 2008a). Humans are a key host for DENV infection, but DENV also can infect sub human primates (Althouse et al., 2014). Species of *Macacus, Pongidae, Certhopicicus, Cercocebus, Papio, Hylobates* and *Pan* are susceptible to DENV infection. Usually the infection in primates is predominantly asymptomatic but the level of viremia is sufficient to infect mosquitoes (Halstead, 2008a).

DENV circulation has two transmission cycles, which are endemic and epidemic cycle and zoonotic/enzootic or sylvatic cycle. Endemic and epidemic cycles is a transmission cycle which involve human hosts and *Aedes aegypti* and *Aedes albopictus* as vectors, while zoonotic and sylvatic cycle involves non-human primates and several other *Aedes sp* as vectors (Wang et al., 2000). The sylvatic cycle (sylvatic means occurring in or affecting wild animals) of DENV was observed in several countries such as West Africa and Malaysia. In West Africa, DENV-2 was identified from *Aedes furcifer, Aedes taylori, Aedes luteocephalus* (Diallo et al., 2003; Franco et al., 2011) while in Malaysia, four serotypes of DENV were detected in *Aedes niveus* mosquitoes and non-human primates (Rudnick et al., 1967). Sylvatic/enzootic cycles could be introduced in epidemic cycles to humans (Gubler, D.J, 2010). Discovery of a new serotype, DENV-5 which was reported in October 2013 could be an introduction of sylvatic cycle to humans, indicating there is a stepping barrier from wild animal (sylvatic) to human host. This new serotype was found in the forest of Sarawak, circulating among non-human primates and then in a 37 year old farmer. It was assumed that DENV-5 was maintained in the forests of South East Asia among non-human primates for centuries before passing the human barrier (Mustafa et al., 2015). The relationship between the sylvatic cycle and epidemic cycle is displayed in Figure 1-12,

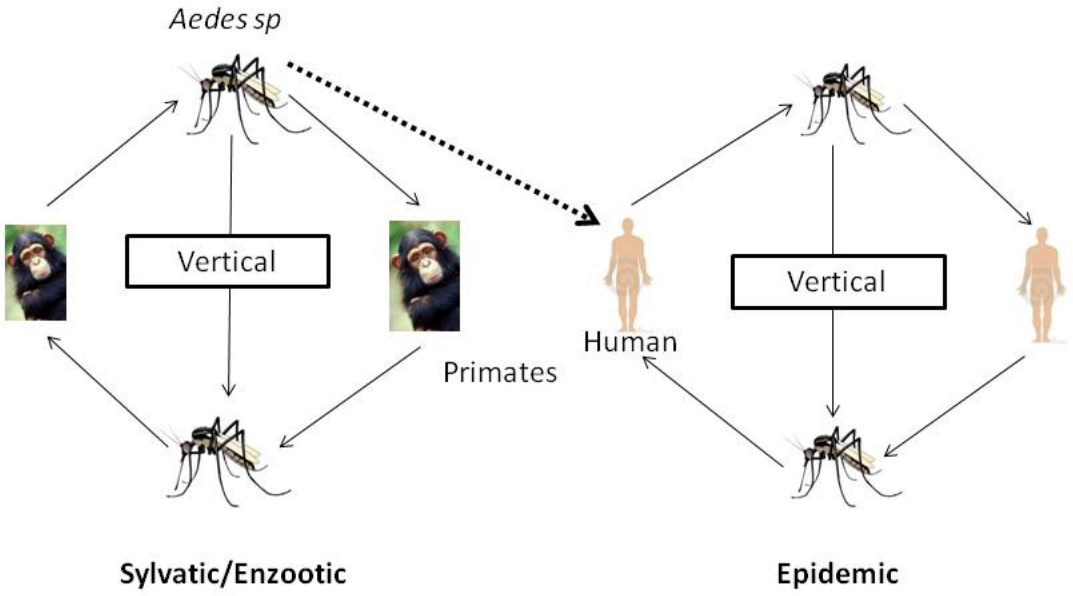


Figure 1-12 Transmission of DENV through slyvatic/enzootic and epidemic cycles. Sylvatic/Enzootic transmission involves primates and mosquito vectors to spread DENV (Left), and epidemic transmission occurs when the dengue virus is spread by *Aedes sp* to humans (Right). Vertical transmission can happened in both cycles, when the virus is transmitted to their offspring. Adapted from previous work (Whitehead et al., 2007).

Vertical transmission can happen both in sylvatic and epidemic cycles, although the importance of vertical tranmission for DENV circulation is still debated. Several studies believe that vertical transmission in *Aedes sp* is important for DENV persistence during unfavourable conditions for virus tranmission (Angel & Joshi, 2008; Guo et al., 2007; Joshi et al., 2002; Martins et al., 2012). However, Adam and Boots (2010) – by using matematical modelling- argue that vertical tranmission at low levels (1-4% infection efficiences) is not important for DENV persistence. Only when infection rates are above 20-30 %, does vertical transmission have a significant role on virus persistence (Adam B & Boots, 2010).

1.3 Dengue in Indonesia

1.3.1 Location, population and geography

Indonesia, the fourth largest country in the world, is located in South East Asia, lying between two continents, Asia and Australia / Oceania at 95° to 141° east longitude and 6° north to 11° south latitude. This vast archipelago country has a total land area of 1,919,440 square kilometres (735,355 square miles), and encompasses an estimated 17,508 islands, only 6,000 of which are inhabited. Being the fourth most populous country in the world, the total population in Indonesia is based on the official national census data, the Central

Bureau of Statistics in Indonesia, in 2010 was 237.641.326. The population density in 2010 was 123.76 people per square kilometer (Indonesia, C. B. o. S., 2015). This means that Indonesia is the 4th largest country and with a high population density too. The rate of population growth during 2000-2010 was 1.49 % and the World Population Prospect predicted that by 2050, the population in Indonesia could reach 366 million (Nations, 2015). The map of Indonesia can be seen in Figure 1-13.

Figure 1-13 The map of Indonesia.
Indonesia is located in two continents, Asia and Australia, and consists of five major islands. Taken from http://www.worldmapof3.xyz/map-of-indonesia-3/

There are five major islands in Indonesia which are : Sumatra with an area of 473.606 square km, Java with an area of 132.107 square km, Borneo/Kalimantan (the third largest island in the world) with an area of 539.460 square km, Sulawesi with an area of 189 216 square km, and Papua with an area of 421.981 square km. Around 58% of Indonesia's population live on the island of Java, making this island the most populous island in the world. Indonesia consists of 34 provinces with 413 regencies, and comprises many ethnic groups. The top three ethnic groups in Indonesia are Javanese (41.71%), Sundanese (15.41%) and Malay (3.45%). The country is also very diverse in culture and has over 300 local languanges, with the national language being Indonesian (Kuipers, 2011).

1.3.2 Environment and demography

Located on the equator, Indonesia has a tropical climate with high humidity and annual wet and dry seasons. Because of its location, Indonesia is affected by monsoons, resulting in two seasons: the dry and rainy seasons. Dry season usually occurs in May-October and rainy season in November-April. The average daily temperature in Indonesia falls within the range of 23 °C and 28 °C, with a variation from minimun 19 °C and maximum 35°C depending on altitude and geographic location (Kuipers, 2011). Generally, relative humidity is between 70-80 % (Copsey, 2012). The average rainfall in Indonesia varies throughout the year, with an average of 2000 - 3000 mm/year (Centre of meteorological, 2015). In the dry season, temperatures vary between 28°-34°C during the day, and between 21°-25°C during the night. While in rainy season the temperature is relatively cooler, at 24 ° -28 ° C during the day, and warmer than the dry season at night, 23 °-26 ° C. These two seasons occur in most parts of Indonesia. However, for some areas in the east and around the Eastern-Sea board, there is a transitional period *i.e.* the transition season. This transition season lasts approximately 1 month in between the two seasons, which is about a month from April to May and in October-November. In this period, rainfall, temperature, windspeed and airflow become erratic, as well as the movement of clouds. Drastic temperature differences are also common in this season (Kuipers, 2011). Indonesia also experinces extreme weather phenomena such as El Nino (associated with drought) and La Nina (associated with heavy rainfall and flooding) (Copsey, 2012).

Indonesia is undergoing four major transformations, which are decentralisation, rapid urbanisation, drastic progression toward democratisation of the political and governance systems and the privatization of various socioeconomic activities (Sarosa, 2006). In the 1970s, mobility, education and urbanization in Indonesia have increased siginificantly. Subsequently, Indonesia has experienced a high urbanisation rate from rural to urban areas. In 1950, only 15% of the Indonesian population lived in urban areas; in the subsequent 40 years, it has doubled reaching 30% in 1990. In 2010, it is estimated that 54% (more than 240 million) of people will be living in urban areas. The main factor that triggered increased urbanisation is the concentration of economic growth and industrialization mostly in major urban areas, while employment in rural areas, focusing on agriculture, has become less attractive (Sarosa, 2006). Rapid urbanisation is often accompanied by several problems such as shortages of productive employment opportunities, urban housing, public services; emergence of squatter settlements, environmental pollution, and socio-

psychological stress (Alatas, 1988). Another concern is the lack of balance development in infrastructure and health service between rural and urban areas (Sarosa, 2006).

1.3.3 Health System in Indonesia at a glance

The organisation of the health sytem in Indonesia follows the governance levels i.e. from Ministry of Health (MOH) central level, to province level and from there to district level. The distribution of health facilities in Indonesia is generally the same among regencies. Goverment hospitals are located in the capital of regency, limiting access to people in rural areas. There are community health centres in every sub district, and posyandus (health centres for children and mothers) in every village but the facilities are limited (Brotowasisto et al., 1988). The organisation of the health system in Indonesia can be seen in Figure 1-14.

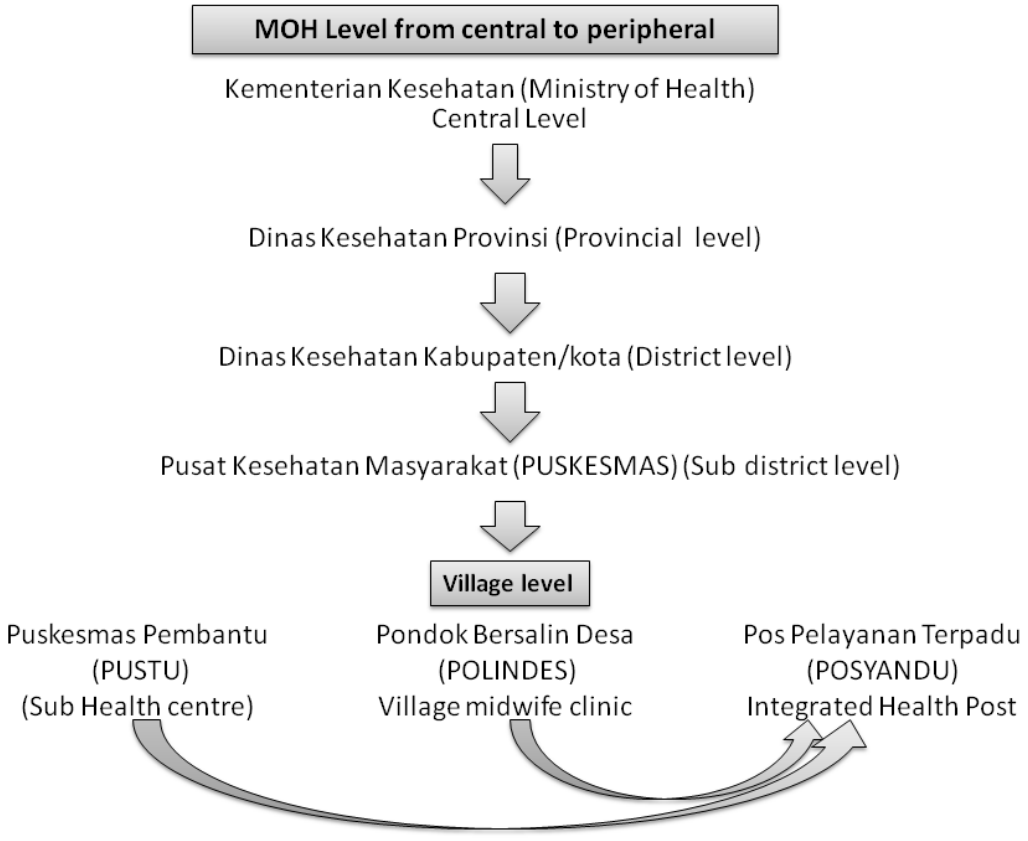


Figure 1-14 The organization structure of health system in Indonesia.
The Health system in Indonesia is under responsibility of the Ministry of Health as a central level, then followed by centres in each province, district, sub district and village. Adapted from (SEARO, 2012).

Since 2001, decentralisation was implemented in Indonesia, causing significant impacts on the national health system. Decentralisation of the system gives responsibility and health care provision to the district/regency. There are differences in each regency's capabilities to serve health facilities and this creates a gap in health services among many areas in Indonesia. Resources for health and service provision, health financing, and health information have been affected by the decentralisation system. In human resources, Indonesia is facing major deficiencies in number and quality of health workers, which affects the health service in this country. In 2006, the ratio of general practitioners and midwives was 19.9 and 35.4 per 100,000 respectively. The distribution of general practitioners and midwives is higher in urban areas than in remote or and rural areas. In terms of health financing, Indonesia spends relatively little on the health service. In 2003, total expenditure in health per capita was $33, of which around 34% was undertaken by public sector and 66% by the private sector. In health information systems, decentralisation resulted in the partial breakdown of this system leading to an unclear division of reporting responsibilities. Consequently, there is no comprehensive database for health issues and disease, resulting in problems developing strategies and monitoring health programmes (WHO, 2010b). However, decentralisation system is believed to be a better system to provide a good health care accesibility, although there are many obstacles due to the gap of resources in each regency and some limitations mentioned above. Decentralization system ideally would help the health function in local goverment to be more simple to administer and manageable since the responsibility of health service is more clear in certain health service coverage. This system should induced local initiative in planning and delivering health services. Indonesia still continue in effort to improve the application of decentralization in the health system, leading to more appropriate and better-targeted health services (Lieberman, 2005).

In 2009, the Indonesian National Health System (NHS) was based on basic principles such as supporting human rights, synergism and dynamic partnership among stakeholders, commitment and good governance, regulation and law enforcement, anticipative and pro-active strategic environmental changes, gender responsive and local wisdom (SEARO, 2012). In recent times many improvements have been made in the health sector in Indonesia (Bank, 2014). Generally, Indonesia has made good progress in health outcomes over the last decade. Infant mortality decreased from 220 per 1,000 births in 1960 to 45 in 2007 and life expectancy increased from 48 years to 66 years. However, geographic inequalities between regions in Indonesia remain large (Bank, 2014). The country havea number of health challenges such as communicable diseases remain a large burden to the

health system, coupled with an increase of non-communicable diseases including cardiovascular diseases, metabolic disease, and cancers. The burden of communicable diseases is one of major concern for the health system in Indonesia. In 2006, around 250 people died from Tuberculosis (TB) every day and over half a million new cases occured every year. Vector borne disease such as Malaria and DHF are reported every year and many Indonesian regions are endemic for Malaria and DHF. HIV infection is also epidemic in Indonesia, where approximately 100,000 to 290,000 Indonesians were living with HIV-AIDS in 2005 (WHO, 2010a). The health problem in Indonesia is also because of the high cases of neglected tropical diseases (NTDs) such as soil-transmitted helminth (STH) infections and lymphatic filariasis (LF), schistosomiasis and neglected bacterial infections, such as yaws and leptospirosis. Viral infection such as Chikungunya and Japanese encephalitis also spread in several areas in Indonesia (Tan et al., 2014).

1.3.4 Burden of dengue disease

Indonesia, the largest country in Southeast Asia by population and area, has been reporting dengue disease since 1968 when DENV infections were detected in the cities of Jakarta (capital city of Indonesia) and Surabaya (East Java) (Nathin et al., 1988; Sumarmo, 1987; Suroso, 1996). Since then, dengue disease has become an important public health problem in Indonesia, characterized by a large infected area and periodic outbreaks with increasing numbers of infections and severity (Setiati et al., 2006). In 1973, the first large outbreak of dengue infection occurred, where 6225 cases were registered in Semarang, the capital of Central Java, from total 10189 cases reported in Indonesia (Sumarmo, 1987). Major dengue epidemics occurred in Indonesia in 1998, during which 72,133 cases and 1414 deaths were reported, and again in 2004 with more than 58,301 cases and 658 deaths in the first 4 months of the year (WHO, 2004). In a 2004 outbreak, 30 of 32 provinces in Indonesia were affected by DENV infection, and the capital city Jakarta was the most affected area. Further to this on 16 February 2004, the Indonesian Ministry of Health declared a national DF/DHF epidemic (Ahmad, 2004; Setiati et al., 2006).

Based on a 45-year registry-based analysis since 1968-2013, the annual DHF incidence increased from 0.05/100,000 in 1968 to ~ 35-40/100,000 in 2013 with the largest epidemic occurring in 2010. In spite of the increase of cases and severity, the case fatality rate (CFR) of dengue disease actually decreased from 41% in 1968 to 0.73% in 2013 (Karyanti et al., 2014). This decline of dengue CFR is a consequence of better clinical management and availability of health care facilities (Suwandono et al., 2006). From 1999 onwards, a shift

of dengue incidence from young children to older age groups was observed (Karyanti et al., 2014; Setiati et al., 2006). The trend of DHF incidence from 1968 to 2013 is shown in Figure 1-15.

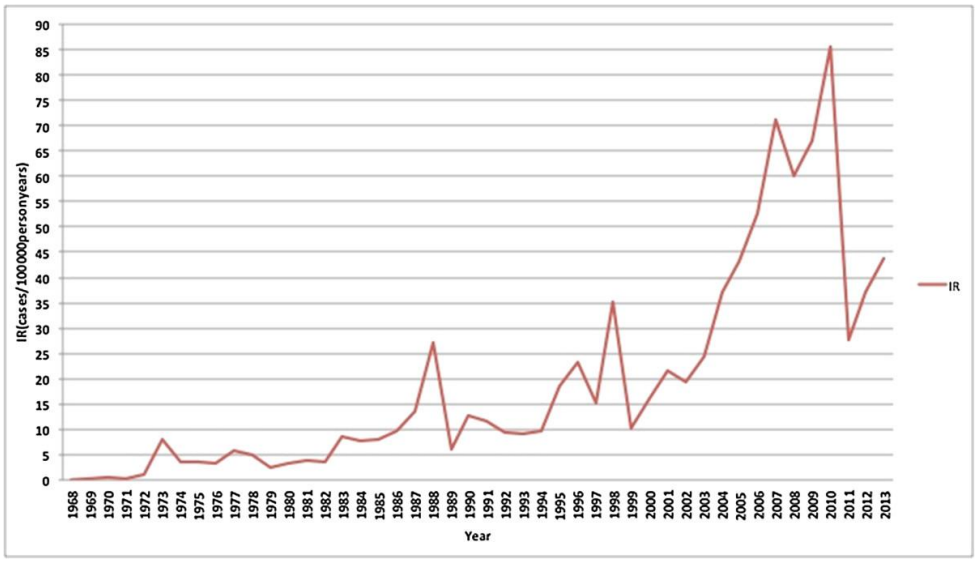


Figure 1-15 Trends in incidence rate of DHF cases in Indonesia from 1968 to 2013.
The incidence rate is measured in number of cases per 100,000 people each year. (Karyanti et al., 2014).

From 2010-2013, Bali and Jakarta were the two provinces with the highest incidence of DENV infection (Karyanti et al., 2014). The five highest incidences in 2013 were Bali (168.5/100,000), DKI Jakarta (104.0/100,000), DI Yogyakarta (96.0/100,000), East Kalimantan (92.7/100,000) and Sulawesi Tenggara (66.8/100,000). The distribution of DENV infection in every province in Indonesia between 2010-2013 is shown in Figure 1-16.

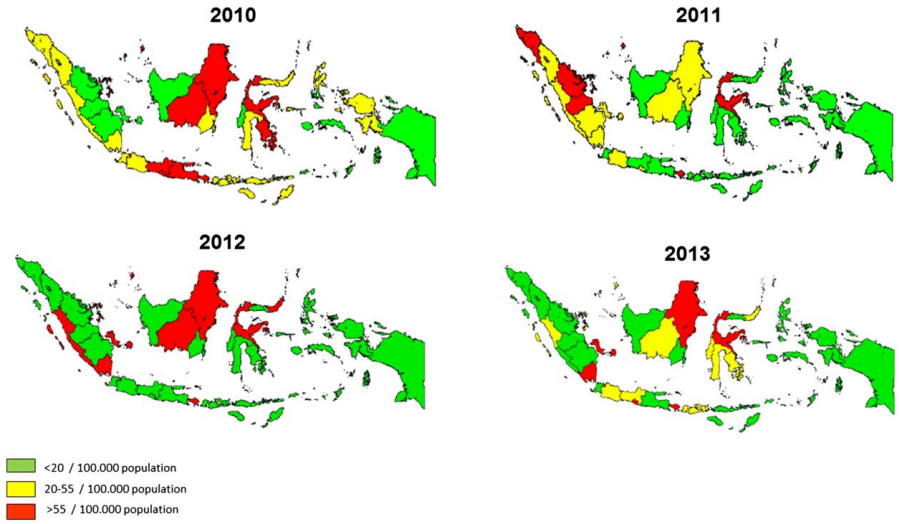


Figure 1-16 Geographical mapping of Indonesian provincial incidence rates of DHF in 2010-2013.
The colours in the map represent dengue cases per 100,000 population. The green colour background represents areas which have <20 dengue cases/100,000; yellow background shows areas with dengue cases of around 20-55 cases/100,000 population and the red background represents the areas with dengue cases over 55 /100,000 population (Karyanti et al., 2014)..

Dengue disease is reported all the year round, with an increase in incidence in the rainy season (Kusriastuti & Sutomo, 2005). The proportion between male and females infected with DENV is almost the same, 53.2% male and 46.8% female in 2012 (Muhadir, 2013). Annually, the cost for dengue exceed $300 million for treatment, prevention and control, indicating it spends large amount of healthcare budgets in Indonesia (Shepard et al., 2013). In Indonesia, dengue disease is primarily endemic in urban areas, as a result of urbanisation, rapid industrial and economic development and human mobility. Tropical climates with high rainfall and favourable temperature and humidity provide a conducive environment for the life cycle of *Aedes sp* (Kusriastuti & Sutomo, 2005). Uncontrolled urbanisation created slum settlements with poor water and sanitation facilities, which that condition will provide breeding sites for *Aedes aegypti*. In addition, urbanisation drives DENV transmission because DENV have adapted to human-mosquito vector-human transmission cycle, which coupled with crowded population and anthropophilic (close/attracted to human) behaviour of *Aedes sp* (Gubler, D., 2011)

1.3.5 Dengue Prevention and Control

The effort to prevent and control dengue disease is carried out globally. The World Health Organization has a goal to reduce the burden of dengue by utilising five technical elements, which are diagnosis and case management, integrated surveillance and outbreak preparedness, sustainable vector control, future vaccine implementation and basic operational and implementation research (WHO, 2012). However, until now the major preventive strategy for DENV transmission is the control of mosquito vectors, since there is no vaccine nor specific drugs for treatment. Initially, control of mosquito vectors focused on space spraying of insecticides for adult mosquito control. Furthermore, it was attempted to also reduce mosquito larvae by larvacides (WHO, 2011). These attempts showed success in certain areas such as in the America from 1946 to 1970, directed by the Pan American Sanitary Board (Brathwaite Dick et al., 2012), and in Cuba in 1981 which applied intensive insecticidal treatment and reduction of larval habitats (Kouri et al., 1989), and in Singapore (Ooi, E.-E. et al., 2006).

There are four classes of insecticide applied for vector control, which are carbamates, organochlorines, organophosphates and pyrethroids (WHO, 2006). Types of insecticides applied for vector control over previous decades include temephos, pyrethroids (deltamethrin and permethrin) and organophosphates (malathion, fenitrothion and pyrimiphos methyl). Organochlorine dichlorodiphenyltrichloroethane (DDT) has also been widely used for several decades. The application of insecticides can be applied as Insecticide Residual Spraying (IRS), space spraying, fogging and treated/impregnated material (Vontas et al., 2012). However, several problems can occur by application of insecticides, such as insecticide resistance, cost and bad odours (WHO, 2011; Yadav et al., 2015). Insecticide resistance against four classes of insecticide has been detected in *Aedes aegypti* (Ranson et al., 2010).

Currently, attempts to prevent and control mosquito vectors include integrated approaches, which combine environmental, biological and chemical factors. The main prerequisite of these methods are to be safe, cost-effective and to have no negative effects on the environment. The purpose of environmental management are to minimize breeding sites for mosquito vectors and to limit contact between DENV, mosquito vectors and human hosts. Environmental management consists of environmental modification (improved water supply, mosquito-proofing of overhead tanks/cisterns or underground reservoirs); environmental manipulation (draining water supply installations, covering domestic water-storage containers, cleaning flowerpots/vases and ant-traps, cleaning incidental water collections) and changes to human behaviour. Behaviour changes attempt to reduce contact between human, vector and virus using protective clothes, repellent, mats, coils, aerosols and insecticide-treated materials such as mosquito nets and curtains (WHO, 2011).

Besides environmental management, biological control is also being considered as an alternative to vector control. Several fish species such as *Lepisosteus tropicus* (Gill), *Astyanax fasciatus* (Cuvier), *Brycon guatemalensis* (Regan), *Ictalurus meridionalis*(Günther) and *Poecilia sphenops (*Valenciennes) are considered as good biological control measures for *Aedes aegypti* by eating the larvae (MartíNez-Ibarra et al., 2002). *Mesocyclops* (*Copepoda*) was also proposed to have capability to eliminate *Aedes aegypti* (Sinh Nam et al., 2012; Vu et al., 2005). Biological manipulation using *Wolbachia pipientis*, an intracellular insect bacterium that has been artificially introduced into *Aedes aegypti*, has also been shown to suppress populations of this mosquito and prevent DENV transmission (Bull & Turelli, 2013; Hoffmann et al., 2011). One of the most prominent capability of Wolbachia manipulate host reproduction is cytoplasmic incompatibility. It is

happened when wolbachia-infected male insects mate with wolbachia-free female insects and resulted non-viable offspring (embryonic mortality) (Lambrechts et al, 2015; Zabalou et al, 2004).

Dengue prevention and control in Indonesia generally applies the strategy proposed by WHO. This programme was led by the Ministry of Health and the Directorate General for Communicable Diseases Control and Environmental Health. The strategies to prevent and control DENV transmission consist of disease management, changing behaviour of communities and establish partnerships in order to reduce mosquito populations (Kusriastuti & Sutomo, 2005). The Indonesian target for DENV control is to reduce morbidity by at least 25% by 2020, with 2010 used as baseline (Muhadir, 2013). Prevention and control of dengue in Indonesia has undergone a long journey. In the 1970s, the "fire-fighting" tactics were applied for vector control. This strategy consisted of peri-focal space spraying of houses within 100 metres of reported dengue cases, health education in a limited area and case management. In 1980, in addition to the "fire-fighting" strategy, mass larvaciding with temephos 1% sand granules at a dosage 1 ppm was applied once a year before the beginning of the transmission season. During 1990 and 1991, mass larvaciding was replaced with selective larvaciding, focusing only on dengue endemic areas. Since 1992, the strategy of vector control focused on community participation to reduce mosquito breeding sites (Suroso, 1996).

Community participation focuses on the village and household levels. The efforts included health education to increase people's knowledge, awareness and practices about dengue diseases and prevention using mass media, door to door visit or visits to women's groups. The 3**M** programme was also introduced to reduce mosquito breeding places at household level. This programme consists of covering water containers (**M**enutup); cleaning water containers (**M**enguras) and burying discarded containers (**M**engubur). Subsequently, the 3M programme was modified with 3M Plus, with the addition of minimizing mosquito bites through behaviour such as wearing long clothes and using repellent (mosquito spray) (Kusriastuti & Sutomo, 2005). Behaviours/practices relating to dengue disease prevention consists of preventing production of adult dengue vectors, preventing exposure to bites of mosquitoes and seeking treatment and patient care (WHO, 2000).

Dengue prevention and control still faces numerous challenges. Horstick et al (2010) in their systematic review identified several limitations of dengue prevention and control such as a shortage of personnel (entomologists, social scientists, operational vector-control

staff), lack of technical expertise at decentralised levels of service, insufficient budgets, inadequate geographical coverage, interventions relying mostly on insecticides, difficulties in engaging communities, little capacity building and almost no monitoring and evaluation (Horstick et al., 2010). Spiegel et al (2005) also mentioned the challenges of vector control in Indonesia including the complexities of ecological and social systems. The key to effective dengue prevention and control is adaptability and sustainability of dengue control programs in public, regional and local areas (Spiegel, 2005). Comprehensive approaches are recommended for prevention and control, involving mosquito control, environmental managament, efficient data collection and development of an early detection system (Beatty et al., 2010)

1.3.6 Limitations of surveillance and molecular research

Dengue surveillance and monitoring is important for identification of dengue outbreaks and monitoring disease trends (Runge-Ranzinger et al., 2014). Dengue surveillance in Indonesia still requires improvement since surveillance systems in Indonesia and in other countries in Southeast Asia, are still based on passive surveillance (Institute, 2009; Ooi, E. E. & Gubler, 2009). Passive surveillance is a type of surveillance which involves no active search for cases, and is solely based on regular reporting of disease data by all institutions that see the patients or test specimens (WHO, 2015b). Passive surveillance has two major limitations, which are inconsistency of standard reporting and reporting without following the WHO case definitions. These limitations lead to underreporting and also overreporting on surveillance systems (WHO, 2011). The greatest weakness in surveillance of dengue cases in Indonesia as mentioned in Dengue Surveillance in the Asia-Pacific Region (2007) was variations in definitions, i.e. lack of virological surveillance, and surveillance not being systematic. These limitations lead to several consequences such as reporting delays and under-reporting of cases (Institute, 2009). Dengue reporting in Indonesia is under the rule of the Epidemic Act (UU wabah No. 4/1984) and the Ministry of Health (regulation no. 560/1989). It was stated that ‘every case of an infectious disease which has the potential to cause an outbreak, should be reported to the district health authority within 24 hour, while awaiting serological confirmation’. However, in reality it is far from well implemented since most areas in Indonesia still do not have good surveillance. The difficulty of rapid diagnosis, low public awareness, a shortage of professionals and laboratory facilities are several obstacles that must be faced (Chairulfatah et al., 2001). Most of the Indonesian population will go to the hospital only when the pain is severe, and

this causes the majority of cases reported are DHF, thus causing underreporting of dengue cases that have not resulted in DHF (Suroso, 1996).

Besides limitations in the surveillance system, Indonesia also lacks molecular research due to limited laboratory facilities, funding and trained staff. Serotyping of DENV in Indonesia has mainly been done for research purposes instead of being used routine procedures in hospitals. Therefore serotyping and genetic information of circulating DENV in Indonesia is inadequate. Most research of molecular epidemiology of dengue virus has been carried out in major Indonesian cities such as Jakarta, Surabaya and Yogyakarta (Graham et al., 1999; Suwandono et al., 2006; Yamanaka et al., 2011). Four dengue serotypes (DENV-1,2,3 and 4) circulate in Indonesia, with DENV-3 and DENV-2 being the dominant serotypes (Sumarmo, 1993). It has also been shown in some studies in Indonesia that there is an important association between morbidity and secondary infection (Graham et al., 1999; Suwandono et al., 2006). Primary and secondary infection were identified based on haemagglutination inhibition (HI) assays (Suwandono et al., 2006). Patients suffering from DENV-1 secondary infection experienced more severe dengue disease than patients with DENV-1 or DENV-2 primary infections (Yamanaka et al., 2011). Several studies also stated that certain serotypes result in more severe clinical manifestations, such as DENV-3 which is most frequently related to severe cases (Corwin et al., 2001; Suwandono et al., 2006). The distribution of DENV serotypes in Indonesia is summarised in Figure 1-17.

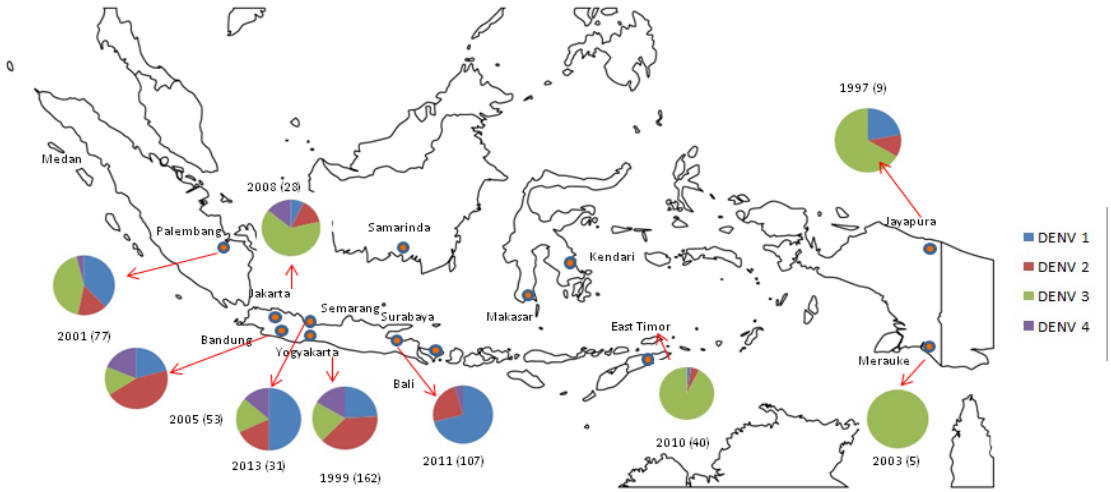


Figure 1-17 The map illustrates the serotype circulating in several areas in Indonesia. The pie chart shows the proportion of DENV serotypes identified in each area, together with information of year of study and the number of dengue cases observed. The map is based on these studies : (Corwin et al., 2001; Fahri et al., 2013; Graham et al., 1999; Ito et al., 2010; Porter

et al., 2005; Richards et al., 1997; Sukri et al., 2003; Suwandono et al., 2006; Yamanaka et al., 2011).

It is clear therefore, that Indonesia requires a lot of improvement in surveillance, research, molecular epidemiology and prevention of DENV transmission. There is an urgent need for research in an integrated context to provide better information of dengue epidemiology in Indonesia. Indeed, it will require participation of many parties, researchers, funders and the government as policy makers.

1.4 Integrated Epidemiology Study

There are several definitions of epidemiology, but the following definition by Last in 1995 as mentioned in Hajat (2011) captures the principles of epidemiology and the spirit of public health:

> "Epidemiology is the study of the distribution and determinants of health related states or events in specified populations , and the application of this study to the control of health problems." (Hajat, 2011)

The epidemiology triangle consists of the agent, a susceptible host and an environment, which interplay between the agent, the susceptible host and a conducive environment which supports the transmission from agent to host (CDC, 2012). In the context of dengue, the agent is the DENV; the host is principally humans, although some primates could be infected by DENV (Kyle & Harris, 2008). The environmental variables involve temperature, rainfall, humidity, socioeconomy and demography etc. The scheme of the epidemiology triangle model is illustrated in Figure 1-18.

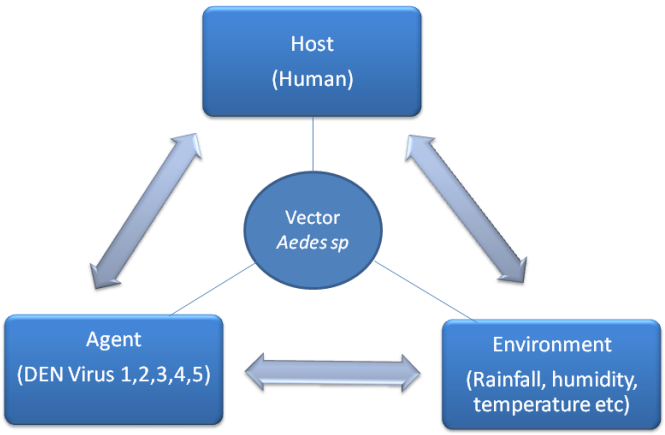


Figure 1-18 Schematic diagram of the epidemiology triangle of DENV.
The epidemiology triangle of dengue disease consists of the infectious agent, the host and environment and also *Aedes sp* as the vector.

In order to study the transmission of DENV, studies must include these three variables. As for all arboviruses, the vector mosquito (*Aedes sp*) also plays an important role in virus (here DENV) transmission. From the diagram in Figure 1-17, it is clear that dengue occurence is caused by multifactorial variables. These include climate change, virus evolution, socioeconomic factors, human travel and uncontrolled urbanization (Murray et al., 2013). For instance, certain genotypes showed higher virulence and caused outbreaks characterized by more severe clinical manifestation (Rico-Hesse, 2003). With regards to environmental factors, temperature is believed to play an important role in adult vector survival, viral replication, and infective periods (Barbazan et al., 2010; Reiter, 2001; Thai, K. T. & Anders, 2011). In addition, the contribution of travel, trade and human mobility may explain the increase of DENV transmission (Gubler, D., 2011; Wilder-Smith & Gubler, 2008). Societal changes such as exploding human populations, unplanned settlement facilititates, conducive breeding sites for dengue vectors and increased human/mosquito contact are also important (Padmanabha et al., 2012). Several studies have also highlighted the importance of socioeconomic factors. A community with a higher income could provide good water and waste infrastructure and use of air conditioning which can reduce the mosquito breeding site and DENV transmission (Astrom et al., 2012; Kikuti et al., 2015; Mulligan et al., 2015). Indeed, DENV transmission is influenced by multifactorial factors which need a comprehensive study in order to have a better understanding of DENV transmission.

1.5 Dengue Spatial Analysis

Application of spatial analysis in infectious diseases has been widely used to predict, prevent and control disease transmission. It is mainly because infectious diseases are caused by multifactorial variables which are interconnected, such as urbanisation, climate, environmental changes, agricultural intensification, and human population changes (Robertson & Nelson, 2014). Among many definitions of spatial analysis, Earth Systems Research Institute, 1996 as mentioned in (Albert & Levergood, 2005) defines that spatial analysis is “the study of location and shapes of geographic features and the relationship between them”. The first recorded use of spatial analysis is the work of John Snow, by creating a map of a Cholera outbreak in Soho, London in 1854 (Koch, 2004). Currently, the application of spatial analysis can be applied in many fields such as geography, politics, social sciences and also health sciences including epidemiology. Spatial analysis

in the context of epidemiology is applied to the analyse of health data with regards to demographic, environmental, behavioural, socioeconomic, genetic and infectious disease risk factors (Elliott & Wartenberg, 2004).

In the late 1950s and early 1960s, development of geographic information systems (GIS) enriched the application of spatial analysis (Goodchild & Haining, 2015). GIS is a system intended to capture data input such as maps, aerial photos, satellites, surveys; to store data and to analyse and present the data as maps, reports, and plans (Foote & Lynch, 2015). Mathematics and statistics also contribute an essential role on the development of spatial analysis particularly on modelling analysis. The first work on mathematical modelling published in 1766, when Daniel Bernoulli developed a mathematical model on the mortality of smallpox in England, which showed that inoculation against the virus will increase the life expectancy at birth by around three years (Blower & Bernoulli, 2004). Currently, many approaches on modelling are proposed to solve the problem from different perspectives. Siettos and Russo (2013) in their review mentioned that there are three general categories of modelling regarding infectious disease dynamics, the first is statistical methods to identify spatial patterns in epidemics and surveillance of outbreaks, the second is the state-space model, a model which is used to forecast the spread of an epidemic and the last is machine learning/expert methods, which are used to forecast the evolution of an ongoing epidemic (Siettos & Russo, 2013). These three categories also consist of different approaches of analysis, which are summarised in the diagram below,

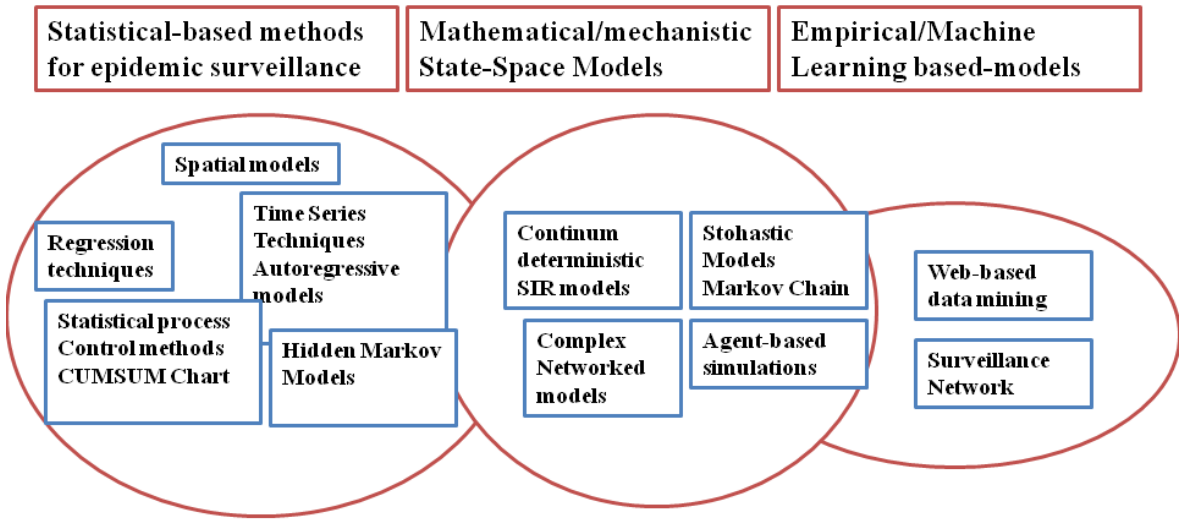


Figure 1-19 Summary of three categories of matemathical modelling
Mathematical modelling consists of three categories, and boxes in each circle represent the type of approaches in each category. Adapted from (Siettos & Russo, 2013).

The application of spatial analysis in DENV transmission provides many benefits such as identifying patterns of spatial and space-time vector and dengue cases, expanding our understanding about how environmental factors influence vector and DENV transmission, and also predicts the changes in spatial risk because of modification of land use or climatic factors. The aims of the application of spatial analysis in infectious disease research is to minimize the burden of disease by providing information to assist public health officers to take preventive action, surveillance and control (Eisen & Eisen, 2011). Many approaches on modelling methods were applied to analyse several variables relating to DENV transmission. Bayesian analysis is one method which is widely used in dengue modelling. Feldstein et al (2015) used a Bayesian approach to assess factors related to the probability of a dengue outbreak (Feldstein et al., 2015). Bayesian model also used to analyse the evolutionary epidemiology of DENV serotypes (Costa, R. et al., 2012) and spatial distribution of dengue incidence and socio-environmental conditions in Brazil (Costa, J. V. et al., 2013). There are many other studies which applied Bayesian analysis on their methods on dengue modelling (Bhattacharjee & Bhattacharjee, 2011; Lowe, Rachel et al., 2011; Malhao et al., 2013; Pan-ngum et al., 2013). Among many approaches in Bayesian analysis, Integrated Nested Laplace Approximation (INLA) is one approach on spatial and spatio-temporal data analysis (Blangiardo M. et al., 2013; Rue H. et al., 2009). Several advantages of INLA methods are providing reliable estimation in a relatively short time, and also its flexibility to conduct Bayesian analysis in an automatic and efficient manner (Blangiardo M. et al., 2013; Rue H. et al., 2009).

1.6 Aims

The main idea of my study is an attempt to combine several important variables such as DENV, mosquito vectors, and environmental factors such as temperature, socioeconomic and demography which are proposed to be crucial role in DENV transmission. The lack of any integrated studies which combine all these variables in a local area, re-emphasize the importance of this study. Indonesia, an endemic DENV country with a long history of DENV infection which is spread in almost all the provinces still has limited information about the transmission of DENV, particulary in local areas. Conducting an integrated study in a local area has become significant due to the difference and uniqueness of each area/region. In this study, I chose Banyumas regency in Central Java as a model area to obtain an in-depth perspective and grasp a more authentic understanding of DENV transmission. Information from this study is expected to improve DENV prevention and control strategies. In more detail, my aims of this study were:

1. To determine the mosquito population in three areas, a DENV endemic, a DENV sporadic and a DENV free area, and to confirm a more reliable measurement technique for an entomology survey to determine the mosquito population and DENV transmission risk level. In addition, I aimed to assess the vertical tranmission possibility in the study area since this tranmission is also proposed to be important in DENV transmission.
2. To find out the distribution of serotypes and genotypes circulating in the study area, in an attempt to understand the molecular virus surveillance and spread of virus in a geographic perspective.
3. To determine the most influencial factors relating to DENV infection in the study area by spatio and spatio-temporal modes based on the reported DENV cases between 2000-2013 and cover several variables such as human population, socioeconomic factors, distance to hospital and land surface temperature etc into both models.
4. To assess the effect of temperature on the mosquito innate immune system, particularly RNA interference (RNAi), since RNAi is proposed to be the most important immune response of the mosquito.

Chapter 2

2 Entomological Factors Relating to Dengue Virus Transmission

2.1 Introduction

Since there is no vaccine available to control DENV transmission, key efforts to prevent the spread of DENV infections are often concentrated on controlling or limiting the DENV transmitting mosquito populations (WHO, 2009). In order to determine the mosquito population in a given area, entomological surveys are conducted by following specific protocols. Standard protocols based on traditional sampling methods (Stegomyia indices) and indices, which are explained in detail further below, have been applied in dengue endemic countries for many years (Focks, D. A., 2003). Traditional mosquito sampling has been carried out based on the calculation of indices such as House Index (HI), Breteau Index (BI) and Container Index (CI) (Focks, D. A., 2003). This protocol was for many years used as a standard to determine local mosquito population. In Indonesia, most entomological studies of dengue vectors still apply these traditional (larvae-based) indices and methods (Mulyatno et al., 2012).

However, these traditional sampling methods have shortcomings in measuring the abundance of adult mosquitoes and are limited in predicting the risk of transmission of dengue virus. Since the traditional sampling methods are only based on larvae, it is not a relevant measurement to predict adult mosquito populations which are relevant as DENV vector. Larval surveys have limitations because of the high numbers of deaths of larvae (Focks, D. A., 2003). Among the other indices, CI is the worst indicator because it only shows the proportion of positive containers in an area, but is unable to determine the number of positive containers per household or per person. The HI is a better index, while the BI is the best of the three because it combines information on both containers and the houses. However, all three indices have limitations for predicting the number of adult mosquitoes in each area (WHO, 2003; Focks, D. A., 2003). Despite their limitations, traditional sampling methods are still used by many entomological studies (Chadee, D. D. et al., 2005; Correa et al., 2005; Gurtler et al., 2009; Sanchez et al., 2010; Sanchez L, 2006; Sulaiman et al., 1996).

Thus, there is an important need to improve and validate the traditional sampling methods in order to better assess protocols to determine mosquito populations. In this study, several

indices were applied to improve the validity of entomological surveys in the Banyumas regency of Java, and relate those to dengue risk. Beside the traditional sampling methods (HI, BI and CI), analysis of pupae indices, adult mosquito measurement and species identification were carried out.

In Indonesia, the country in which this study took place, dengue disease has been reported from most of all the provinces in this country with various endemicity status (Karyanti et al., 2014). Yet this neglected tropical disease is poorly investigated despite the importance of Indonesia in the region (Tan et al., 2014). To further understand the differences in areas and locally, this study assessed areas in the Banyumas regency of Java with three reported DENV statuses: endemic areas (area which regularly reported number of DENV cases in the last three years), sporadic areas (area which irregular reports and numbers of DENV cases in the last three years) and free areas (area with no reported number of DENV cases in the last three years) (Indonesia, M. o. H., 1992). Information of differences between these three different areas will give better understanding of mosquito population and how these and derived mosquito indices relate to local DENV transmission risk.

In addition, vertical transmission is believed to be important to DENV maintenance (Guo et al., 2007; Joshi et al., 2002; Martins et al., 2012) and was therefore assessed. Besides by horizontal transmission through mosquito bites, dengue virus can also be transmitted by vertical transmission, as dengue virus in mosquitoes can be transferred into the egg at the time of fertilization through the fallopian tubes during the process of embryogenesis (Khin & Than, 1983). These eggs will be infected by dengue virus and produce infected larvae which later mature and produce adult mosquitoes containing DENV with infection rates of more than 80% (Beaty, 1996).

The research questions in this chapter are as follows:

a. Is there any difference in the density and biology of mosquitoes in DENV endemic, sporadic and free areas of Banyumas regency, Java,?

b. What are the dominant containers where mosquito larvae are found?

c. Which mosquito species are dominant in the survey area?

d. Whether there has been vertical dengue virus transmission which allows dengue virus to persist in certain areas?

e. Are the traditional larvae indices which are used for the entomology survies still relevant today to predict dengue virus transmission risk, or are combinations of indices useful in this study area?

The result of this study will give information on the most representative measurement(s) to determine mosquito population and how these relate to DENV transmission risk, so that the surveys which are routine procedures locally can be carried out more efficiently and possibly inform the use of these indices in other countries affected by DENV.

2.2 Study Area and Methods

2.2.1 Study area description

The study site for this study is the Banyumas Regency, located in the southwest of Central Java Province, Indonesia. Coordinates for this location are as follows: 108 " 39` 17`` - 109" 27` 15`` east longitude, and between 7" 15` 05``- 7" 37` 10`` south latitude. The total area is estimated to be 1, 327, 60 km^2 (132, 759, 56 ha), with 1, 847, 216 inhabitants, 932, 183 male and 915, 033 female. Banyumas Regency consists of 27 sub districts, and contains 39 community health centres. These community health centres are located in every sub district, most of the sub districts have one community health centre, but several sub districts have two community centres depending on the coverage area. Every sub district also consists of several villages; Banyumas Regency consists of a total of 331 villages (Health Office of Banyumas Regency, 2010).

Banyumas Regency was chosen as a study area based on its appropriateness: dengue cases happened each year in this regency and its good accessibility; Wijayanti is based here and has strong links to the local vector-borne disease research infrastructure. The entomology surveys were carried out twice, in the dry and rainy seasons, to determine the differences in mosquito population densities between both seasons in sample areas of research. In the dry season, three villages were chosen: Tanjung Village in South Purwokerto Community Health Centre (DENV endemic area), Village Panusupan, Cilongok I community health centre (DENV sporadic area) and DENV free area (Gunung Lurah Village, Cilongok II community health centre). One village, Sokanegara Village, East Purwokerto Community Health Centre (DENV endemic area), was added during the rainy season survey in order to obtain more larvae/mosquito samples for DENV immunohistochemistry assays. Endemicity status criteria are based on “The technical Manual Eradication of dengue Mosquito-Borne Diseases, Indonesian Ministry of Health (Indonesia, M. o. H., 1992). The determination of DENV endemicity status of each area has been done prior to the survey, based on reported DENV cases 2009-2011 from Banyumas Regency Health Office.

The locations of the entomology survey are displayed in Figure 2-1.

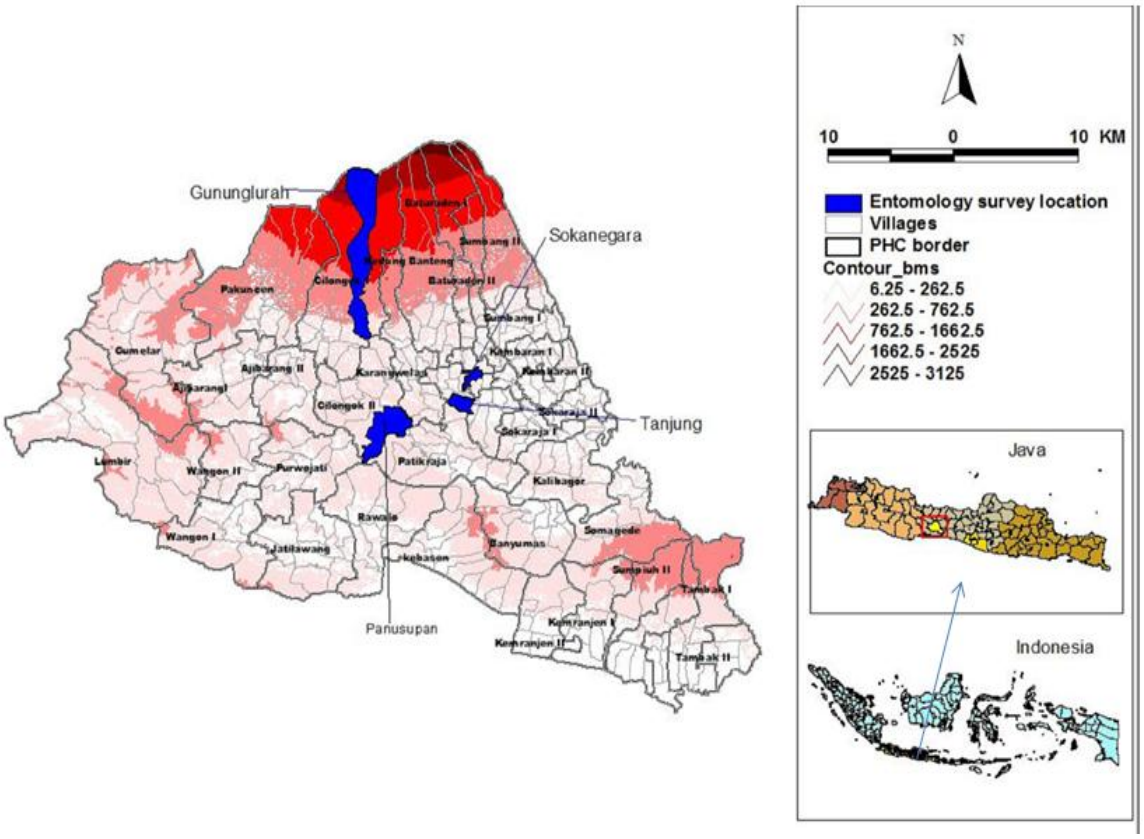


Figure 2-1 Location of Entomology Survey.
The map shows the area of regency which consist of 29 sub districts. Blue areas are the locations of the entomology survey: Tanjung, Sokanegara, Panusupan and Gunung Lurah. This map also shows the contours of the area (in meters). Lower right, map of Java with Banyumas Regency also whole map of Indonesia.

Data on the ecological conditions of each village were collected in order to have a deeper understanding about the conditions of the area of study. These are described in the Table 2-1.

Table 2-1 Description of the study sites in Banyumas Regency, Java.

Details of the ecology and population of the four study sites are provided.

Study sites	Ecological description.						
	Urban /rural	Population	Elevation (metres above sea level)	Coverage area (ha)	Annual rainfall (mm)	Range of temperature (in °Celsius)	Range of humidity (%)
Tanjung (DENV endemic)	Urban	9696	65	149	2654	28-32	73-91
Sokanegara (DENV endemic)	Urban	7987	75	119	2436	30-38	57-83
Panusupan (DENV sporadic)	Rural	7627	200	775	2950	29-36	70-87
Gunung Lurah (DENV free)	Rural	7120	400-700	878	2550	25-34	57-83

The survey in the dry season was conducted May-June 2012 while the rainy season survey was conducted in January-February 2013. In each village, 100 houses were chosen by simple random sampling, resulting in a total of 300 houses in the dry season and 400 houses in the rainy season being studied. The survey consisted of larvae surveys, captures of adult mosquitoes, ovitrap instalment and measurement of temperature and humidity in each house. Determination of the coordinates of houses with GPS (Global Positioning System) for spatial analysis were done. Before the field survey, permissions were obtained from health authorities, community health centres and head of villages in the area of study. Moreover, for all work done in this study, ethical approval has been obtained from the University of Glasgow (project number: 2012082) and Indonesian public health authorities (Medical and Health Ethics Committee, Faculty of Medicine, University of Gadjah Mada, Indonesia) Number : KE/FK/323/FC.

2.1.1. Larvae, Pupae, container, and adult mosquito surveys

Larval collections were carried out in every containers observed in surveyed houses. Larval surveys conducted in the following manner:

a. All places or containers/water reservoirs (traditional bath-tub, buckets, flower pot etc) that can be assumed to act as mosquito breeding sites were assessed to determine the presence/absence of mosquito larvae.
b. If at first sighting larvae no larvae are detected, wait approximately 30 seconds -1 minute to ensure that no larvae/pupae escaped initial observations.
c. To check the smaller breeding sites such as vases/potted plant, water/bottled water they often needed to be moved for better observation.
d. To check for presence of larvae in dark spaces, or cloudy water, flashlights were used.

To support larvae collections, the materials required are vials/bottles, pipettes, flashlight, stationary, larvae collection form and larvae key for identification. All containers, both artificial containers (water containers, buckets, flower pots, drums or other water-holding) and also natural containers (bamboos, tree holes) in selected houses were observed, assessed and recorded. To measure mosquito index the following indices were used: House Index (HI : percentage of houses infested with larvae and/or pupae), Container Index (CI : percentage of water-holding containers infested with larvae or pupae), Breteau Index (BI : number of positive containers per 100 houses inspected), and Free Larvae Index (Focks, D. A., 2003). Calculations of indices are performed as follows:

HI (House Index) = Number of locations positive (house) for mosquito larvae per 100 locations/house.
CI (Container index) = Number of container positive for mosquito larvae per container inspected
BI (Breteau index) = Number of container positive for mosquito larvae per 100 locations/houses.
Free Larvae Index = Number of houses inspected without mosquito larvae (negative) per number of houses inspected.

For the interpretation of risk transmission levels based on the mosquito index, we referred to WHO guidelines (Focks, D. A., 2003). The details are indicated in the table below:

Table 2-2 *Aedes sp* density figure of Stegomyia index following AHA Brown (Focks, D. A., 2003).

Density figure	House Index	Container Index	Breteau Index
1	1-3	1-2	1-4
2	4-7	3-5	5-9
3	8-17	6-9	10-19
4	18-28	10-14	20-34
5	29-37	15-20	35-49
6	38-49	21-27	50-74
7	50-59	28-31	75-99
8	60-76	32-40	100-199
9	>77	>41	200

Density Figure = 1 = Low density figure

Density Figure = 2-5 = Medium density figure

Density Figure = 6-9 = High density figure

WHO has tabulated the density figures 1-9 to predict the mosquito density in certain area (Focks, D. A., 2003). Density Figure determined based on the results of HI, CI and BI.

Identification of mosquito larvae was carried out with larvae collected in the areas of entomological survey based on the key identification described earlier (Stojanovich, 1965). The results will define the dominant larvae, mosquito species and the dominant type of containers in the area of study. The main difference between larvae of *Aedes aegypti* and *Aedes albopictus* is the presence of a comb scale with a stout median and lateral spine in *Aedes aegypti* and a comb scale with a fringe in *Aedes albopictus* (Stojanovich, 1965). A description of these differences is shown in Figure 2-2.

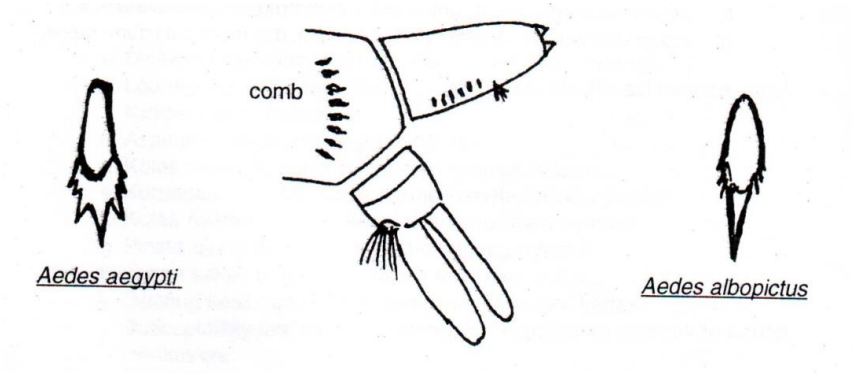


Figure 2-2 The differences in identification between *Aedes aegypti* and *Aedes albopictus* larvae.
Larvae of *Aedes aegypti are* identifed by the presence of a comb scale with a stout median and lateral spine (left) whereas Larvae of *Aedes albopictus* identified by a comb scale with a fringe (right) (Stojanovich, 1965)

Pupae collection also carried out during the entomology survey. The indices for pupae calculations are performed as follows :

Container Pupae Index (CPI) (Pupae index) = number of container positive with pupae per number of inspected houses
House Pupae Index (HPI) = number of houses positive with pupae per number of inspected houses
Pupae per person = total number of pupae per total number of person in the house inspected during the survey
Pupae per house = total number of pupae per number of house inspected
Pupae per container = total number of pupae per number of inspected container

To facilitate adult mosquito collections, back-pack aspirators were used to capture adult mosquitoes in resting and flying positions. We focused on places where mosquitoes are known to normally rest inside the house. *Aedes sp* prefer to rest in dark, shielded, humid places and in hanging objects such as clothes, curtains and walls. Adult mosquito capture was carried out between 8-11 AM for around 20 minutes per house. We put adult mosquitoes which were succesfully captured into plastic containers covered by muslin and added several banana rod sticks in the box to preserve the humidity before sending mosquitoes to the laboratory for further identification. There, identification of adult mosquitoes was conducted based on key identification as described by (Stojanovich, 1965). The differences of adult mosquito between *Aedes aegypti* and *Aedes albopictus* are visible on the scutum. *Aedes aegypti* has a scutum with lyre-shaped white markings whereas *Aedes albopictus* has a scutum with a long median longitudinal white stripe extending from anterior margin to about the level of the wing root (Stojanovich, 1965). These detailed descriptions can be seen in Figure 2-3.

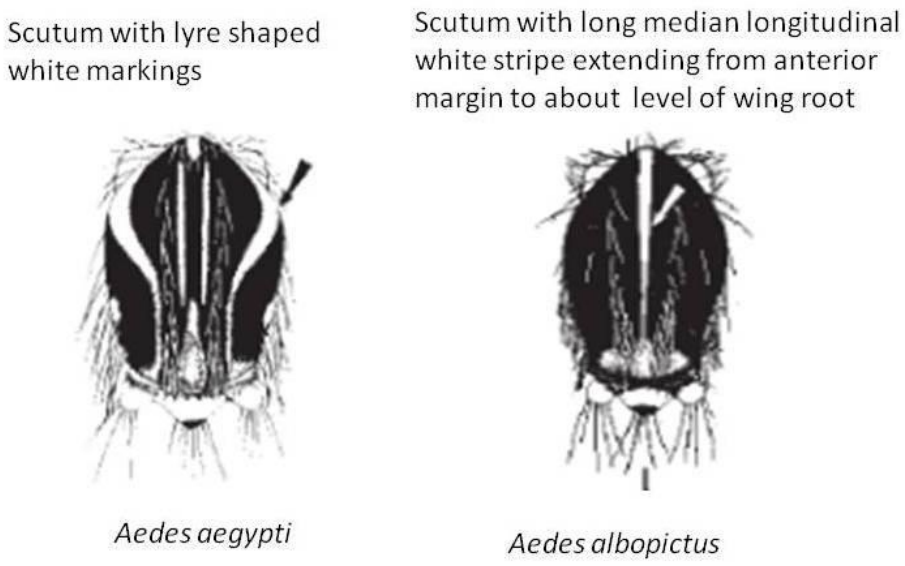


Figure 2-3 The difference between *Aedes aegypti* and *Aedes albopictus* adult mosquito. Adult *Aedes aegypti (left)* shows a scutum with lye-shaped white markings whereas *Aedes albopictus* (right) has a scutum with a long median longitudinal white stripe extending from anterior margin to about the level of the wing root (Stojanovich, 1965).

2.2.2 Ovitrap installations

Oviposition traps (ovitraps) were installed inside and outside of houses in the study area (100 houses each villages). Ovitraps are a simple device used to attract the female *Aedes* sp mosquitoes to oviposit their eggs. The ovitraps used in this study are made from a 250 ml plastic container, with the outer wall of container painted black in order to attract the mosquitoes. A glass with tap water filled to around one-third and filter paper was put into the inside of the glass and placed into the trap. The filter paper above the surface of water will provide a place for female gravid mosquitoes to lay their eggs. Ovitraps were placed both indoors and outdoors of houses in the study area. Indoors referred to inside the house, usually places which are safe from the reach of children (near bathroom, bedroom) while outdoors refers to outside but around 1-5 metres from the house. Six days after installation, ovistrips (filter paper) from each of the ovitraps were collected for eggs calculation. The Ovitrap Index (OI) was determined by calculation the percentage of ovitrap positive with eggs from the total number of ovitrap.

2.2.3 Temperature and humidity determination

As enviromental factors such humidity and temperature contribute to the mosquito life cycle, humidity and temperature were measured by hygrometer and in addition the temperature in each house recorded.

2.2.4 Rearing of *Aedes sp*

Headsquashes from F1 mosquitoes (Filial 1; first generation of mosquito hatched from the eggs in ovistrips) were used for headsquashes and assays on vertical transmission. In order to obtain F1 mosquitoes for headsquashes, eggs were obtained from the ovistrips and then grown to larvae, pupae and adult mosquito stages in rearing rooms. Ovistrips had been placed in trays containing water for around 1-2 days until larvae hatched. Larvae were maintained with dog food in trays until pupae developed. Pupae were then collected in water containers, placed in cages (size 30 x 30 x 30 cm) and grown to adult mosquitoes. Mosquitoes with a minimal age of 5 days were then processed for head squash samples. The rearing room of mosquito is displayed in Figure 2-4.

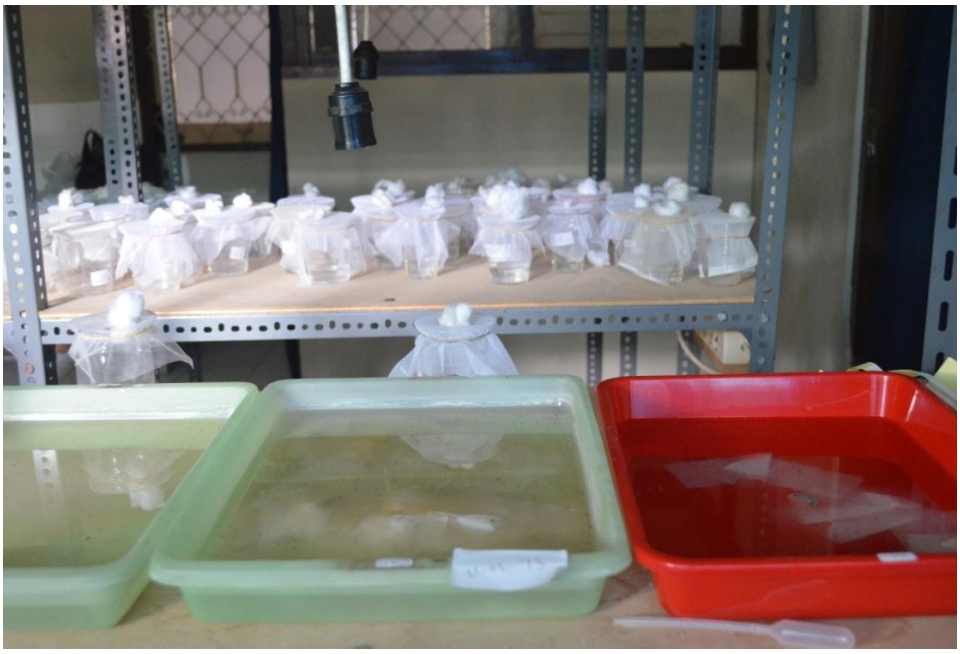


Figure 2-4 Rearing room of mosquitoes from eggs to adult mosquitoes.
Ovistrips collected in villages were placed in trays. After they reached pupae stage, specimens were transferred to cages and reared to become adult mosquitoes.

Species and sex identification of mosquitoes was carried out before the head squash analysis based on previously published taxonomic guidelines (Stojanovich, 1965). Both

male and female mosquitoes were included in the immunohistochemistry test. The immunohistochemistry assay was carried out following a procedure developed locally (Umniyati, SR 2009). The virus infection rate (VIR) was calculated as (number of mosquitoes by species infected with dengue virus ÷ total number of that species tested) x 100.

2.2.5 Head squashes preparations

An immunohistochemistry assay based on streptavidin-biotin-peroxidase-complex (SBPC) was conducted to confirm vertical transmission of DENV in Banyumas Regency on aedine mosquitoes caught locally (see above). Each headsquash preparation was made from the caput/head part of mosquitoes. The caput part was separated from the body, and then put in the slide covered by poly-l-lysine. The detailed procedure of headsquash analysis is as follows:

a. Paper cups which contained Filial 1 mosquitoes (adult mosquito from eggs collected from the field) were placed into a cryofreezer for 15 minutes or until the mosquitoes died.
b. Then the mosquitoes were taken by pince and needle to separate the abdomen, thorax and caput parts of the body. The thorax part was stored in an eppendorf tube, whereas the caput placed was in a glass slide covered by Poly L-Lysine. Each slide contained caputs of 10 mosquitoes.
c. The mosquito caputs were arranged in rows to provide enough space between each specimen.
d. Then caputs were closed over by cover glass slip and the tip of dissecting needle in order to obtain a good headsquash.
e. The glass cover slide dropped in cold methanol (-20°C) and incubated for 5 minutes.
f. Following fixation, the slide was rinsed with running water and dried.
g. The headsquashes were stored in -80°C before being processed.

Below are head squashes which are ready to be processed in the imunohistochemistry assay (Figure 2-5).

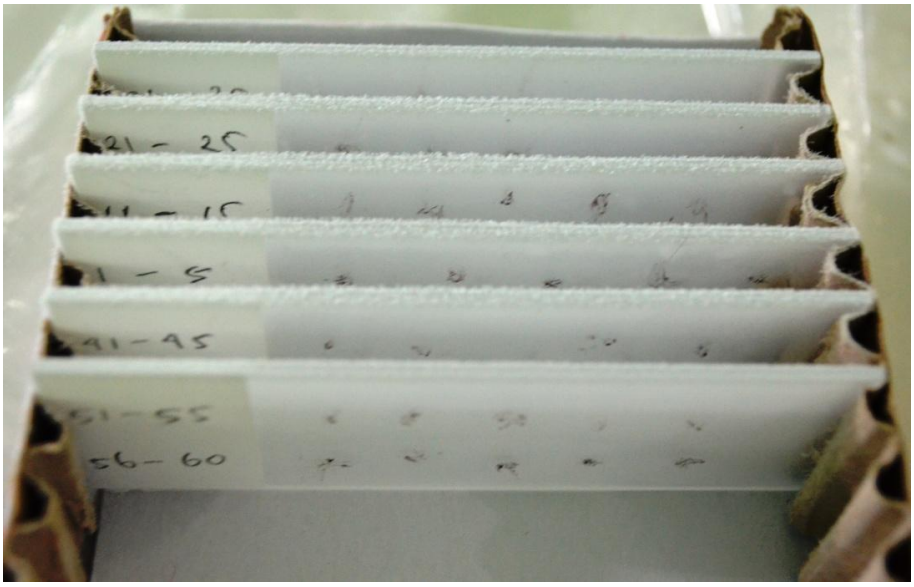


Figure 2-5 Head squashes of mosquitoes.
This picture showed that each slide contains caputs of 10 mosquitoes, which ready for immunohistochemistry assay. Each slide was marked by the number of each mosquitos to provide an easier process in result analysis.

2.2.6 Immunohistochemistry assay

The immunohistochemistry (IHC) assay for dengue virus was conducted based on a locally developed protocol (Umniyati, SR, 2009) using the Starr Trek Universal HRP Detection Kit. The kit contains five reagents ready for use, ie universal trekkie links containing biotin-labeled secondary antibody, trekAvidin-HRP conjugate containing streptavidin-labeled peroxidase enzyme HRP, DAB chromogen betazoid, Betazoid diaminobenzidine tetrachloride (DAB) buffer, a protein Background Sniper blocking solution containing non-immune serum. This IHC test used primary antibody from Monoclonal Antibody DSSC7 (Class IgG) produced by the dengue team, Gadjah Mada University which detects NS1 dengue antigen (does not crossreact with chikungunya virus, Japanese enchephalitis). Biotinylated secondary antibody was acquired from Biocare Medical. The test was performed as follows:

a. Headsquash slides were washed in PBS (phosphate buffered saline) for 2 minutes and then the slides were soaked in peroxidase blocking solution with a mixture of H_2O_2 in a ratio of 1:9 at room temperature for 10 minutes.
b. The slide was then incubated in Background sniper (Protein blocking solution) for 10 minutes at room temperature.
c. 20 µl primary antibody monoclonal antibody DSSE (1C7) diluted 1:10 in PBS was added to each slide and incubated at room temperature for 1 hour.

d. Each slide was rinsed with PBS for 2 minutes, three times.

e. 20 µl Trekkie universal link (secondary antibody) was added to each slide and incubated at room temperature for 15 minutes , then rinsed with PBS twice for 2 minutes.

f. The slide was then incubated with TrekAvidin-HRP for 10 minutes and rinsed with PBS twice for 2 minutes.

g. The Kromogen DAB (Biocare Medical) substrate was prepared as follows: dilute 1 µl Betazoid DAB Chromogen with 600 µl Betazoid DAB substrate buffer.

i. Incubate the slide with 20 µl Kromogen DAB substrate for 10 minutes, followed by rinsing the slide with running water.

j. Mayer hematoxylin (counterstrain) was added to the slide and then incubated for 1-3 minutes, then rinsed in running water and dried.

k. The slide was dipped in alcohol, dried and cleaned up.

l. Mounting media was added and the slide covered with cover glass. After drying, the slide can be viewed under a microscope and staining analysed.

If the central nervous ganglion cells in the caput stains brown in colour in the cytoplasm, and brown granules are also surrounding the cell which indicates the mosquito is positive for dengue antigen (positive result). Negative result as the central nervous ganglion cells appear blue or pale and there are no brown granules surrounding the cells; this means the mosquito is negative for dengue antigen.

2.2.7 Questionnaire for knowledge, awareness and practices related to dengue in endemic, sporadic and free areas

In order to find out about Knowledge and Awareness of DENV and Practices in DENV prevention (KAP) in the surveyed villages, a questionnaire collection was carried out. The data collection was done at the same time as the entomology survey during the dry season. Each house (a total of 300 houses) is represented by one head of family who is required to answer a standardised questionnaire about knowledge, awareness and practices towards dengue disease and prevention. The respondent must be over 15 years old, in order to ensure clear communication with the interviewer. Personal identification such as age, gender, social economy status and education level were identified. The details of the questionaire can be seen in Appendix 1. The questionnaire answers were assessed and put into two categories: good or bad for knowledge, awareness and practices. The first step uses a total sum score of the value of each variable and then a normality test was conducted using Kolmogorov Smirnov. Based on the normality test, the total score

indicates the data is not normally distributed (p-value <0.05), therefore the median value for basic categorization was used. Good knowledge means the total score of knowledge ≥ median, and bad knowledge means the total score < median. Data was analysed using SPSS 16.

2.3 Results

2.3.1. Larvae Indices in Dry and Rainy Seasons in the Banyumas study area

Over the course of the entomological survey of the study area, we verified the presence of mosquito larvae in containers of a total of 300 houses in the dry season and 400 houses in the rainy season. The single larvae standard method (larvae from each container which had larvae were taken and the species identified) was applied in this survey (procedure based on recommendations of the Directorate of Communicable Disease Control in Indonesia and WHO as reviewed elsewhere (Focks, D. A., 2003). We observed all containers, both artificial and natural. Following sampling, larval indices were measured as HI, CI, BI and FLI as described in Methods. The results for the larval indices in all four villages across the seasons compared to DENV endemicity status are shown in Table 2-3.

Table 2-3 Risk level of dengue transmission compared on larvae indices in the 4 study areas in the dry and rainy seasons.

The following table summarises House index (HI), Breteau Index (BI), Container Index (CI) and Free Larvae Index (Flipse et al.). Risk level determination according to AHA Brown (Focks, D. A., 2003); civ: confidence interval.

Village	DENV status	Larvae index, dry season					Larvae Index, rainy season				
		HI (95% civ)	BI (95% civ)	CI (95% civ)	FLI (95%civ)	Risk Level	HI (95%civ)	BI (95% civ)	CI (95% civ)	FLI (95% civ)	Risk Level
Tanjung	Endemic	16 (8-23)	16 (8-23)	9.6 (5-14)	84 (76-91)	Medium	19.44 (11-27)	18 (10-25.53)	3.73 (2-5)	75 (66-83)	Medium
Sokanegara	Endemic	-	-	-	-	-	18.27 (10-25)	18 (10-25)	4.6 (2-6)	81.72 (74-89)	Medium
Panusupan	Sporadic	25 (16-33)	35 (25-44)	21.8 (15-28)	75 (66-83)	Medium	44 (34-53)	71 (62-79)	12.7 (9-15)	56 (46-65)	High
Gunung Lurah	free	3 (0-6)	3 (0-6)	2.5 (0-5.2)	97 (93-100)	Low	16 (8-23)	19 (11-26)	3.44 (1-4)	84 (76-91)	Medium
Average		15	18	11	85		24	31	6	73	

Based on the larval indices from the field survey in dry and rainy seasons from four villages, we can determine that the larvae index in the rainy season are mostly higher than in the dry season. House Index (HI) and Breteau Index (BI) in all villages are higher in the rainy season, but the Container Index (CI) are lower in rainy season because more containers were found in the rainy season. Surprisingly, Panusupan (DENV sporadic) showed the highest HI, BI and CI among other villages. Meanwhile, Tanjung and Sokanegara (DENV endemic) showed almost similar percentages for the various indices in the rainy season. Gunung Lurah (DENV free) was confirmed to have the least larvae indices, excepted the BI in both seasons.

The risk level of DENV transmission corresponding to these indices was determined following WHO criteria (Focks, D. A., 2003). Tanjung and Sokanegara (DENV endemic) were classed as medium risk for DENV both seasons, while Panusupan (DENV sporadic) was classed as medium risk in dry season and high in the rainy season. Gunung Lurah (DENV free) was classed as low risk for DENV transmission risk in dry season and medium in rainy season. This suggests a clear disparity between reported DENV and risk levels calculated by using standard indices.

In order to allow improved comparisons of the larvae indices, risk level and dengue cases in the field, we gathered updated dengue cases data from Banyumas Health Office, December 2013 and compared the larvae indices with the number of dengue cases registered after the survey (Table 2-4).

Table 2-4 Mosquito larvae density in dry and rainy seasons, and updated dengue cases in 2012 and 2013.
Updated dengue case numbers were obtained from the Banyumas Health Officer's Report and compared to larval indices. *Despite one case, classed as sporadic based on years preceding surveys.

Village	Endemicity status	Mosquito larvae density, dry season (May-June 2012)	Mosquito larvae density, rainy season (Jan-Feb 2013)	Dengue cases, 2012	Dengue cases , 2013
Tanjung	Endemic	Medium	Medium	2	15
Sokanegara	Endemic	-	Medium	13	9
Panusupan	Sporadic	Medium	High	0	0
Gunung Lurah	Free area	Low	Medium	0	1*

Based on the results shown in Table 2-4, mosquito larvae density and predicted risk is not always in concordance with the number of dengue cases which happened in a given area. In Tanjung (DENV endemic) which categorized as medium risk level by using larvae indices, 2 and 15 cases happened in 2012 and 2013 respectively. Moreover 13 and 9 dengue cases respectively were also detected in Sokanegara (DENV endemic) in 2012 and 2013. Surprisingly, Panusupan (DENV sporadic) was classed medium and high mosquito density but in fact in 2012 and 2013 there were no dengue cases reported in this area. Gunung Lurah village which has medium mosquito density in rainy season is has one case, however this case could have been imported cases rather than a indicator of local transmission. These results indicated that mosquito larvae densities and derived indices in a region are not always comparable to the incidence of dengue fever and in this study a poor indicator of risk.

In addition to the calculation of larvae indices, species identification was carried out with the larvae obtained from the entolomogy survey. The larvae species found in each village and distribution are shown in Figure 2-6.

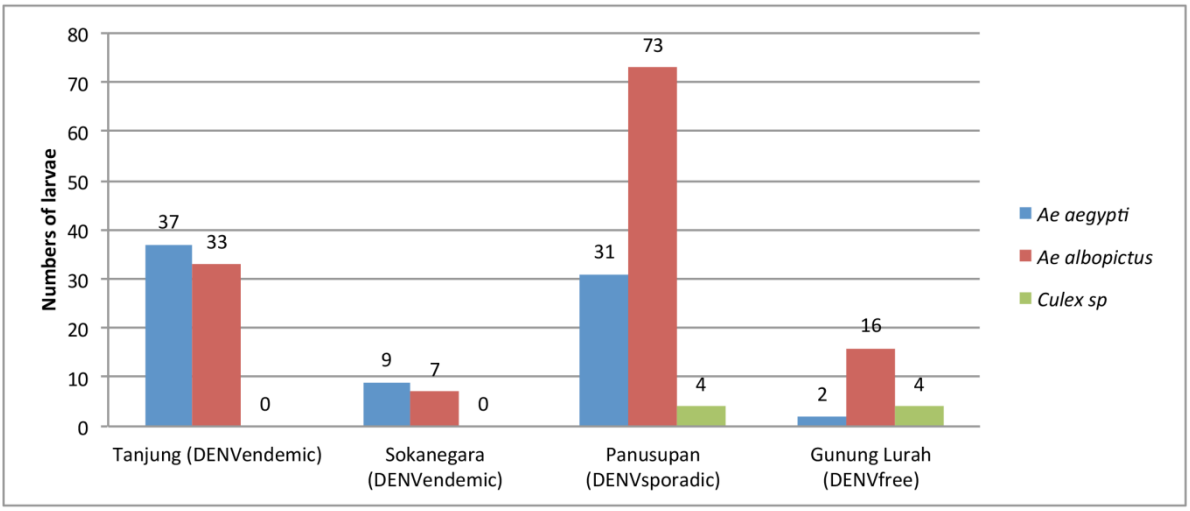


Figure 2-6 Number of mosquito species within the larvae identified in each of the four villages in dry and rainy season.
Each bar represents the mosquito species as indicated in the legend.

The charts above illustrate that Tanjung and Sokanegara (DENV endemic) have almost the same proportion of *Aedes albopictus* and *Aedes aegypti*, while no *Culex sp* were identified. In Panusupan (DENV sporadic) and Gunung Lurah (DENV free), *Aedes. albopictus* was found to be dominant species, and *Aedes aegypti* and *Culex sp* were discovered in fewer

number. The high number of *Ae.albopictus* in sporadic and free area in accordance with the preference of this species to live in rural areas with more vegetation.

A pupae survey was also conducted during the entomology survey to complement these data. Result of pupae indices are indicate in Table 2-5.

Table 2-5 Pupae indices for the four villages included in this study.
*CPI = Container Pupae; HPI =House Pupae Index. Data for dry/rainy seasons are indicated.

Name of Village	status	CPI (%)		HPI (%)		Pupae/ person		Pupae/ house		Pupae/ container	
		dry	rainy	dry	rainy	dry	rainy	dry	rainy	dry	rainy
Tanjung	endemic	4.22	3.81	5	9	0.02	0.061	0.09	0.26	0.05	0.05
Sokanegara	endemic	-	4.85	-	11	-	0.04	-	0.21	-	0.05
Panusupan	sporadic	6.25	1.43	7	5	0.03	0.02	0.11	0.11	0.07	0.01
Gunung Lurah	free	3.31	0.5	2	2	0.01	0.007	0.04	0.03	0.03	0.005

Based on these data, CPI and HPI in DENV endemic and sporadic areas were higher than DENV free area. Pupae index in dry and rainy season appear to be no specific patterns or differences.

2.3.2 Container survey results

To complete the entomological surveys, container observation were carried out in each house taking part to obtain information about the dominant containers are in the area of study. Container surveys are essential to provide information about breeding preferences of *Aedes sp*, so this information will valuable for dengue prevention program. Several components such as pH, O_2, sun exposure, temperature, level of organic matter are cannot be measured in this study due to limited time and resources. Other components which related to the breeding site of mosquito is the water condition because *Ae aegypti* and *Ae albopictus* have different preferences. More details observation including the component above in futher studies will give more benefit information.Details of the result of container survey in the area of study, by village, are shown in Table 2-6.

Table 2-6 Proportion of water-holding containers infested with larvae and/or pupae in the four villages of the study area.
Both rainy and dry seasons are taken into account and indicated. Containers are described by type, as indicated.

Type of water-holding container	Container observed									Container positive for larvae								Total container positive with larvae	Total container without larvae	Proportion infested (%)
	Tanjung		Sokanegara		Panusupan		Gunung lurah		Total observed	Tanjung		Sokanegara		Panusupan		Gunung Lurah				
	dry	rainy	dry	rainy	Dry	rainy	dry	rainy		dry	rainy	dry	rainy	dry	rainy	dry	rainy			
Traditional bath tub	75	75		88	63	63	48	48	460	3	8		11	4	12	1	0	39	421	8.48
Buckets	55	198		152	73	344	62	212	1096	6	5		2	17	24	1	7	62	1034	5.66
Dispenser	8	16		16	0	8	0	1	49	2	2		0	0	3	0	1	8	41	16.33
Leaf Midrib	0	10		1	1	13	1	49	75	0	0		0	0	2	0	5	7	68	9.33
Used bottles	0	57		61	1	14	2	105	240	0	0		0	0	5	0	3	8	232	3.33
Refrigerator	8	10		17	0	3	0	0	38	1	0		0	0	1	0	0	2	36	5.26
Flower pot	7	11		1	18	6	3	0	46	1	0		0	9	1	1	0	12	34	26.09
Water storage/container	10	57		47	2	91	4	130	341	3	2		1	4	16	0	2	28	313	8.21
Aquarium	3	5		4	0	4	0	0	16	0	0		1	0	1	0	0	2	14	12.50
Discarded Tires	0	3		4	1	4	0	7	19	0	0		3	1	5	0	1	10	9	52.63
Drum	0	40		0	0	0	1	0	41	0	1		0	0	1	0	0	2	39	4.88
coconut shells	0	0		0	1	7	0	0	8	0	0		0	0	0	0	0	0	8	0.00
	166	**482**		**391**	**160**	**557**	**121**	**552**	**2429**	**16**	**18**		**18**	**35**	**71**	**3**	**19**	**180**	**2249**	**7.41**

It is clear from the container survey that more artificial containers than natural containers are found in the study area (independent t test, $p<0.05$). Buckets, traditional bath-tub, and water storage/container were identified as the most abundant containers in all the villages. Furthermore, discarded tyres were found to be the most dominant container infested with larvae (52.63%). Flower pots, dispensers, and aquarium also showed a high proportion of infestation with mosquito larvae.

Adult mosquito numbers are believed to be the most representative measure for information on mosquito abundance (Focks, D. A., 2003). In order to measure adult mosquito populations, we used a backpack aspirator to capture adult mosquitoes. This tool can catch the mosquito in either resting or flying positions. After identification of the mosquito species, we compiled species percentages as shown in the chart Figure 2-7.

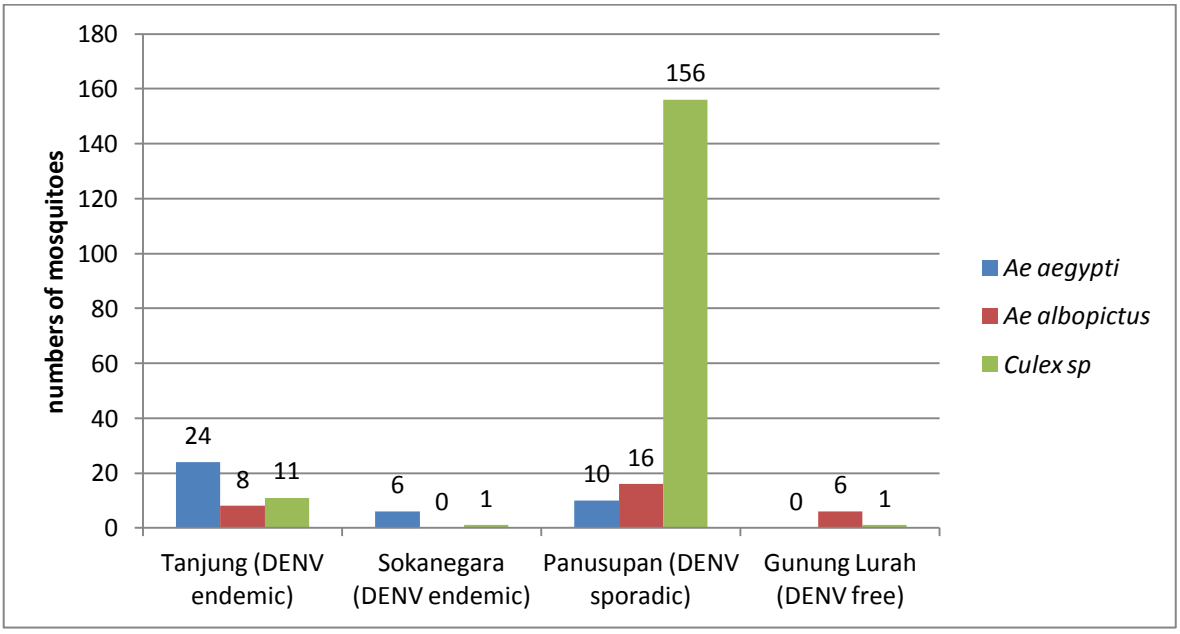


Figure 2-7 Number of adult mosquito species collected in the each of the four villages studied in the dry and rainy seasons.
Each bar represents the mosquito species indicated as described in the legend. Dengue endemicity of the capture area is also indicated.

Aedes aegypti was the dominant adult mosquito captured during the survey in Tanjung and Sokanegara (DENV endemic). In Panusupan (DENV sporadic) *Culex sp* was present in high number compared to other mosquito species. In Gunung Lurah (DENV free), very few mosquitoes were observed during the survey , and these were mostly *Ae. albopictus*.

2.3.3 Temperature and humidity in the study area.

As environmental factors such humidity and temperature contribute to mosquito life cycles, we measured humidity by hygrometer and temperature by thermometer in each house taking part in the mosquito survey. The results of these two variables are compiled in Table 2-7.

Table 2-7 Temperature and humidity (percentage) in the study area.
All four villages were included in this survey and DENV status is indicated.

Village	Dengue Status	Range of temperature	Average Temperature	Range of humidity	Average Humidity
Tanjung	DENV endemic	28-32	30,74	73-91	82,69
Sokanegara	DENV endemic	30-38	33.75	57-83	70.15
Panusupan	DENV sporadic	29-36	32.93	70-87	76.71
Gunung Lurah	DENV free	25-34	31.37	57-83	67.19

From the temperature measurements of the four villages (Table 2-7), we found that the temperature can vary from 25°C-38°C with highest temperature recorded in Sokanegara village. From the average temperature in the four villages, it would appear that the range of temperatures seems to condusive for the mosquito life cycle (Rowley and Graham ,1968), with optimum temperature for *Aedes sp* ranging between 27-32°C and less effective in temperatures between 17-25°C. The average humidity in the study area varied between 67.19 % in Gunung Lurah to 82.69 % in Tanjung Village.We can conclude that the temperatures and humidity in the four villages support the mosquito life cycle. Th.

2.3.4 Geographical mapping of larvae and mosquitoes in entomological survey areas.

In order to provide better description about the mosquito population in a geographical context, we visualised our data in a map. By using Geography Information System (GIS) support, we can capture, manage, display and analyse large volumes of spatial data in a geographical context. In the entomological survey, we recorded the coordinates of houses selected by GPS (Global Positioning System) and developed a map showing the locations of houses that were positive or negative for larvae or mosquitoes. This map also shows characteristics of land use in the village. The detailed map shown in Figure 2-8.

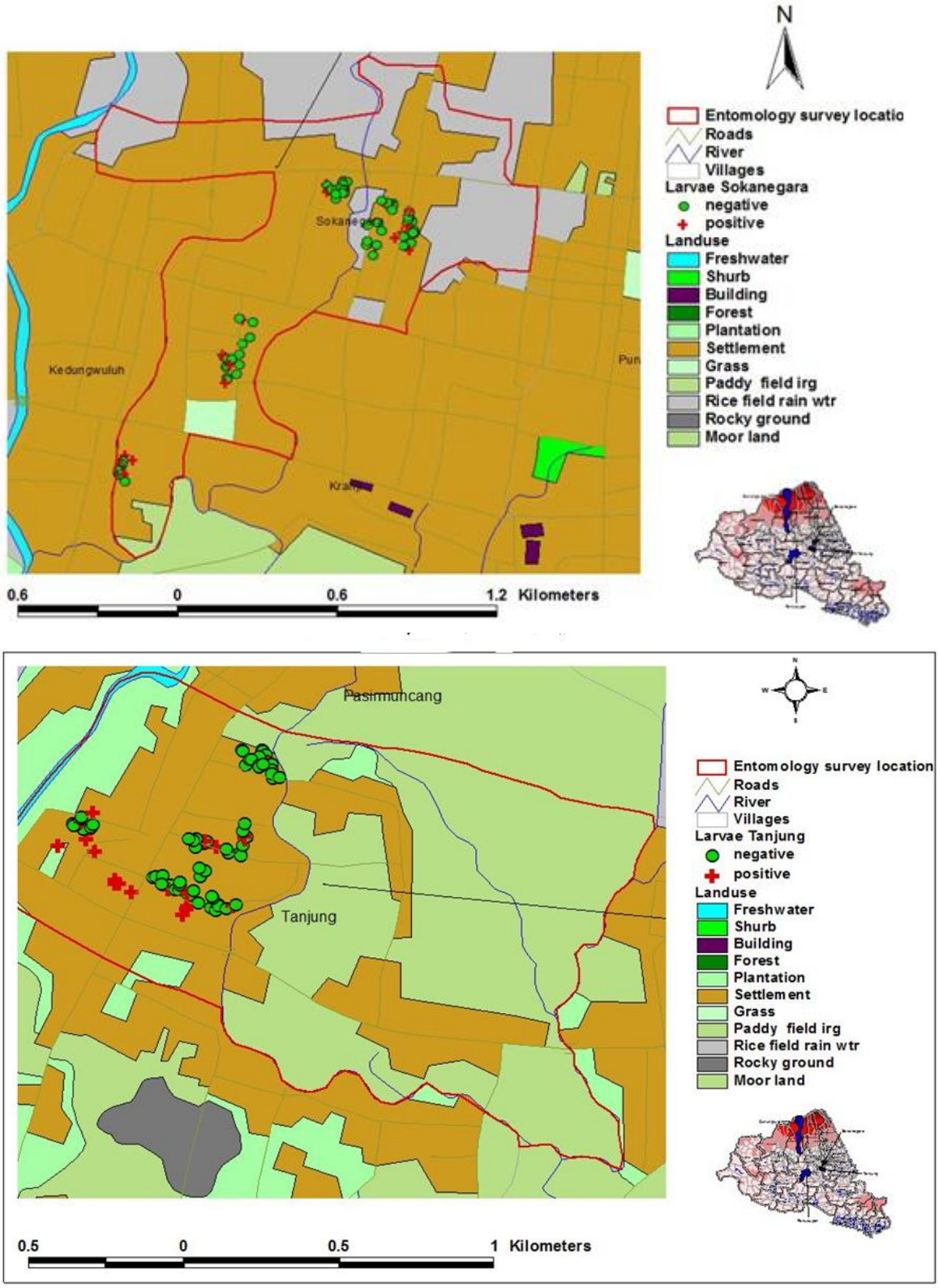
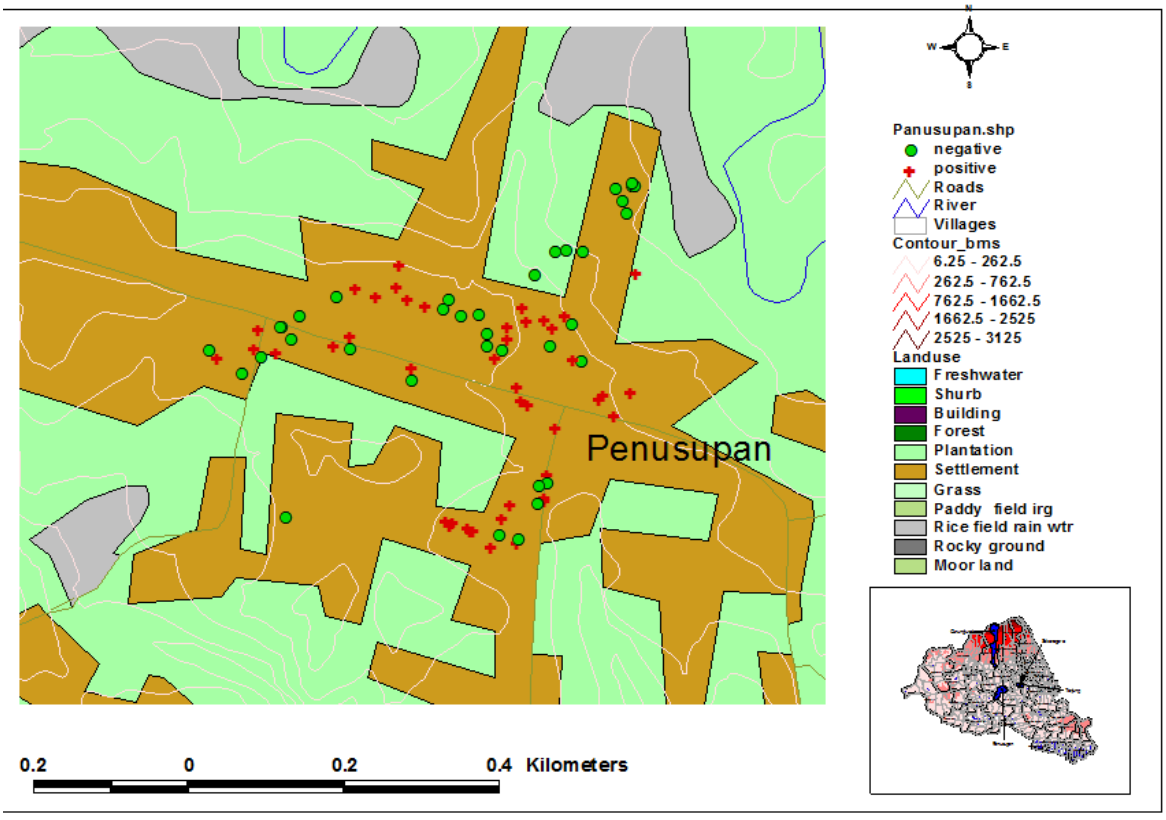


Figure 2-8 Map and analysis of geographic characteristics and mosquito/larvae distribution in two surveyed villages, Sokanegara (above) and Tanjung (below).
Locations of houses that were positive or negative for larvae and adult mosquitoes in Sokanegara and Tanjung Villages are shown. Plus symbol in red represents houses that were positive for larvae or adult mosquitoes. Small green circles represent houses without larvae or mosquitoes. The red line shows the borderline of the village. The map also displays land use in the area, as described in legend.

Based on the map of Sokanegara village, this area mainly consists of settlements and rice fields, whereas in Tanjung village (bottom) the land mostly consists of settlement and plantation areas. The distribution of positive and negative houses shows two different patterns, which are: areas consisting of a mixture of positive and negative houses and areas with only positive houses or only negative houses (for larvae/adult mosquitoes). One possible explanation for the presence of areas with mostly negative or positive larvae and mosquitoes probably relates to the same behaviour within neighbourhoods. Based on previous work (Espino et al., 2012) that analysed responses to water container management in the control of dengue vectors in Philiphinnes the authors stated that changes of behaviour relate to response in the surrounding community. Sokanegara and Tanjung villages are classified as DENV endemic areas and mostly settled area increases the possibility of contact between humans and mosquitoes. It is interesting to compare land use on the map in Panusupan (sporadic) and Gunung Lurah (free area) and also the distribution of houses with positive and negative larvae/adult mosquitoes. The area maps of Panusupan and Gunung Lurah are shown in Figure 2-9.



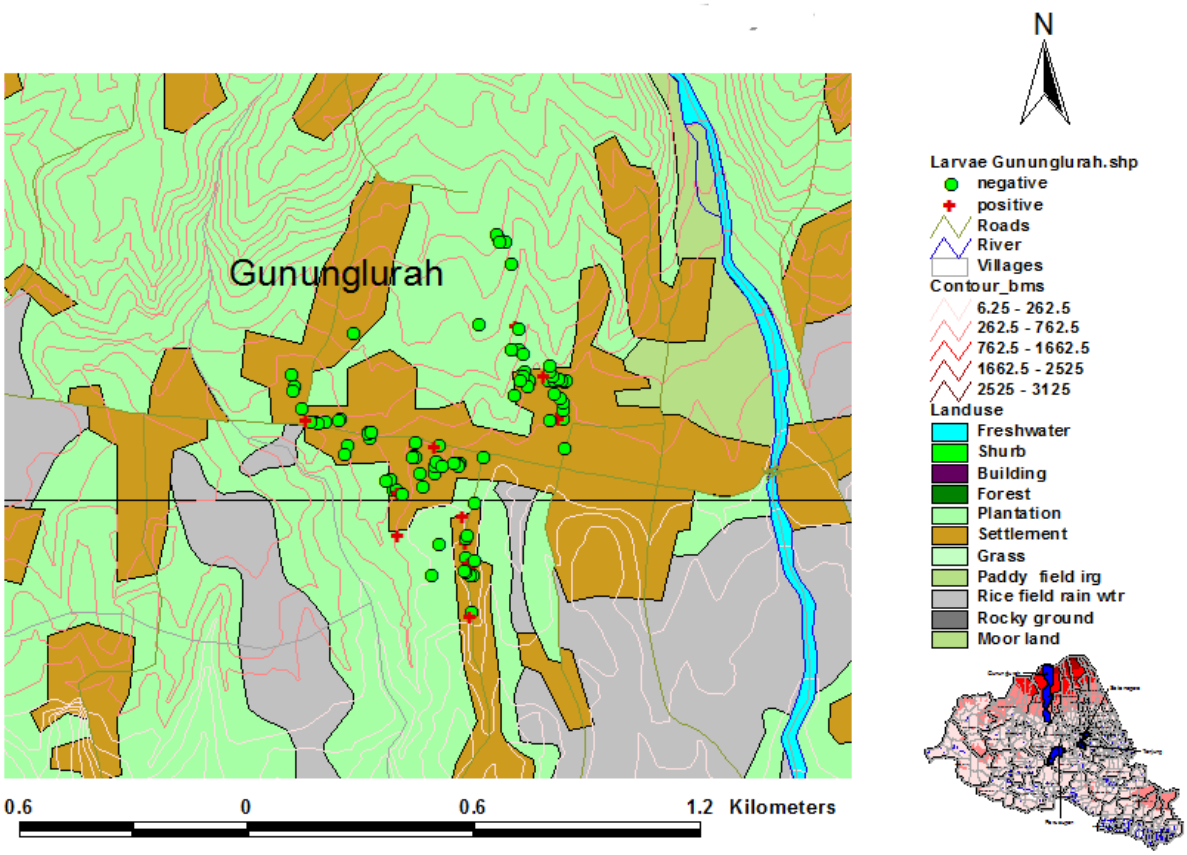


Figure 2-9 Map and analysis of Panusupan and Gunung Lurah villages.
Location of houses that are positive or negative for larvae or adult mosquitoes in Panusupan (above) and Gunung Lurah villages (below). Red crosses represent houses with positive larvae or adult mosquitoes. Green circles represented houses without larvae or mosquitoes. The map also shows land use in the area, as described in the legend.

We also built maps that describes the location of dengue cases in DENV-affected areas. The aim of this map is to show the distribution of dengue cases. Addresses of dengue patients between 2012 and 2013 in Tanjung, Sokanegara and Gunung Lurah villages were obtained from Banyumas Regency Health Office. The map can be seen below.

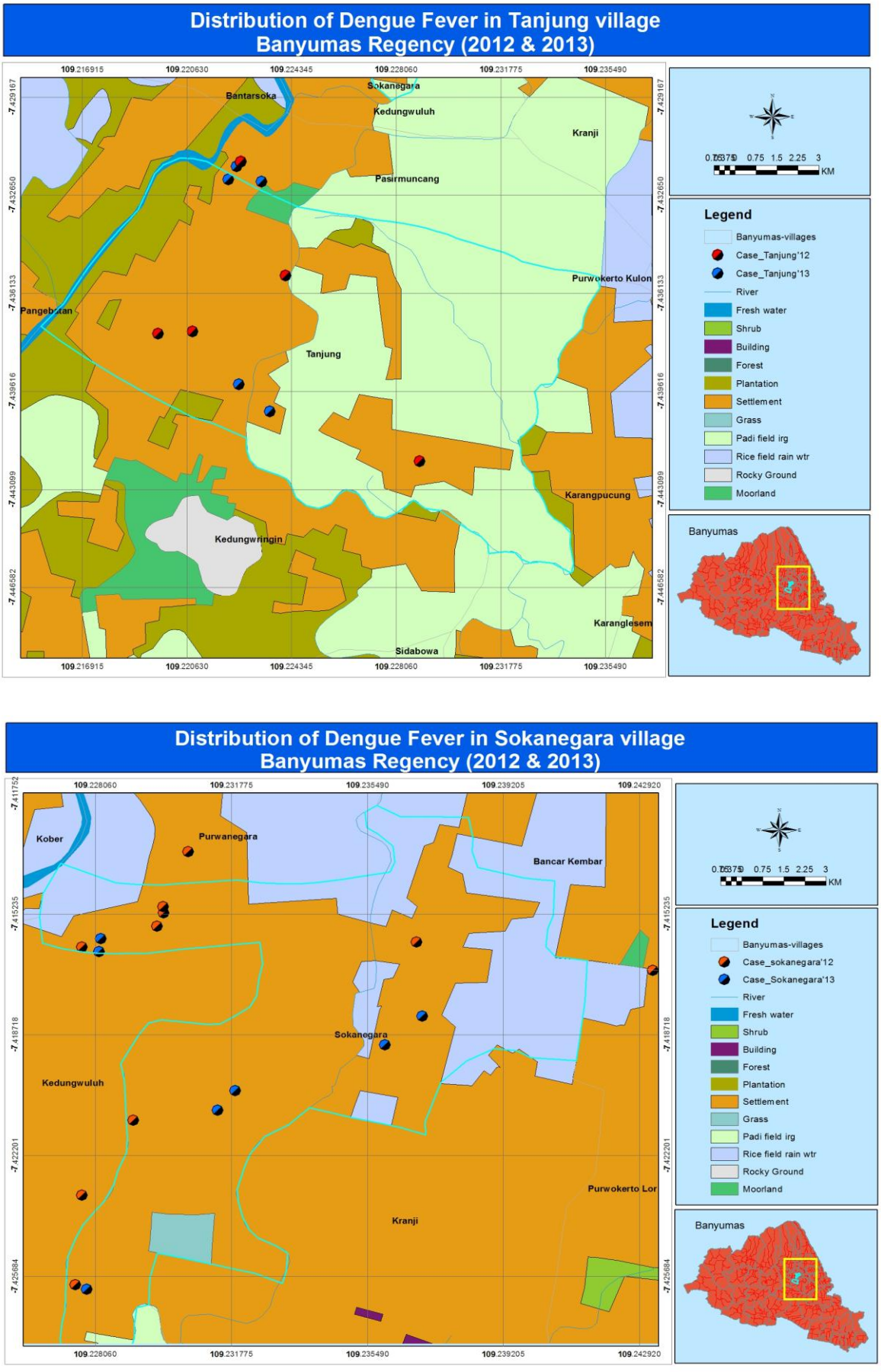


Figure 2-10 Map of the distribution of dengue fever in Tanjung (top) and Sokanegara villages (bottom panel) in 2012 and 2013.

Red circles represented dengue cases in 2012 whereas blue circle showed dengue cases in 2013.

From the map in Figure 2-10, it appears that the spread of dengue cases happened in clusters and also in single cases separated by considerable distance. Dengue clusters can be defined as when two or more dengue cases occur within 14 days of each other, and the homes of the dengue patients are within 150 m of each other (Loh, 2001). Dengue cluster cases happened in Sokanegara in 2012 when three people in the same house developed dengue disease consecutively (shown in Sokanegara village as three red dots in very close proximity). These dengue clusters corroborate the statement that *Aedes sp* have a multiple host feeding characteristic where they tend to bite many times on different people (Higa, 2011).. A single dengue case is possibly caused by transportation and human mobility (Teurlai *et al*, 2012). This results should lead a better awareness on dengue transmission to people who live close to dengue patients.

2.3.5 Ovitrap indices and DENV risk.

After 6 days, oviposition traps (ovitraps) were collected from the study area and the ovistrips were inspected visually in the laboratory. Several of the ovitraps were not used further because the water spilled out - possibly due to children playing or animal interference- and some ovitraps were also lost due to unknown factors. Observation of mosquito eggs was carried out for each ovitrap recovered from the field and the number of eggs counted. Subsequently, we determined the Ovitrap Index (OI), the percentage of positive ovitraps against the total number of ovitraps recovered for each site. The result of OI can be seen in Table 2-8.

Table 2-8 The ovitrap indices from four villages included in the study.

Village	position	ovitrap observed	ovitrap positive with eggs	ovitrap index (%)	total number of eggs	average of eggs/ovitrap
Tanjung	indoor	83	24	28.91	551	22.95
	outdoor	81	46	56.79	1753	38.10
Sokanegara	indoor	53	10	18.86	263	26.3
	outdoor	47	26	55.31	721	27.73
Panusupan	indoor	58	13	22.41	477	36.69
	outdoor	67	45	67.16	1614	35.86
Gunung lurah	indoor	68	17	25	406	23.88
	outdoor	63	46	73.01	2026	44.04

From the results shown in Table 2-8, it is apparent that the ovitrap index in outdoor positions is higher than in indoor positions among all the villages observed. The term outdoor in this study means outside the house but in near proximity (1-5 metres). Female gravid mosquitoes in the area of study prefer to lay their eggs outside the house. A clearer description of the OI can be seen in the graph below:

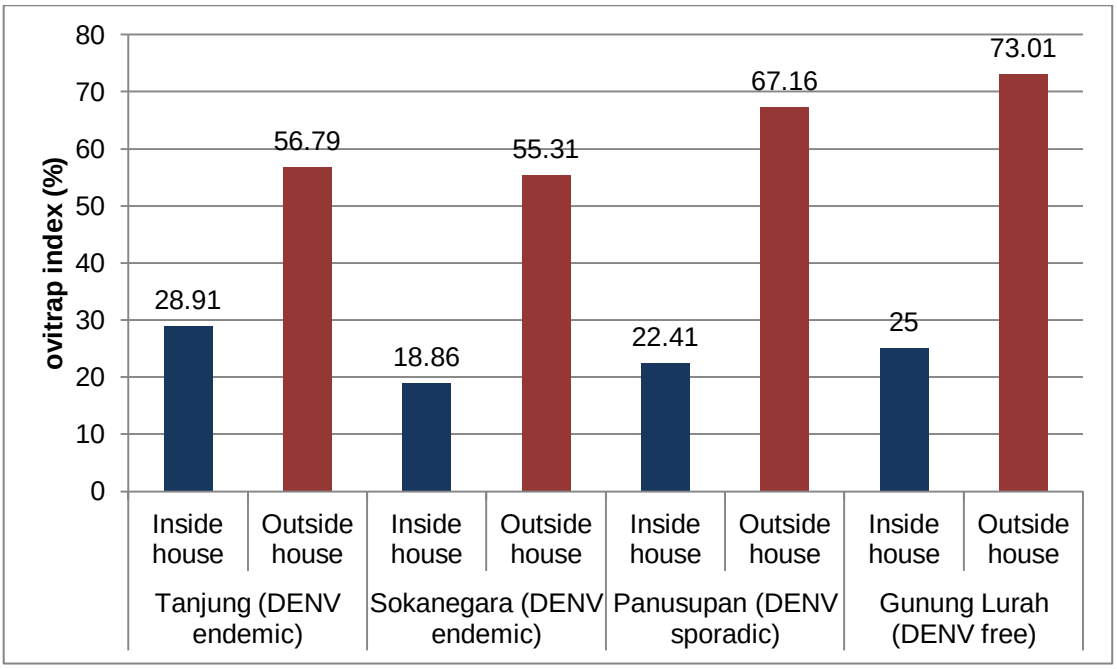


Figure 2-11 Graph showing the ovitrap indices of four villages, either indoors or outdoors. Blue represents the ovitrap index indoors (in percentage) and red represents ovitrap index outdoors (in percentage).

From Figure 2-11, it is clear that the OIs in outdoor position were higher than in indoor among four villages observed (independent t test, $p<0.05$). All OIs in outdoor position showed more than 50%, while in indoor position less than 30%. Furthermore, the eggs form the ovistrips were used to be grown in a rearing room to adult mosquitoes. This filial 1 were then processed for IHC assay to assess the vertical transmission in the area of study.

2.3.6 Immunohistochemistry assay and demonstration of vertical transmission of DENV.

In order determine whether there is vertical transmission of DENV occurring in the study area, we carried out immunohistochemistry assays on head squashes from F1 (filial 1; first generation) mosquitoes. Immunohistochemistry assays was chosen as a method because the lack of supporting system to conduct PCR test in the area of study. Eggs were collected from the oviposition strips (ovistrips) and grown in a rearing room to undergo

their four phases of metamorphosis. Adult mosquitoes were then processed as head squashes in poly-L lysine coated slides as described in Methods. The immunohistochemistry test was carried out based on the method described by Umniyati *et al* (2009) using Streptavidin-Biotin-Peroxidase-Complex (SPBC) method which only needs a light microscope for analysis. Monoclonal antibody DSSC7 (class IgG), which detects DENV NS1 antigen but does not cross react with chikungunya virus, was used as primary antibody. Biotinylated secondary antibody was used as secondary antibody to detect the primary antibody (Umniyati, 2009). DENV vertical transmission research in Indonesia is still being carried out using this method due to the limited number of laboratories having PCR machines. The main principle of this method is a very strong bond occurs between streptavidin and biotin (Umniyati, 2009). Streptavidin is a protein isolated from *Streptomyces avidinii* which can bind to biotin. A secondary antibody labelled with biotin is then added which recognises the primary antibody. The application of streptavidin labelled with horseradish peroxidase enzyme and a chromogenic substrate (from Novostain Universal Detection Kit) to detect antigen in cells or tissues is very sensitive and this method can detect even a low level of antigen (Umniyati, 2009). A schematic figure of the immunohistochemistry principle is shown in the figure below:

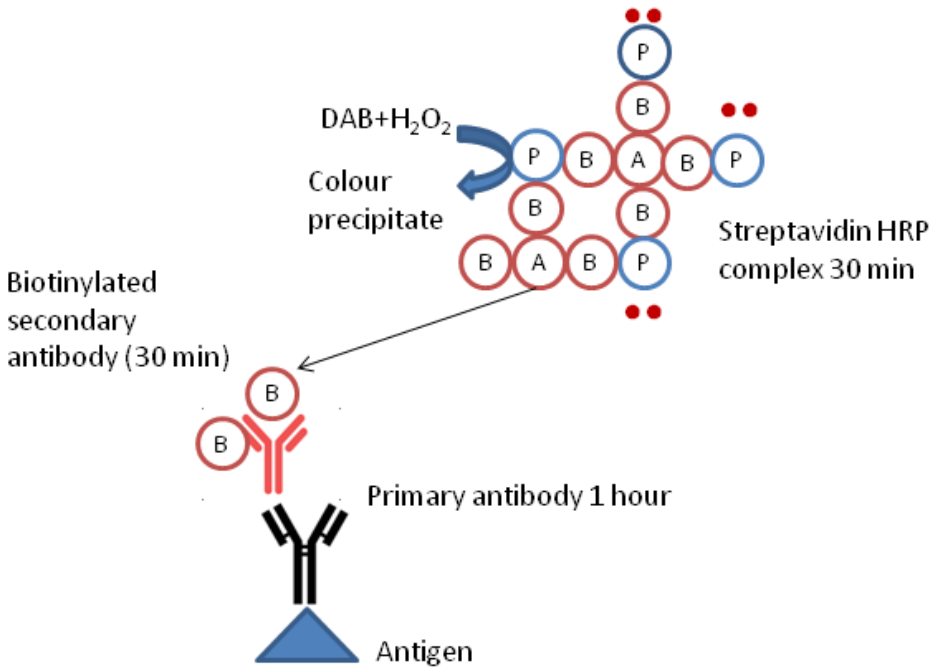


Figure 2-12. A schematic picture of the immunohistochemistry SPBC.

We were eager to confirm whether vertical transmission of DENV occurs in Banyumas Regency because many researchers have hypothesized that this mode of transmission may allow maintenance of DENV during inter-epidemic periods (period between two epidemics) (Angel & Joshi, 2008; Cruz et al., 2015; Joshi et al., 2002). We predicted that vertical transmission may take place in Banyumas Regency as DENV endemic areas are present. However, there have been no previous studies performed in this area to determine this mode of transmission and maintenance.

165 head squashes of mosquitoes from four villages were tested by immunohistochemistry. The virus infection rate (VIR) was calculated as (number of mosquitoes by species infected with dengue virus ÷ total number of that species tested) x 100. The result of immunohistochemistry assay and infection rate is shown in the table below:

Table 2-9 Immunohistochemistry (IHC) assay results of mosquito headsquashes.

DENV infection rates in mosquito headsquashes from the four villages of the study area are indicated. Positive results indicate vertical transmission. VIR: virus infection rate.

Village	DENV Status	IHC result							
		Aedes aegypti				*Aedes albopictus*			
		Positive	Negative	Total	VIR (%)	Positive	Negative	Total	VIR (%)
Tanjung	Endemic	8	31	39	20.51	3	9	12	25.00
Sokanegara	Endemic	13	15	28	46.43	11	12	23	47.83
Panusupan	Sporadic	7	27	34	20.59	0	13	13	0.00
Gunung Lurah	Free	0	6	6	0.00	1	9	10	10.00

Positive and negative results of the immunohistochemistry test can be distinguished from the image slide under a light microscope. An example of a positive and negative result is shown in the Figure 2-13.

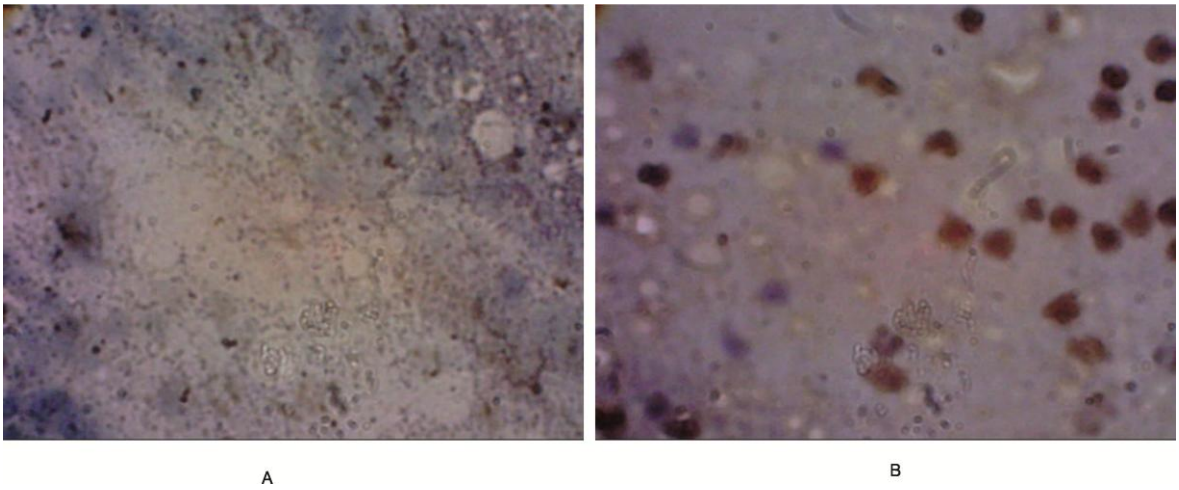


Figure 2-13 Positive (A) and negative (B) result of the DENV immunohistochemistry test of head squash samples.
(A) Picture shows a positive result for DENV, the central nervous ganglion cells in the caput stains brown in the cytoplasm, and brown granules are also surrounding the cell which indicates the mosquito is positive for antigen dengue (1000 x magnification). (B) Picture shows a negative result as the central nervous ganglion cells appear blue or pale and there are no brown granules surrounding the cells; this means the mosquito is negative for dengue antigen (1000 x magnification).

Based on IHC assay results, the vertical transmission occurs in four villages observed, both in *Aedes aegypti* and *Aedes albopictus* (exclusively in this mosquito in one specimen in the DENV free village). Sokanegara (DENV endemic) has the highest infection rate among other villages, 46.43% in *Aedes aegypti* and 47.83% in *Aedes albopictus*. Tanjung (DENV endemic) showed 20.51% infection rate in *Aedes aegypti* and 25% in *Aedes albopictus*. Panusupan (DENV sporadic) and Gunung Lurah (DENV free) were detected to have lower VIR, compared to endemic areas (VIR less than 20%). This finding support the statement that vertical transmission is important for maintenance of DENV, indicated by the high VIR in DENV endemic areas

2.3.7 Questionnaire of knowledge, awareness and practices related to dengue in endemic, sporadic and free area

Based on the questionnaire data, sociodemographic profiles of respondents from the three villages surveyed are displayed in Table 2-10.

Table 2-10 Key sociodemographic profile in three villages

Sosiodemographic variables	Tanjung	Panusupan	Gunung Lurah
Gender			
Female	70	72	79
Male	30	28	21
Average age (years)	45.23	43.57	37.61
Education			
Never go to school	1	0	0
Not finished elementary school	3	12	22
Elementary school	10	52	44
Junior high school	14	22	18
Senior high school	41	12	15
Graduate or postgraduate school	31	2	1
Job			
Not working	10	19	1
Farmer	4	13	7
Bussiness	15	13	18
Employee	6	1	2
Labour	4	16	11
Others (mainly houseviwes)	48	35	61
Economy level			
< minimum district wage	18	74	64
≥ minimum district wage	82	26	36
Water Resources			
Well	45	92	10
Pipe water	54	1	11
Others	1	7	79
House Construction			
Good	87	51	57
Bad	13	49	43

Table 1-10 shows the sociodemographic characteristics of the 300 respondents in Tanjung, Panusupan and Gunung Lurah villages. Most respondents in the three villages were female, perhaps because many women stay at home while the survey was performed while males go to work. From 100 respondents interviewed in Tanjung village, 70 were female and 30 were male with ages between 18-84 years old. 82% of respondents were of good economical status (income above minimal regency salary standard) and 31% of respondents had received a good education (diploma/bachelor degree). From these observations, it was found that 54% of water resources were obtained from a water supply company and 45% from wells. 87% of respondents lived in a house of good or appropriate construction. The characteristics of respondents in Panusupan village showed that the distribution of age was from 16 to 69 years old, with 72% of them female. 52% had an education level of primary school and 74% had an income under minimal regency salary standards. They mainly use wells (92%) as their water resources. 51% of respondent houses were of good construction but 49 % was poorly constructed. 80% of respondents in

Gunung Lurah village were female and 20% male, with the dominant education level primary school. 61% of them were housewives and 64% had an income under minimal regency salary standard. Their water resources were different from other villages, as they mainly used piped water from natural resources (79%), so they did not store water in traditional bath-tubs (water containers), buckets etc to cover their water consumption. 57 % of houses were of good quality, but 43% were poorly constructed.

Based on data analysis as explained in the methods, the result of KAP survey can be seen in Table 2-11.

Table 2-11 Knowledge, awareness and practices of dengue disease prevention in Tanjung, Panusupan and Gunung Lurah villages.

No	Variables	Tanjung		Panusupan		Gunung Lurah	
		Good (%)	Bad (%)	Good (%)	Bad (%)	Good (%)	Bad (%)
1	Knowledge of dengue disease and prevention	51	49	53	47	60	40
2	Awareness of respondent to dengue disease and prevention	66	34	68	32	52	46
3	Practices of respondent to prevent dengue disease	57	43	50	50	52	48

Based on the KAP results, more than 50% of respondents in all the three villages have a good knowledge about dengue disease and prevention. Based on the questionnaire answers, there were two questions about knowledge from total 10 questions which have many wrong answers. 96 % respondents in Tanjung did not know the symptoms of dengue disease and 95 % did not know that eradication of mosquito breeding places can be done by draining of bath-tubs, closing water containers and burying unused potential water reservoirs around the house. In Panusupan village, the respondents also did not know the symptoms of dengue (96 % give false answers) and 99% did not know that eradication of mosquito breeding places can be done by draining of bath-tubs, closing water containers and burying unused potential water reservoirs around the house. These observations also occurred in Gunung Lurah village. From these results, it can be observed that although respondents generally showed a good knowledge, but they have little knowledge particularly about the symptoms and dengue prevention effort. These results about

knowledge should be an important concern when giving counselling to focus on the symptoms and prevention of dengue disease.

Based on DENV prevention practice questionnaire results, 83% of respondents in Tanjung village do not bury or put into waste storage any unused potential water containers surrounding their house, 70 % hang up clothes and 63 % do not use long clothes to protect their body from mosquito biting. In Panusupan, 84% do not bury or put into waste storage any unused potential water containers surrounding their house and 66 % hang up clothes. In Gunung Lurah villages, 89 % do not bury or put into waste storage any unused potential water containers surrounding their house, 68% hang up clothes and 52 % do not use long clothes to protect their body from mosquito biting. The interesting thing revealed here in DENV prevention practice results is that the practice, which does not support the dengue prevention in three villages are almost the same. The majority of respondents in Tanjung, Panusupan and Gunung Lurah villages do not bury or put into waste storage any unused potential water containers surrounding their house which are a condusive places for mosquito breeding sites and also hang up the clothes behaviour (the preferences of mosquito to rest).

The correlation between knowledge and awareness to the household practices of people of preventing dengue disease is important. By chi square test, the correlation between the knowledge with household practice of people was analysed and its results shown in Table 2-12.

Table 2-12 Chi square test results of correlation of knowledge and practice prevention of dengue fever.

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.418[a]	1	.518		
Continuity Correction[b]	.269	1	.604		
Likelihood Ratio	.420	1	.517		
Fisher's Exact Test				.611	.303
Linear-by-Linear Association	.416	1	.519		
N of Valid Cases[b]	300				

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 36.54.

b. Computed only for a 2x2 table

Based on Table 2-12, there is no influence of knowledge to respondents's practice to prevent dengue disease (p-value=0.604). Analysis on correlation between awareness to

practice prevention of DENV infection also carried out, and its results shown in Table 2-13.

Table 2-13 Chi square test results of correlation of attitude and practice prevention of DENV infection.

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.340[a]	1	.560		
Continuity Correction[b]	.211	1	.646		
Likelihood Ratio	.339	1	.560		
Fisher's Exact Test				.620	.323
Linear-by-Linear Association	.339	1	.561		
N of Valid Cases[b]	300				

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 43.65.

b. Computed only for a 2x2 table

Based on the results, there is no influence of awareness to practice DENV prevention (p-value = 0. 646). So, both knowledge and awareness did not influence the practice of DENV prevention

Linking between knowledge, attitude and practice of people with the mosquito infestation is also interesting to find out. Data analysis was carried out in an attempt to find out the correlation of KAP with mosquito infestation. The analysis results can be seen in Table 2-14.

Table 2-14 Correlation between knowledge, awareness and practices in three villages with mosquito infestation.

Variables in the Equation

		B	S.E.	Wald	df	Sig.	Exp(B)	95.0% C.I.for EXP(B)	
								Lower	Upper
Step 1[a]	totalattkat(1)	.563	.341	2.717	1	.099	1.756	.899	3.428
	totalprackat(1)	.580	.384	2.287	1	.130	1.786	.842	3.789
	Constant	-1.919	.198	93.976	1	.000	.147		
Step 2[a]	totalattkat(1)	.575	.340	2.860	1	.091	1.778	.913	3.463
	Constant	-1.792	.170	110.951	1	.000	.167		

a. Variable(s) entered on step 1: totalattkat, totalprackat.

Based on the results, there is no influence of knowledge, awareness and practice with mosquito infestation (p-value = 0.091) in the three villages.

2.4 Discussion

Entomology surveys are applied by many dengue endemic countries as a way of measuring to determine mosquito populations. The result from such entomology surveys can be used as basic supporting information for prevention of DENV transmission and risk assessments (Ooi, E.-E. et al., 2006; Scott & Morrison, 2010). In Indonesia, areas with high mosquito populations will be treated with larvicides such as temephos or organophospates. The Indonesian Ministry of Health also calls for community participation to conduct routine entomology surveys, in attempt to prevent DENV transmission (Ministry of Health, 2011). For many years, traditional sampling methods i.e. larvae indices such as HI, BI and CI were applied, despite many of its limitation (Barbazan et al., 2008; Bowman et al., 2014; Focks, D. & Alexander, 2006; Focks, D. A., 2003). To assess the validity of traditional sampling methods and indices in an effort to improve the quality of local entomology surveys, we carried out traditional sampling combined with additional methods such pupae index, species identification and adult mosquito collections and also IHC to assess DENV vertical transmission.

The result from traditional sampling and indices reveal that the larvae indices are not always correlated with the number of dengue cases. Thus the risk level DENV transmission in this study area cannot be concluded from traditional larvae indices as they do not correlate with the reported dengue cases. We also found that most of the larvae indices are higher in rainy season than in dry season. This is possibly correlated with the rainfall as the containers outside the house were filled with rain water which provided breeding sites for mosquitoes. Rainfall is an important environmental factor associated with *Aedes sp* breeding site and DENV incidence (Karim et al., 2012; Waldock et al., 2013; Wee et al., 2013). This finding also suggest that health officers and the community should focus their efforts of DENV prevention at the beginning of the rainy season.

Free larvae indices/FLI (number of houses inspected without larvae (negative) per number of houses inspected) in this survey were in the range of 75-97% in the dry season and 56-84 % in the rainy season. Gunung Lurah showed the highest FLI both in dry and rainy seasons; this finding supports the status of Gunung Lurah as a free dengue area as this village shows low mosquito density and also a low risk level of DENV transmission. The free larvae index is the index generally used by the Indonesian Ministry of Health to measure the risk of dengue transmission. The target for each area in Indonesia is to have an FLI index of 95% or above. Of the four villages, only Gunung Lurah has an FLI index of

97% in dry season, indicating that the three other villages have a risk of dengue virus transmission. Meanwhile, Panusupan village (sporadic area) is considered as a high risk level transmission area and Tanjung and Sokanegara (endemic area) village are suggested to have a medium risk level of dengue transmission. Thus although these findings can provide information for health authorities and also to the heads of the villages to implement preventive measures in their area, our results also indicate that larval indices alone cannot explain local risk (Sanchez L, 2006)

We also performed pupae index-based surveys as suggested by Focks (2006), who proposed that pupae indices are better indicators to measure mosquito populations than traditional sampling (Focks, D. & Alexander, 2006). However the results of pupae indices revealed that this index also does not well correlate with the dengue cases in the area of study. Instead, we found that the adult mosquito surveys are better marker of mosquito population and DENV transmission risk. There is an interesting phenomenon in which sporadic areas and free areas also have a medium or high mosquito density. However, when we observe further the dominant adult mosquito species that is found in Panusupan village, *Culex sp*, which is not a vector of dengue virus is dominant while in Gunung Lurah Village we found very few of adult mosquitoes. We conducted species identification to complete the entomological surveys. Indeed, species identification is important procedure but rarely applied in the field, due to the need for trained staff. Routine programs in several villages in Indonesia which conducts the larvae survey every month, only observe the presence or absence of larvae but do not identify the species which makes the level of risk determined from there not reliable, by our findings. Without species identification, we would have assumed that Panusupan has a high risk DENV transmission due to the high number of mosquito, but from the species identification we confirmed that most of them are *Culex sp*, ie not vectors of DENV.

From the result of this study, we can also support the previous findings that *Aedes aegypti* and *Aedes albopictus* play key roles. *Aedes aegypti* tend to live in urban areas whereas *Aedes albopictus* prefer to live in rural areas (Focks, D. A., 2003; Higa, 2011). In Indonesia, *Aedes aegypti* is still the primary vector for dengue virus, while *Aedes. albopictus* is a secondary dengue vector (Ministry of Health, 2011). In addition, in a recent review on the vector competence differences between *Aedes aegypti* and *Aedes albopictus* it was stated that *Aedes. aegypti* has higher vectorial capacity than *Aedes albopictus*. They stated that although that the midgut of *Ae. albopictus* may be more susceptible to DENV infection than *Aedes aegypti*, but the dissemination of virus to the saliva gland is lower

than in *Aedes aegypti* (Lambrechts, L et al., 2010). Higher presence of *Aedes aegypti* than *Aedes albopictus* in endemic areas compared to sporadic and free area in this study would thus correlate with the dengue status. Vector competence of *Aedes aegypti* is higher than *Aedes. albopictus*, so the higher distribution of *Aedes aegypti* in endemic areas is likely result in better transmission of DENV and more cases as a consequence. The existence of *Culex sp* in Panusupan and Gunung Lurah Village is an interesting fact compared to endemic areas. Calderon-Arquedas et al, (2009) also identified *Culex sp* as well as *Aedes sp*. in a field study in the urban area of Greater Puntarenas, Costa Rica (Calderón-Arguedas et al., 2009). Although *Culex sp* has not been documented to transmit dengue virus it could be a vector of other pathogens. Vazeille *et al* (2003) stated that *Aedes aegypti* is the most effective vector for dengue viruses and is highly receptive to oral infection (Vazeille M, 2003). Another study suggested that infection of *Culex quinquefasciatus* by the parenteral route with dengue virus type 2, but found very low levels of replication, and the authors concluded that *Culex. quinquefasciatus* should not be considered a biological vector of dengue viruses (Vazeille-Falcoz et al., 1999).

The adult mosquito percentage results in Sokanegara Village are in accordance with previous work about the differences of distribution between *Aedes aegypti* and *Aedes albopictus* as reviewed by (Higa, 2011). That study concluded that *Aedes aegypti* prefers to live in urban areas, domestic/indoors with close contact to humans, whereas *Aedes albopictus* usually lives in rural areas, with high vegetation. the differences of distribution between *Aedes aegypti* and *Aedes albopictus* is based on the different response of these two species to a domestic environment, but changes in the environment by human activities such as urbanisation, climate change etc. also can change their distribution (Higa, 2011).

From container survey, we found that discarded tyres were the most frequent container infested with larvae (52.63%). We also found that more artificial containers were found in the four villages surveyed compared to natural containers. This finding shows that people can minimize the potential containers for breeding mosquitoes by reducing artificial containers such as traditional bath-tubs and buckets. Therefore, a program to provide clean water by pipeline is expected to reduce the number of dengue cases. Provision of clean water by pipelines will reduce mosquito breeding places and will reduce the number of dengue cases. It is also be possible to prevent other diseases such as diarrhoea, dysentery, cholera, typhoid, hepatitis, leptospirosis, malaria, scabies, chronic respiratory diseases and intestinal parasites (Ministry of Health, 2011). Schmidt et al., 2011 who analysed the

interaction between human population density and the lack of tap water suggested that improving water supply in a high human population density area could make better vector control efficiency. Better water storage systems and a reduction of these containers should reduce *Aedes sp* breeding sites (Schmidt et al., 2011).

Buckets, water storage containers and traditional bath-tubs were found to be the dominant breeding container observed in all four villages. This finding is correlated with the specific behaviour of the local people who usually store water in buckets for several days. They use it for cooking, washing dishes or boiling it to prepare drinking water. Traditional bath-tubs were the dominant container found because people mostly take a bath twice a day, and they store water inside the house to do this. This behavior is different from people who live abroad or in higher economy areas of Indonesia because mostly they use bath-tubs or showers where the water is automatically discarded after bathing. This finding is similar to the research carried out in Surabaya, Indonesia which found that the traditional bath-tub is the productive container for mosquito breeding sites found inside in house followed by the traditional water container and other water containers (Mulyatno et al., 2012). From work with Ovitrap Indices (OIs), we observed more ovitraps positive with eggs in outdoor position than indoor position. This result corresponds with the study performed in West Java, Indonesia by Syarifah *et al* (2008) which concluded that 25.18% more *Ae. aegypti* eggs were found outdoors compared to indoors. The choice by the gravid female mosquitoes to lay their eggs is adaptive and influenced by several factors. Some authors (Spencer et al., 2002) stated that female mosquitoes will seek oviposition sites that are safe from predators to enhance the survival of their offspring. Work by others (Kittayapong & Strickman, 1993) showed that several factors such as the presence of conspecific larvae or pupae, sun exposure, container size and the presence of a lid can also influence the preference of the female mosquitoes in their choice of oviposition sites. The preferences of female mosquito to lay eggs outside/outdoor should give input information about how to control the mosquito. Since most of the larvacide usually applied inside the house such as put in bath tub, bucket etc, while the fact is most of the mosquito eggs found outdoor. Application of larvacide in the container located outside the house must be considered.

The average number of eggs is about 22-44 eggs per ovitrap in this study. This finding is supported by previous studies by others (Chadee, D D. et al., 1990) who reported that mosquitoes lay around 11–30 eggs per container. Following these results for the ovitraps in our area, indices can be classified into level 3 (ovitrap index 20-40%) and level 4 (≥40%) (Food and Environmental Hygiene Department, Hongkong, 2013). Ovitrap indices are

differentiated into 4 levels and each level has suggested action. The area with OI level 3 for example is suggested to have regular weekly programs to eliminate all potential breeding places, whereas in level 4, employment of larvacides or adulticidies is recommended (Food and environmental hygiene department, Hongkong, 2013).

The headsquashes samples from all four villages tested positive for DENV by the immunohistochemistry test, indicating that vertical transmission may occurs in Banyumas Regency in both *Ae. aegypti* and *Ae. albopictus*. All four villages had larvae that when raised to adults gave DENV positive results in immunohistochemistry tests, although we did not attempt to isolate infectious virus from these mosquitoes and formal proof of vertical transmission by PCR is still required. These data suggest however that vertical transmission should be assessed further in the context of local risk assessments. Tanjung and Sokanegara villages, both DENV endemic areas both showed potentially higher positive infection rates compared to the other villages, and if further observations confirm high vertical transmission rates these could be a factor explaining why DENV is endemic in these areas as vertical transmission reportedly plays a role in viral maintenance in nature (Mulyatno et al, 2012). The data in this study show a higher percentage of vertical transmission than other previous studies (Martinez et al, 2014; Le Goff et al, 2011; Espinoza et al, 2014). However, this study used individual mosquitoes as a sample and other studies used pooled mosquitoes (several mosquitoes in one pool). Other studies looking at individual mosquitoes have indicated the possibility of higher levels of vertical transmission (Joshi et al, 1996; Cecilio et al, 2009).

Dengue case records from the Banyumas Health Office in 2013 showed that there are 15 and 9 dengue cased in Tanjung and Sokanegara, respectively. The high vertical transmission rate could be one of a number of factors explaining why dengue virus is endemic in these areas because vertical transmission plays a role in viral maintenance in nature and potential transmission to humans mainly in the rainy season (Mulyatno et al., 2012). The high infection rates in these areas may be caused by better vector competence of *Aedes aegypti* or insecticide resistance possibly caused by regular application of fogging in this area or due to environmental factors shown above. Interestingly positive result of DENV IHC were also detected in Panusupan (sporadic area) and Gunung Lurah (free area) villages although with lower virus infection rates (VIR). The occurrence of vertical transmission in Banyumas Regency is an important finding and Health Officers should be made aware of this. This transmission may explain why dengue virus persists in populations and transmission of DENV regularly occurs in endemic areas.

The importance of vertical transmission to dengue virus transmission is still debated. Several studies assumed that vertical transmission shows no clear significant correlation with dengue cases. For example, (Adam B & Boots, 2010) used a mathematical model to analyze the significance of vertical transmission and stated that this is not a crucial factor for long term virus persistence. They predicted that vertical transmission would become significant for dengue persistence when the vertical transmission rate reaches more than 20-30% in an endemic area. So, while several studies argue that vertical trasnmission does not have a significant influence on the incidence of dengue infections, others (Lee, H. & Rohani, 2005) investigated the occurrence of dengue disease and vertical transmission of DENV in *Aedes albopictus*, and found that vertical transmission of DENV happened prior to the reporting of human cases.

From the questionnaire results, there was no correlation between knowledge and attitude to practice of DENV prevention in three villages. This finding was also observed in several studies about KAP in many countries such as Jamaica, Malaysia, Thailand and Philipphines (Hairi et al., 2003; Koenraadt et al., 2006; Shuaib et al., 2010; Yboa & Labrague, 2013). This indicates that good knowledge or awareness does not necessarily lead to better practice. There is a gap between knowledge, awareness and practice which needs to be addressed for the prevention of DENV. There are many possible reasons why people who had a good knowledge score did not improve their practice on DENV prevention, such as there are no contiunous monitoring and the lack of action consistency (Yboa & Labrague, 2013). It emphasizes the need for strategies to ensure the translation of knowledge into practice. In addition, there are some important findings on details of answers in this study which showed that most of the respondents in all three villages still have a lack of knowledge of the symptoms of dengue disease and also how to eradicate mosquito breeding places. People do not bury or put into waste storage any unused potential water containers surrounding their house, hang up clothes and do not use long clothes to protect their body from mosquito biting. These findings could give advice to the prevention strategies, so that the educational campaigns could be more effective. Surprisingly, the KAP survey also does not correlate with the mosquito infestation in each village. Conflicting results between the correlation between KAP and mosquito infestations were also found in several previous studies (Anand et al., 2014; Koenraadt et al., 2006). This finding showed that there are a lot of other things that affect mosquito infestation rates in an area such as environmental factors (humidity, rainfall, temperature)(Walker et al., 2011) and socioeconomic factors (Dowling et al., 2013).

From the entomology survey, we can conclude that there are several differences in mosquito populations in endemic, sporadic and free areas, but here this is not well correlated with the dengue incidence. Mosquito populations are higher in the rainy season than dry season and discarded tyres were found to be the dominant containers which are potential mosquito breeding sites. It can be assumed that the reduction of these containers will minimize the incidence dengue of cases. *Aedes aegypti* is still considered to be the primary vector of dengue virus based on being the dominant species found in endemic areas. With vertical transmission happening in Banyumas Regency, as based on immunohistochemistry assay, this finding may explain the fact that this regency has regular dengue cases. From the spatial analysis, we found several clusters of dengue cases, and the distribution of dengue cases may be caused by human mobility, multiple host feeding characteristics of *Aedes sp* and better transportation facilities. The result of this study, suggest that additional survey such as adult mosquito collection, species identification and vertical transmission test are essential to have accurate measurements of mosquito population and DENV risk transmission. From questionaire result, there is a gap between knowledge, awareness and practice, leading to the need of effort to ensure the translation of knowledge into practice. KAP survey also does not correlate with mosquito infestations, this reflected that there are other factors such as environment, socioeconomic etc which possibly influence the mosquito infestatation in certain area.

Overall, the limitation of this research is that the entomology survey has been done only once in a season, and more in depth analysis of the dynamics of mosquito population density would benefit if surveys were conducted each month per year. However it requires more labour, time and money to do so. However we limited with time and material for the field surveys in Indonesia. Our results suggest already improve ways of correlating mosquitoes with DENV risk in this regency, and this can influence practice in Indonesia and potentially elsewhere were mosquito-based indices are applied.

Chapter 3

3 Molecular Epidemiological Study of DENV circulating in Banyumas Regency

3.1 Introduction

There have been a limited number of reports on the genetic diversity of DENV isolated in Indonesia. As an endemic DENV country with a long history of DENV transmission in almost all provinces, Indonesia still has a lack of studies focused on molecular aspects of circulating DENV. Four DENV serotypes (DENV 1, 2, 3 and 4) circulate in Indonesia and DENV-2 and DENV -3 were observed as the dominant serotypes (Karyanti et al., 2014; Sumarmo, 1993). Published studies about circulating DENV in several cities in Indonesia are summarised in Chapter 1 (Figure 1-15). The limited number of studies is mainly due to the lack of laboratory facilities, professional staff and funds. Indeed, it is important to carry out molecular studies on DENV as this virus circulates continuously in many areas of Indonesia.

Obtaining good molecular surveillance data on DENV is important to understand the transmission dynamics of this virus and support prevention and control programs. As mentioned in several studies, some DENV serotypes and genotypes can cause more severe clinical manifestations (Nisalak et al., 2003; Rico-Hesse, 2003). The first observation about differences in virulence among serotypes or genotypes came from patients with hemorrhagic disease (Barnes & Rosen, 1974; Gubler, D. J. et al., 1978). Vaughn et al (2000) found that more severe disease correlated with DENV-2 infection in Bangkok (Vaughn et al., 2000). Similar results reported DENV-2 to be a more severe serotype compared to DENV-1 infection (Fried et al., 2010). Thus, detection of the serotype and genotype could predict the severity of the clinical manifestations and be useful information for DENV prevention strategies.

An association between secondary infection with different serotypes of DENV and more severe clinical manifestations has also been documented in several publications (Guzman, M. G. et al., 2013; Suwandono et al., 2006; Thomas et al., 2008; Vaughn et al., 2000). This phenomenon could be predicted by having a good database of existing local DENV serotypes in patients. To date, DENV surveillance and diagnosis in Indonesia generally still relies on serology tests such as enzyme-linked immunosorbent assay (ELISA). This assay is easy to use and inexpensive but is unable to determine DENV serotypes, particularly after secondary infections because antibodies formed in this type of infection

are cross-reactive within other serotypes and also other flaviviruses (Innis et al., 1989; Peeling et al., 2010). Other flavivirus infection known to occur in Indonesia are Japanese encephalitis (Liu et al., 2010) and Zika virus (Kwong et al., 2013). Indeed, it is important to have good molecular DENV data based on more reliable assays such as Polymerase Chain Reaction (PCR), and therefore more accurate epidemiology DENV data could be available in time.

Evolution of DENV by increasing genetic diversity within each serotype is also observed due to the error-prone nature of the viral RNA-dependent RNA polymerase (Bennett et al., 2006; Foster et al., 2003; Klungthong et al., 2004). This genetic change could impact on DENV virulence and epidemiology of DENV transmission. Based on complete E gene sequences, there are five genotypes of DENV-1 (Goncalvez et al., 2002; Rico-Hesse, 1990); five genotypes of DENV-2 (Lewis et al., 1993); four genotypes of DENV-3 (Lanciotti et al., 1994) and also four genotypes of DENV-4 (AbuBakar et al., 2002; Klungthong et al., 2004; Lanciotti et al., 1997). Molecular surveillance of genotype diversity provides the possibility to track the geographic origin of DENV and provides an opportunity to analyse the route of DENV transmission across time and place. Several publications also hypothesized that certain serotypes or genotypes are displaced by a more virulent strain associated with a dengue outbreak. In Myanmar, there is displacement of DENV-2, -3 and -4 by DENV1 which correlated with a dengue outbreak in 2011 (Thu et al., 2004). Yamanaka et al (2011) also reported an increase of about three times the number of dengue cases in Surabaya, Indonesia after displacement of the predominant DENV from type 2 to type 1 (Yamanaka et al., 2011). Other studies also documented the displacement of serotypes, indicating this phenomenon occurs regularly (Balmaseda et al., 1999; Li, D.-s. et al., 2010; Rico-Hesse et al., 1997; Usuku et al., 2001). The genotype evolution of DENV could cause phenotypic changes in the viruses, increasing their potential to cause a dengue outbreak (Grenfell et al., 2004). Therefore, conducting molecular studies on DENV will provide information which could lead to a better understanding of pathogenesis, outbreak potential and epidemiology of DENV transmission.

Most studies on molecular DENV in Indonesia were carried out in important cities such as Jakarta, Semarang and Surabaya (Fahri et al., 2013; Suwandono et al., 2006; Yamanaka et al., 2011). This is possibly because most dengue outbreaks occur in major cities (urban), relating to high human populations, poor sanitation, and high mobility (Karyanti et al., 2014). However, there is a need to do more molecular DENV research across Indonesia, since DENV is spread through almost every province. Banyumas Regency, the area of this

study, experienced regular DENV tranmission. Based on surveillance data from Banyumas Health Office from 2000-2013, DENV cases tend to show significant increase since 2000, with outbreak notified in 2008 and 2010. 57 of 331 villages in this regency are categorized as DENV endemic villages (an area that has regularly reported DENV cases in the last three years), while 156 are DENV sporadic villages (an area which has had an irregular number of DENV cases reported in the last three years), and the rest are free areas (an area that has had no reported DENV cases in the last three years) (Regency, 2013). Despite the annual dengue incidence in Banyumas Regency, there is no previous molecular DENV data available. In this study, I collected patient serum samples from the local government hospital (RS Margono Soekarjo) in Purwokerto, Banyumas Regency in order to obtain molecular serotyping and genotyping data on DENV.

3.2 Methods

3.2.1 Ethical approval and research authorization

Ethical approval was obtained from both Indonesia and Glasgow. Ethical approval from Indonesia was accepted from Medical and Health Research Ethics Committee (MHREC) Faculty of Medicine Gadjah Mada University (Number KE/FK/ 323 /EC), while in Glasgow, ethical approval was achieved from MVLS College Ethics Committee University of Glasgow (Project No 2012082). A letter of permission to conduct research was also accepted from the Research committee of Margono Soekarjo Hospital, Purwokerto (Number 420/13054/VI/2012).

3.2.2 Informed consent

Informed consent from the patients or their representative (for children) was taken before sample collections. An informed consent form was included in the application request to Ethical commitees in both Indonesia and Glasgow. Detail of informed consent can be seen in Appendix 2.

3.2.3 Serum patient collection

Serum samples were collected in Margono Soekarjo Hospital under the supervision of the head of laboratory (Ms. Subowati Amk). Samples were obtained from patients suspected to have a DENV infection by medical assesment. 5 ml blood samples were taken by a nurse and collected in a 5 ml BD EDTA vacutainer tube. The blood samples were centrifuged to

separate the serum and cells at 1600 g for 15 minutes and stored at -20°C. The sample collection was carried out from 17 July 2012-25 May 2013.

3.2.4 Serology test

A rapid dengue IgG/IgM (PT Fokus Diagnostik kit) test based on immunochromatography assay was performed as routine diagnosis for dengue in Margono Soekarjo Hospital on each sample.

3.2.5 Patient data collection

Patient data such as gender, age, address and also their serology results were collected from the Medical Record section of RS Margono Soekarjo Hospital.

3.2.6 RNA Extraction

RNA extraction was performed using the High Pure Viral Nucleic Acid Kit (Roche) following manufacturer's protocol. This work was carried out in Microbiology department, University of Gadjah Mada, Indonesia

3.2.7 Material Transfer Agreement

The RNA extraction was conducted in Indonesia, then the extracted products were sent to Glasgow. In order to obtain a Material Transfer Agreement (MTA), several documents were required such as Memorandum of Understanding (MoU) between my previous institution, Faculty of Medicine and Health Sciences, University of Jenderal Soedirman and The University Court of the University of Glasgow. A MoU between these institution was signed on 3 October 2013. The review and decision process for the MTA application took a long time, until the letter of permission for receiving delivery of the samples in Glasgow was obtained from Ministry of Health, Indonesia (LB.02.02/I.5.I/8160/2014) on 5 August 2014. Samples arrived in Glasgow on 29 September 2014.

3.2.8 DENV Serotyping

Complementary DNA (cDNA) synthesis was carried out using SuperScript III reverse transcriptase (Invitrogen) following the manufacturer's procedure. Primer D2 was used for reverse transcription. The primers and procedure were conducted following the work by

Wijayanti et al (2006), corresponding to the C/prM region of DENV (Wijayanti et al., 2006). The PCR reaction used combinations of primers designed by Lanciotti et al (1991) and multiplex nested PCR by Harris et al (1998), D1 and D2 were used for identifying flavivirus infection (dengue, St.Louis encephalitis and West Nile virus), while TS1, TS2, TS3 and DEN4 were used for DENV serotyping (Harris et al., 1998; Lanciotti et al., 1992). Details of the primers can be seen in Table 3-1.

Table 3-1 Nucleotide sequence of primers

Primer	Nucleotides sequences	PCR Program
D1	5' TCA ATA TGC TGA AAC GCG CGA GAA ACC 3'	First PCR : 95°C for 2 min, followed by 35 amplification cycles of 95 °C for 30sec, 55°C for 30 sec and 72°C for 45sec min and final extension 72°C for 7 min
D2	5' TTG CAC CAA CAG TCA ATG TCT TCA GCT TC 3'	
TS1	5' CGT CTC AGT GAT CCG GGG G 3'	
TS2	5' CGC CAC AAG GGC CAT GAA CAG 3'	Second PCR :95ºC for 2 min, followed by 20 amplification cycles of 94ºC for 30 sec, 62ºC for 30 sec and 72ºC for 45 sec min, with a final extension at 72ºC for 7 min. Template for the second PCR used the dilution of first PCR product (1/10)
TS3	5' TTA CAT CAT CAT GAG ACA GAG C 3'	
DEN4	5' TGT TGT CTT AAA CAA GAG AGG TC 3'	

The PCR reaction were done on the samples using the primers and the program in Table 3-1, The PCR products were then analysed on a 1% agarose gel run in 1x TAE buffer. The expected sizes for RT-PCR products using consensus primers D1 and D2 is 511 bp, and for the multiplex nested PCR amplification products using the specific oligonucleotide primers TS1, TS2, TS3 and DEN4 are 482 bp (DENV-1), 119 bp (DENV-2), 290 bp (DENV-3), and 389 bp (DENV-4).

3.2.9 DENV Genotyping

Genotyping was performed based on Envelope (E) gene sequence (Fahri et al., 2013). Genotyping based E gene is most widely used by many studies of phylogenetic analysis (Klungthong et al., 2008; Luo et al., 2013; Ong et al., 2008).

3.3 Results

3.3.1 Sample Collection Results

To investigate the prevalence of DENV in the chosen study area serum samples were obtained with permission to analyse the serotype and genotype of the infecting DENV. 156 serum samples were collected during the sample collection period in Figure 3-1, a graph represent the number of reported DENV cases monthly. There is fluctuation of cases with the peak in January and April, during the rainy season in Indonesia.

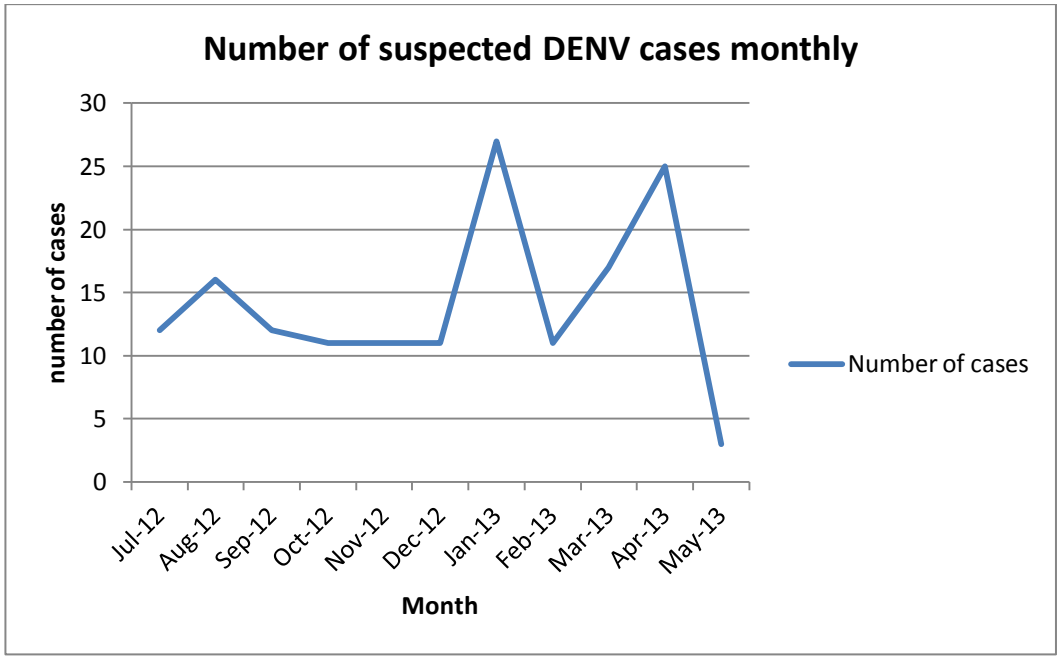


Figure 3-1 Number of suspected DENV cases monthly from July 2012 to May 2013.
The blue line represent the fluctuation of DENV cases number throughout the period of samples collection in Margono Soekarjo Hospital.

3.3.2 Patient data based on gender and sex

Based on the medical records, the dominant age group among the suspected dengue patients is between 30-44 years old (32.7%), and the lowest number of patients is in the over 75 years age group (3.8%). The number of patients within in each age group is illustrated in Figure 3-2.

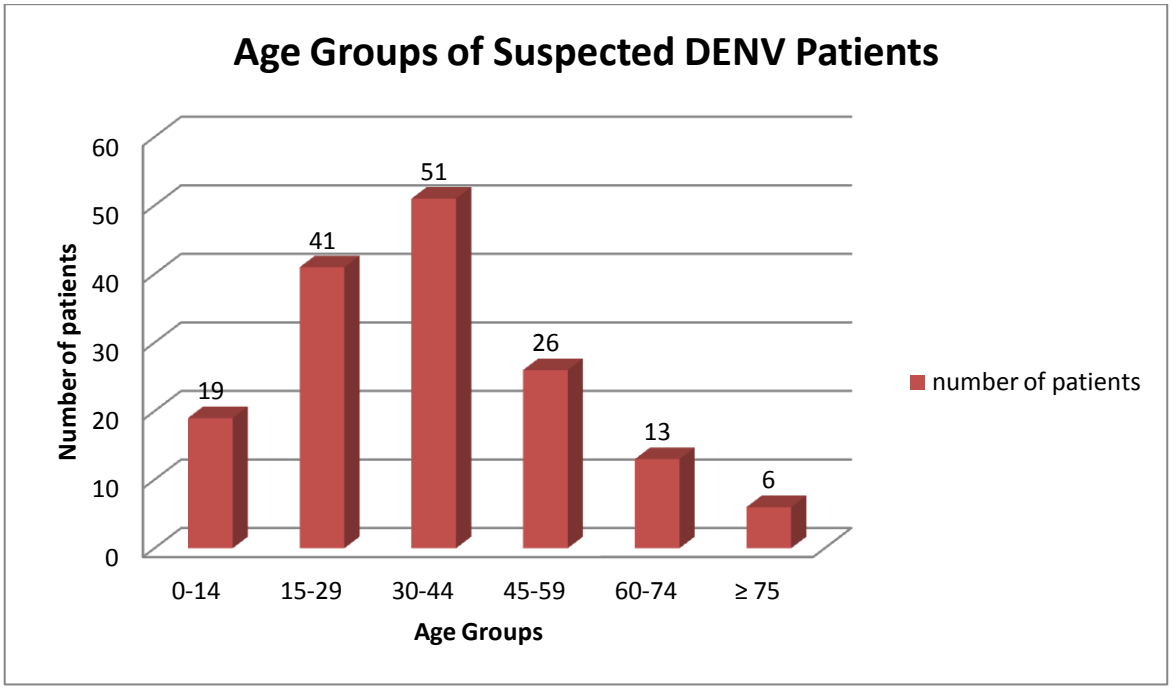


Figure 3-2 Age group distribution of suspected DENV patients.
The bar chart represents the total number of patients in each age group.

Based on gender, 56% of suspected dengue patients are female, while the remaining 44% are male, as shown in Figure 3-3.

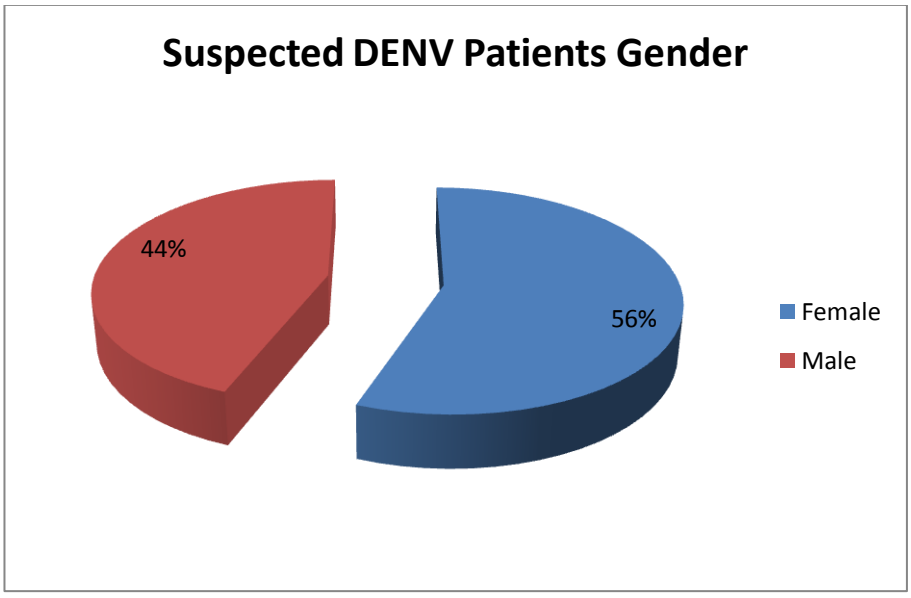


Figure 3-3 Gender distribution of DENV patients.
The percentage of female patients is represented in blue and the percentage of male patients is represented in red.

3.3.3 Serological data for DENV infection

In order to determine if the patients were infected with DENV and whether it is a primary or secondary infection a rapid test dengue IgG/IgM check device (PT Fokus Diagnostik kit) based on an immunochromatography assay was performed. The aim of this test is to differentiate between primary and secondary infections based on Immunoglobulin G (IgG) and Immunoglobulin M (IgM) responses. People who have never had a previous DENV infection (primary infection) respond to infection by becoming IgM positive, usually at low antibody titres. However people with a secondary infection (had previous DENV infection) will show high titres of IgG in the acute phase of infection, and the IgM response is usually lower (CDC, 2010; Peeling et al., 2010). The benefits of this test are to monitor DENV infection and also to predict the risk of developing the severe form of DENV. The results from the serological test are shown in Table 1-2. Our results indicated that more than half of the samples were found to have no recent DENV infection (58%). The total number of positive samples, 89% of patients had a recent secondary infection. However, there is a possibility of false negative and false positive results of the serology test. False negative can be the result of early sample collection ie too early in the infection, and also the low response of IgM in secondary infection, while false positives can happened due to the cross-reactivity between flaviviruses (Hanna, 2015).

Table 3-2 Dengue serological result.
(n =156 patient). IgM : Immunoglobulin M; IgG =Immunoglobulin G; (+) : reactive; (-) : non reactive

Serology results	male	female	Total	%male	%female	Interpretation
IgG positive and Ig M negative	20	23	43	46.5	53.5	Secondary infection
IgG negative and IgM positive	2	5	7	28.6	71.4	Primary infection
IgG positive and IgM positive	10	6	16	62.5	37.5	Secondary infection
IgG negative and IgM negative	37	53	90	41.1	58.9	No recent infection
Total samples	69	87	156	44.2	55.8	

3.3.4 DENV serotyping

To determine which DENV serotype was prevalent in the DENV positive patient samples, RT-PCR serotyping was carried out using the method described previously by (Wijayanti et al., 2006). 21 of 156 samples were tested by nested PCR using primers as described in Table 1-1. Unfortunately, all tested samples returned no positive bands by gel

electrophoresis, indicating the samples were negative DENV RNA. A representative image of PCR analysis of 5 samples is shown in Figure 3-4.

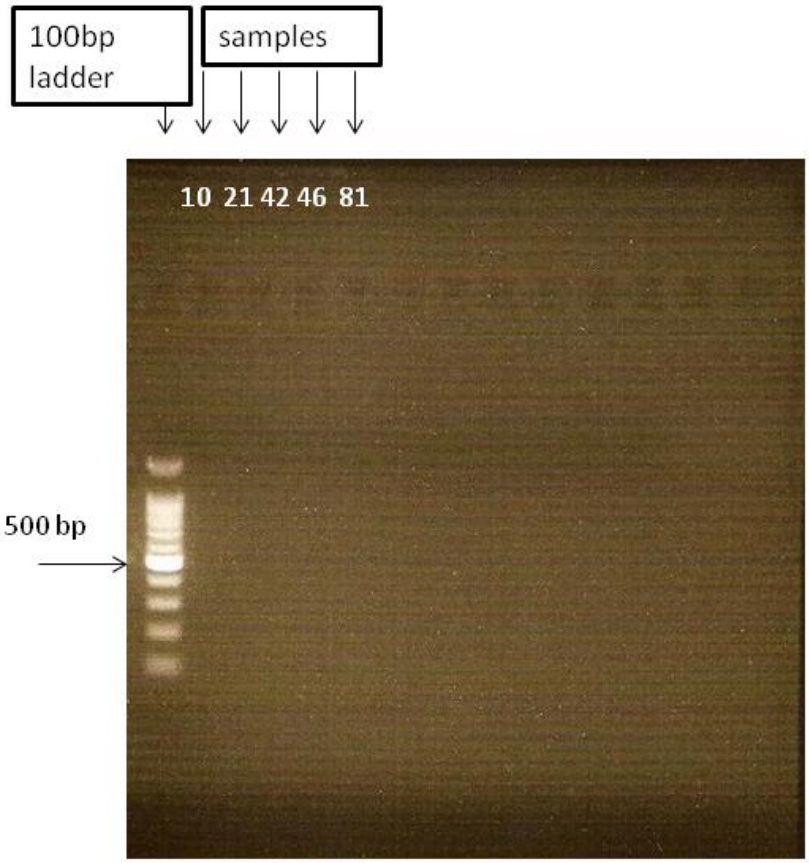


Figure 3-4 Serotyping nested PCR from DENV detection in samples 10, 21, 42, 46 and 81. Patients samples were analysed for the presence of DENV RNA by nested RT-PCR and products electrophoresed on a 1% agarose gel. Lane1: 100bp ladder (Promega) Lane 2: sample 10; Lane 3: sample 21; Lane 4: sample 42; Lane 5: sample 46; Lane 6: sample 81.

A DENV-2 positive control RNA sample from Dr. Andrew Davidson (University of Bristol) was obtained, however the sequence of this virus contains too many mismatches in the C/PrM region for the primers to bind, therefore it cannot be used as a positive control. Therefore, due to the time constraint of this study, the rest of the samples were not processed for serotyping and genotyping.

3.4 Discussion

It is critically important to monitor circulating DENV in Indonesia in order to establish a useful molecular DENV database. Limited research has resulted in a lack of information regarding the molecular epidemiology of DENV in Indonesia. A good molecular database

of DENV is undoubtedly important to monitor DENV infection, track the spread of DENV strains and support prevention and control programs. In this study, 156 patient samples were collected from Margono Soekarjo Hospital, Purwokerto in order to analyse the circulating DENV serotypes and genotypes in that area.

Based on the age grouping analysis (Figure 1-1), adult patients in the 30-44 age range are the majority of suspected dengue patients during the period of sample collection (32.7%), and the least number of suspected cases were in patients over 75 years (3.8%). These results are in agreement with the general age shift pattern of dengue patients which is observed in Indonesia and other DENV endemic countries in South-East Asia with adults becoming the predominant age group (Cummings et al., 2009; Gupta et al., 2006; Karyanti et al., 2014; Kittigul et al., 2007; Setiati et al., 2006; Sirisena & Noordeen, 2014; Tantawichien, 2012; Thai, K. T. D. et al., 2011). Historically DENV infection is predominantly detected in children, however since the beginning of 1980s, several studies observed more dengue cases in older age groups (Guzman, M. G. et al., 1990; Ooi, E. E. et al., 2001; Rigau-Perez et al., 2001). In Indonesia, an increase in the number of adult dengue patients was observed for the first time in surveillance data from 1975 to 1984 (Sumarmo, 1987). Higher activity and mobility in adults than children or older age groups, could possibly explain this pattern change (Barmak et al., 2011; Reiner Jr et al., 2014). Human movement is also believed to play an imporant role in the spread of DENV (Stoddard et al., 2009; Teurlai et al., 2012), since adults tend to be more active in work, travel, and social activities, thus their risk is higher than for children. Less reported dengue cases in children who spent most their time at home may indicating that it could be a change of location where DENV is acquired, for instance in work places or public areas (Ooi, E. E. et al., 2001). In addition, demographic changes such as birth and death rates may contribute to the shifting age pattern of dengue cases (Cummings et al., 2009). This shifting age pattern must be considered by respective health offices in their planning on DENV prevention strategies. Since more adults are observed in reported dengue cases, education about the clinical signs of dengue and when to seek professional health care in adult age group must be focused on this population. In addition, prevention and vector control program also should cover working areas or public areas, not only houses (Karyanti et al., 2014).

Based on the gender proportion results, the number of female patients (56%) is slightly higher than male (44%) patients. This is also represent by the higher proportion of female who had primary infections (71.4%), and secondary infections (IgG positive, IgM

negative) by 53.5%. The reported DENV cases in females is in accordance with Whitehorn and Simmons (2011) who stated that more females reported DENV infections than males. This may be because of differences in health care seeking behaviour, physiology or immunological differences (Whitehorn & Simmons, 2011). Indeed a study conducted by Azami et al (2011) about epidemic dengue in Malaysia also stated the that gender and ethnicity did not influence dengue infection rates in that area (Azami et al., 2011). Several studies in South America also resulted no significant difference between male and female on reported dengue cases. (Garcia-Rivera & Rigau-Perez, 2003; Gunther et al., 2009; Trravassos da Rosa et al., 2000). The result of this study is also in contrast with other studies of male-female differences in the number of reported dengue fever cases in six Asian countries (The Lao People's Democratic Republic, the Philippines, Singapore, Sri Langka, Malaysia, Cambodia) which found a consistent pattern of male predominance among persons 15 years or older (Anker & Arima, 2011). However, the small number of samples (156) in this study is one possible explanation of this discrepancy, and it would require more samples to draw general conclusions about gender and DENV cases in Banyumas over the long term.

The DENV serological results illustrate that most patients had secondary infection (89.4%), while only a small proportion (10.6%) had a primary infection. Correlation between secondary infections by different DENV serotypes and development of more severe clinical manifestations was documented in several publications (Guzman, M. G. et al., 2013; Rothman, 2010; Suwandono et al., 2006; Thomas et al., 2008; Whitehorn & Simmons, 2011; Yamanaka et al., 2011). Antibody-dependent enhancement (ADE) has been hypothesized as an explanation of the correlation between secondary infection and severe dengue disease (Flipse et al., 2013; Guzman, M. G. et al., 2013; Halstead, S B., 2014). Unfortunately, confirmation of the secondary infection by certain serotypes leading to more severe DENV infection cannot be conducted since no previous DENV serotype data are available but this study sets a baseline. There is acorrelation finding between the age group shift of reported DENV cases from childreen to adult patients and the fact of higher secondary infection, because secondary infection usually happened in more middle-age people because it acquires time between first infection and next infection.

. The absence of good positive controls makes it difficult to assess the success of the PCR. If this part of my work can be completed, it would provide a lot of information regarding the circulating serotypes and genotypes in Banyumas Regency. It would lay the foundation for a DENV database in Banyumas Regency since there is no previous information about

serotypes and genotypes for future work. In addition, phylogenetic analysis based on genotyping results could show the geographical movement and divergence of DENV.

Chapter 4

4 Spatial Analysis of Risk Factors for Dengue Transmission in Banyumas Regency.

4.1 Introduction

The complexity of various factors related to DENV transmisson such as human population, DENV evolution, mosquito population, human mobility, urbanisation, socioeconomic factors, demography and climate change lead to the need for a type of analysis which is able to cover many variables in an integrated manor. Spatial analysis has been widely used to analyze the factors associated with DENV infection. The rapid development of spatial analysis is mainly enhanched by more sophisticated applications of geographic information systems (GIS) and mathematic modelling (Goodchild & Haining, 2015; Siettos & Russo, 2013). Currently, many approaches are available to perform spatial analysis which provides more opportunities to solve problems in infectious diseases. General categories and the many approaches for mathematic modelling are summarised in Chapter 1 (Figure 1-18). In the context of DENV infection, the application of spatial analysis helps to understand the relationship between vectors, humans and DENV cases in space and time, the risk factors related to the incidence and outbreaks and also predicts the area with high risk of DENV infection (Eisen & Eisen, 2011). Information gathered from spatial analysis studies are undoubtedly important for DENV prevention and control strategies.

Spatial analysis studies on risk factors of DENV infection have been done by analyzing several variables proposed to influence dengue occurrence. Louis et al (2014) reviewed 26 studies on dengue risk mapping and grouped the studies into four categories based on the different types of variables the predictor used: population, demographic and socioeconomic, climatic and environmental, as well as entomological variables (Louis et al., 2014). Several spatial analysis studies proposed that weather and climate including temperature, precipitation and humidity was the most important risk factor for DENV occurrence (Gharbi et al., 2011; Hii et al., 2009; Hii et al., 2012; Morin et al., 2013; Thai, K. T. D. et al., 2010; Yang et al., 2009). However, other studies identified demography and socio-economic changes, human behaviour and population as being the main driving forces behind DENV occurrence and outbreaks (Costa, J. V. et al., 2013; Ibarra et al., 2014; Mondini & Chiaravalloti-Neto, 2008; Teixeira & Cruz, 2011). Therefore, there are different results observed in various countries or local areas with different variables and

predictors. We conclude that unique characteristics of the environment, demography, economy and other variable predictors in each region lead to the different results on DENV risk factors. Therefore, it is important to conduct a spatial analysis study on the DENV risk factors locally in order to obtain a better perspective on what the main risk factors are in a certain area, and help advise prevention and control strategies.

Several approaches for spatial analysis have been used to analyse DENV risk factors. Naish et al (2014) outlined several approaches that have been used to determine the relationship between variables such as temperature, climate and dengue for example cross correlations, logistics and multivariate regression, seasonal auto-regressive integrated moving average (SARIMA) time series and wavelet time series (Naish et al., 2014). Besides those approaches, many publications used Bayesian analysis as a base of their method to conduct spatial analysis related to DENV (Bhattacharjee & Bhattacharjee, 2011; Costa, J. V. et al., 2013; Costa, R. et al., 2012; Feldstein et al., 2015; Pan-ngum et al., 2013). The main benefit of using a Bayesian analysis is its ability to incorporate information from previous studies and to control for both known and unknown confounding factors, and it is also easy and flexible in computational analysis (Dunson, 2001). One of the recent developments of Bayesian analysis is Integrated nested Laplace approximation (INLA). This approach has enchanced Bayesian analysis by its ability to analyse complex random effects in a short time without loss of accuracy (Blangiardo M. et al., 2013; Martino S., 2010).

DENV risk factors in Indonesia are still poorly understood, since there are only a limited number of studies available. From the review of the 26 DENV risk factor studies carried out by Louis et al (2014), there are no studies from Indonesia. It is important to conduct spatial analysis on DENV risk factors to determine most significant factor relating to DENV infection, so then prevention and control programs can be applied efficiently. In this study, long term analysis based on the dengue cases in Banyumas regency during 2000-2013 was conducted using demographic, socioeconomic and environmental variables to determine the most influential factors related to DENV transmission. INLA appoaches were applied to generate two models, spatial only and spatial-temporal models to determine risk factors for DENV transmission in Banyumas Regency. Based on my knowledge, this study provides the first in depth analysis of DENV risk factors by spatio and spatio-temporal models in Indonesia. The information from this study will have a direct benefit in understanding the drivers of local dengue risk, thus it will help the local DENV prevention and control program.

4.2 Methods

4.2.1 Dengue cases data collection

The dengue cases data used in this study is based on data available from Banyumas Regency Health office, from January 1st 2000 to December 31st 2013. This data recorded all hospital-reported dengue cases in Banyumas Regency as part of the routine dengue surveillance in this regency. There is no distinction between DF, DHF and DSS; all reported cases in the hospital are categorized as dengue cases.

4.2.2 Population and socioeconomic data

Population data for 329 villages in the Banyumas Regency was obtained from the Indonesian census conducted by Central Bureau of Statistics of the Republic of Indonesia, carried out in 2010. National population data is not available every year, since the census is carried out every 10 years. The latest census data, carried out in 2010, was used with the assumption that the population at risk remained stable during the study period. To cover the potential bias in the risk population, land cover data for the year 2000 and 2010 was extracted from the South Asian Centre for Remote Imaging, Sensing and Processing (CRISP) of the National University of Singapore (http://www.eorc.jaxa.jp/SAFE/LC_MAP/) (CRISP, 2011).

Several descriptors (n=53) in each village was gathered from the census data such as the age structure, working status and education level of the population. To simplify a large number of data, reduction of variables has been carried out. Categorization of gender, employment type, age-group and education level were carried out as can be seen in Table 4-1.

Table 4-1 Categorization on socio-economy factors.
* variables transformed prior to analysis

Variable	Description
Female*	Proportion of females
Employment1*	Employment: Agriculture / Livestock / fisheries. Includes the following: working in agriculture (farmer), horticulture, plantations, fisheries, livestock, forestry and other agriculture.
Employment2*	Employment: Industry related. Includes working in mining and quarrying, processing industry, electricity and gas and construction.
Employment3*	Employment: Business related. Includes working in trade, hotels and restaurants, transportation and warehousing, information and communication and finances and insurances.
Employment4*	Employment: Public / Civil Servant. Includes working in educational services, health services, social services and other (such as real estate, water providers, etc.)
Age1	Age 0 to 5
Age2	Age 6 to 16
Age3*	Age 17 to 25
Age4	Age 26 to 35
Age5	Age > 36 including up to age 98.
Education1	Little / No Education: Includes responses never going to school and not finished / not yet finished elementary school
Education2	General Education: finished elementary, junior high or high school
Education3*	Higher Education: Vocational school, diploma, bachelor or post graduate degree

Because these variables have a strong correlation with each other, principal component analysis (PCA) has been carried out to summarize the socio-economic conditions in the Banyumas Regency into few composite variables, also named axis. A similar procedure was adopted in previous research (Antony & Rao, 2007; Hightower, 1978; Sekhar et al., 1991) (Principal components analysis was run on the data matrix using PC-ORD software version 6.03 (MJM software Design, Gleneden Beach, OR). The final set of components was determined using stopping procedures developed by Peres-Neto et al., 2005.

Two significant axes were observed could explain a total of 60.2% the variation of data, the first axis explains 42.2% and the second explains 18.2% of the variation. The axes are standardised between 0 and 100 for the purpose of analysis using the formula:

$$sPCA = \frac{PCA - Min\ PCA}{Max\ PCA - Min\ PCA} \times 100$$

where PCA is the axis score, sPCA is its standardised value and, minPCA and maxPCA are the minimum and maximum value of the axis score. The first principal component axis (sPCA1) provides information regarding the structure in employment type and education level in each village. A value near 0 informs on villages where people are more likely to have little to no education and are employed in the employment categories 1&2, whereas 100 indicates villages where people are more likely to be better educated, and are employed in the employment categories 3&4. The second principal component axis (sPCA2) provides information regarding the age structure in each village. A value near 0 informs on villages with high proportion of retired people, whereas 100 indicates villages with high proportion of working families.

4.2.3 Distance to hospital

To cover the accesibility of health care (hospital, health centre), the distance to the nearest hospital was measured by computing the shortest Euclidean (shortest line) distance from the centroid of each village. Distance to hospital is included in the analysis based on assumption that the longer distance to hospitals will increase the under-reporting of dengue cases.

4.2.4 Environmental data

Several variables of environmental data were collected from various sources. Elevation covering the whole of the regency was extracted from a Digital Elevation Model (DEM) freely available from Consultative Group on International Agricultural Research-Consortium for Spatial Information at http://www.cgiar-csi.org/data/srtm-90m-digital-elevation-database-v4-1 (CGIARCSI, 2015). The mean altitude was computed for each village by averaging all values provided by the DEM that were encompassed by the village's administrative boundaries.

A similar procedure was carried out for summarising all environmental information for each villages. Precipitation data covering the whole of the regency was extracted from 30 arc-seconds resolution WorldClim data tile 39 freely available at http://www.worldclim.org/. The vegetation index was sourced from the Moderate Resolution Imaging Spectroradiometer (MODIS) website (http://modis.gsfc.nasa.gov/). In

this study, we used the enhanced vegetation index (EVI) data, which provides an 'optimized' vegetation index designed to enhance the vegetation signal with improved sensitivity in high biomass regions and a reduction of both the canopy and atmosphere influences.

For EVI, all available spatial data, i.e. between February 2000 and December 2013, were extracted from the product Terra MOD13Q1 Version-5, totalling 319 maps for each variable (https://lpdaac.usgs.gov/products/modis_products_table/mod13q1). Spatial data on Land Surface Temperature daytime (LST) and nLST (night-time temperatures) were collected from the Moderate Resolution Imaging spectroradiometer (MODIS) website (http://modis.gsfc.nasa.gov/). LST data for the period March 2000 to December 2013 were available (634 maps) and downloaded from the product Terra V5 MOD11A2 version 5 (https://lpdaac.usgs.gov/products/modis_products_table/mod11a2). MOD11A2 is comprised of day-time and night-time LSTs. Consequently, all variables were computed for both situations. The LST and nLST data including average mean, average maximum, average minimum and disturbance rate for both whole year, dry season and wet season for all remote sensing information (GIS) in the models

4.2.5 Statistical analysis

To analyse the factors influencing the number of people reporting DENV infection in Banyumas Regency from 2000 to 2013, a Bayesian Poisson geostatistical analysis was conducted. The unit analysis for this study is based on village level. Integrated nested Laplace approximations (INLA) was used to do fast approximate Bayesian inference (Rue et al., 2009). Analyses were carried out in R (version 3.1.1) and the INLA package. Two models have been developed, Model 1 is a purely spatial model developed upon the cumulative number of cases recorded during the study period per village. This model determines the correlation between the number of dengue cases in each village and analyses several variables which are hypothesized to influence DENV transmission throughout the period of interest. Model 2 is a spatio-temporal model developed upon the number of cases recorded per year in each village. Aim of the Model 2 is to test the resilience of the correlation by considering both temporal and spatio-temporal structures. The implementation of spatial and spatio-temporal geostatistical analysis using INLA following the work of Blangiardo et al (2013) (Blangiardo M. et al., 2013). The analysis was conducted by Margo Chase Topping and Thibaud Porphyre, University of Edinburgh.

4.3 Results

4.3.1 Spatial Description of DENV cases in Banyumas Regency

Based on data obtained from Banyumas Regency Health office 2000-2013, DENV cases occured every year with fluctuations in the number of cases. Outbreaks were observed in 2008 and 2010. The total number of reported DENV cases each year in Banyumas Regency throughout the study period can be seen in Figure 4-1.

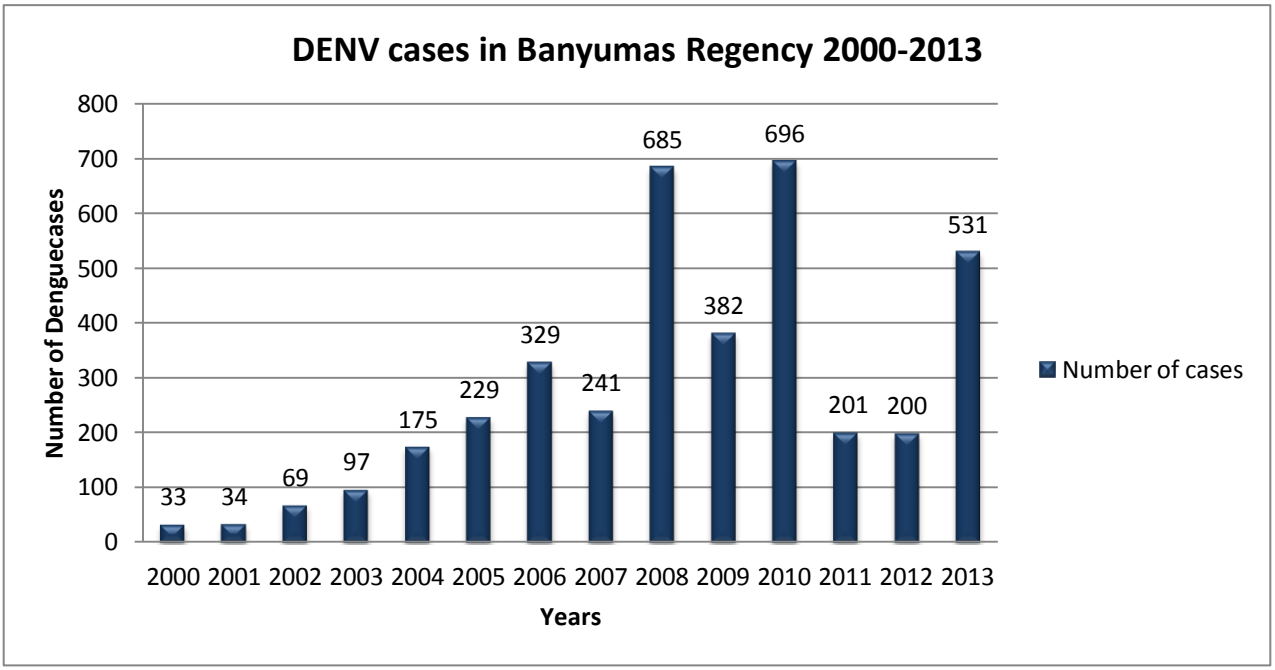


Figure 4-1 Total number of reported DENV cases per year in Banyumas Regency from 2000-2013.
Dark blue bars show the number of reported DENV cases every year in Banyumas Regency.

A map of the distribution of the reported dengue cases throughout the regency was created from 2000-2012. It is obvious that there is a clustering of reported dengue cases in the regency. The endemic areas are mostly located in urbanised zones, with a clustering of cases in Purwokerto city, the capital city of Banyumas Regency (shown in area where most dots observed in Figure 4-2). The dengue-free areas tend to locate to the fringe areas of this regency, of which most of them are rural. It is also apparent that most dengue cases occur in lower altitude, although a small number of cases also occurred at high altitude. This distribution is also possibly influenced by the distance with the hospital as can be seen in Figure 4-2.

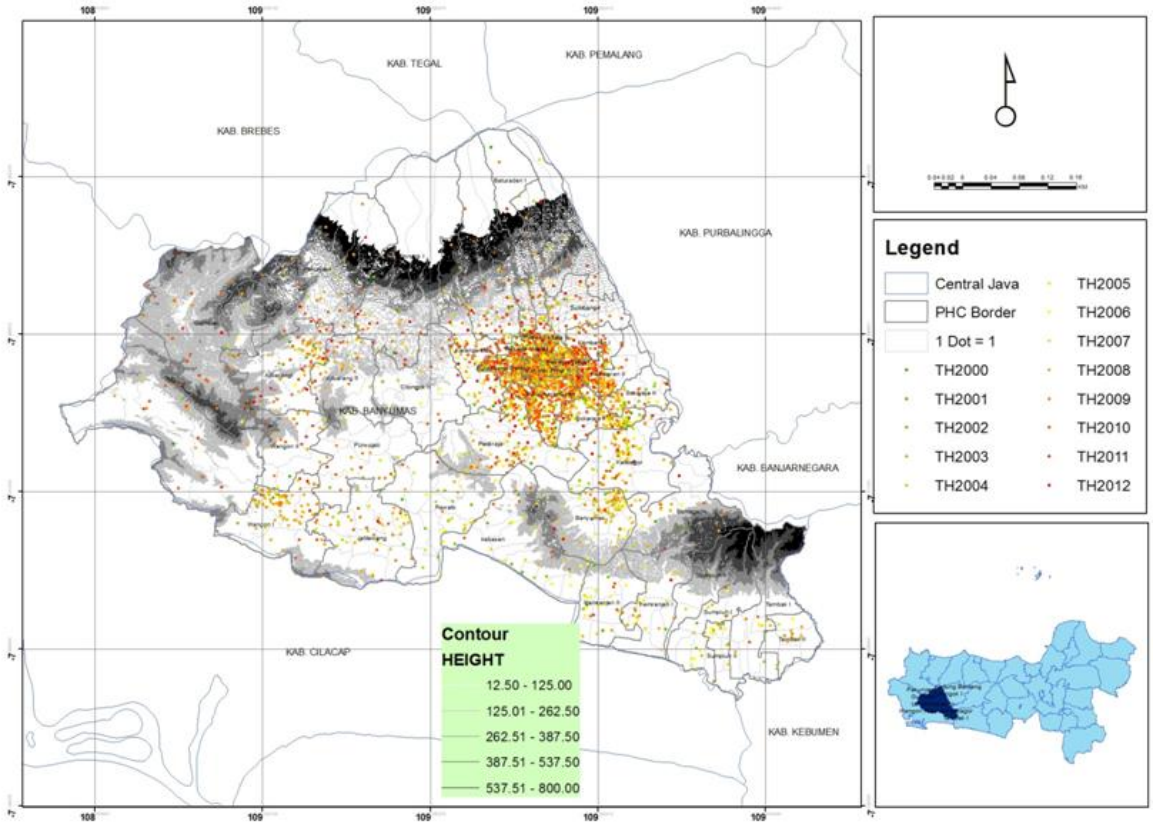


Figure 4-2 Distribution of dengue reported cases in Banyumas Regency 2000-2012
Dots with different colour represent one dengue cases reported in each year. The contour (elevation) is also shown in the map, where the light colour represents the low land, and the dark colour represents more high altitude area. The insert map at the bottom right illustrates the location of Banyumas Regency (dark blue) in Central Java, Indonesia.

4.3.2 Spatial Modelling Framework (Model 1)

Based on the spatial-only Poisson regression model (Model 1), which analysed the cumulative number of dengue cases during 2000-2013 in each village, there are several variables which showed assocation with reported dengue cases. The final model 1suggests that variables observed to associate with reported dengue cases in Banyumas Regency were the structure in employment type and education level, distance to hospital and average minimum of the night-time land surface temperature. The level of association (informed by the incidence risk ratio, IRR) and influence (indicated by the changes in Deviance Information Criterion, ΔDIC) of all included factors in the final spatial-only multivariate model is shown in Table 4-2.

Table 4-2 Estimates of the posterior distributions of the final spatial-only model (Model 1) for the risk of dengue infections in the regency of Banyumas, Indonesia.

IRR=incidence risk ratio. ΔDIC=Changes in Deviance Information Criterion (DIC) due to the removal of a risk factor from the full model. The incidence risk ratio (IRR) is calculated as the exponential of the posterior estimates and represent the proportional increase in incidence risk per unit increase of the predictor. For each unit increases of the predictor, a value IRR>1 indicates that the risk would increase, whereas a value IRR<1 indicates that the risk would decreases. A value IRR=1 indicates that a lack of influence of the variable in the risk of dengue. The DIC indicates the performance of the model to explain the observed disease process, whereas ΔDIC is showing how much each risk factors influence such performance. If ΔDIC=0, this would indicate a variable with little influence in the full model to explain the observed disease process. In contrast, large values of ΔDIC would indicate the important variables in explaining the observed disease process. †Variance of the posterior distribution of the spatial process. This variance measure indicates the degree of variability in the disease process that is not explained by the included predictors but is specific to situations in village.

	Median	**95% credible interval**	**IRR**	**ΔDIC**
Intercept	-2·08	-2·56 to -1·60	-	-
PCA1	0·04	0·03 to 0·05	1·04	28·0
Distance to hospital (km)	-0·08	-0·12 to -0·03	0·93	1·5
Average minimum of the night-time land surface temperature (^{o}C)				4·6
Less than 10^{o}C	0·50	0·18 to 0·82	1·64	
10^{o}C to 15^{o}C	0·25	0·04 to 0·46	1·28	
15^{o}C and more	Ref.			
Spatial variance	0·95	0·71 to 1·27	-	-

Based on Table 4-2, the socioeconomic variable, particularly the level of education and employment structure in each village, was the most important risk factor in Model 1. These variables were found to have a positive association with the village-level risk throughout the study period. Besides the level of education and employement, distance to hospital and average minimum night-time temperature also showed a close correlation with the number of reported dengue cases. In the area with night time temperatures betweeen 10°C and 15°C, the risk of DENV transmission will increase by 1.28, while in areas below 10°C the risk will increase up to 1.64.

4.3.3 Spatial-Temporal Modelling Framework (Model 2)

Model 2 was carried out to test the resilience of the results to spatio-temporal changes in Banyumas Regency from 2000-2013. Similar to Model 1, the level of association and influence of all included factors in the final spatio-temporal multivariate model (Model 2)

is shown in Table 1-2. Model 2 confirmed the results from Model 1 ie that the the socio-economic variables (the employment type and education level) were the main risk factors relating to DENV transmission as can be seen in Table 4-3.

Table 4-3 Estimates of the posterior distributions of the final spatio-temporal model (Model 2) for the risk of dengue infections in Banyumas Regency Indonesia.

PCA1 = employment type and education level; IRR=incidence risk ratio. ΔDIC=Changes in Deviance Information Criterion (DIC) due to the removal of a risk factor from the full model. Interpretations for IRR and ΔDIC can be found in table 1; †Variance of the posterior distribution of the structured spatial process. ††Variance of the posterior distribution of the spatio-temporal process. *Variance of the posterior distribution of the unstructured spatial process. These variance measures indicate the degree of variability in the disease process that is not explained by the included predictors.

	Median	**95% credible interval**	**IRR**	**ΔDIC**
Intercept	-2·38	-2·84 to -1·93		
PCA1	0·04	0·03 to 0·05	1·04	27·1
Distance to hospital (km)	-0·06	-0.10 to -0.02	0·94	0·42
Minimum of the night-time land surface temperature (^{o}C)				1·13
Less than 20^{o}C	0·29	0·08 to 0·50	1·33	
20^{o}C and more	Ref.			
Spatial variance†	0·61	0·41 to 1·10		
Spatio-temporal variance††	0·42	0·35 to 0·51		
Village effect*	0·0008	0·0001 to 0·0055		

After adjusting for associated variables, both models identify the same areas for which the risk of dengue was not explained but showed some spatial structure. A map was generated to represent how the models could explain the DENV transmission by tested variables (Figure 4-3).

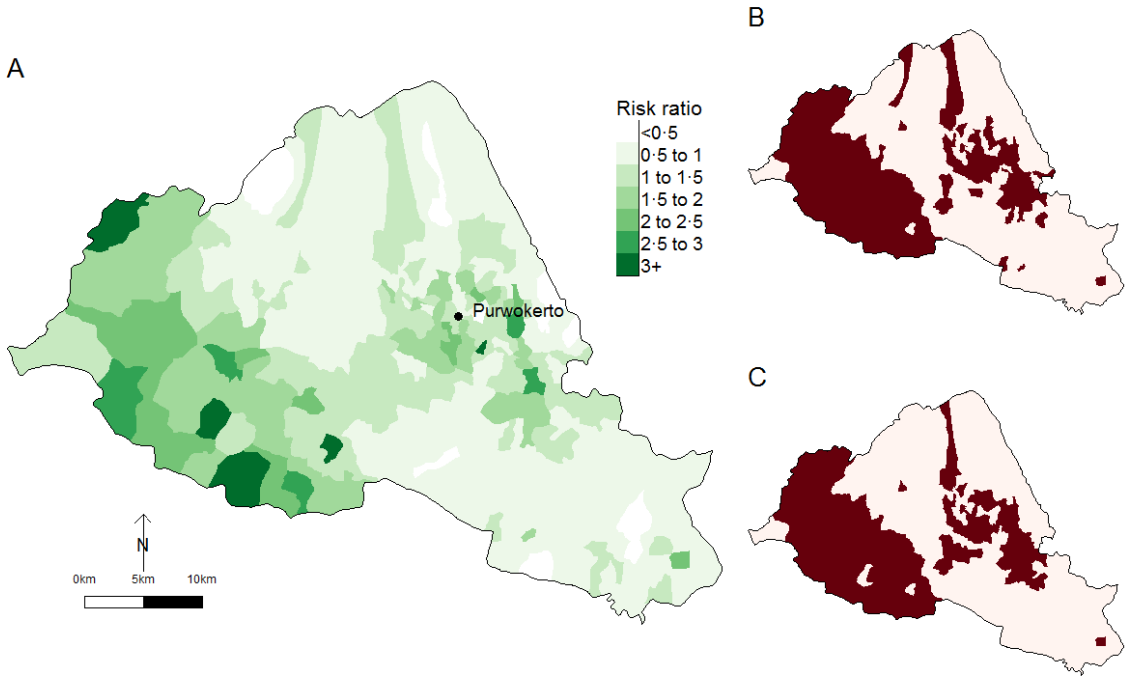


Figure 4-3 Adjusted village-level risk of DENV for the period 2000-2013.

(A) Map of the spatial pattern of the unexplained risk for DENV infection, as identified in Model 1. The risk ratio takes the value one if no deviation exist between the model's inferences based on the included, known risk factors and observations. Values of less than 1 (white and lightest shade of green) indicate villages that have a lesser risk of infection than predicted, whereas darker shade of green indicates villages for which the model did not account for all the risk of infection. (B) Distribution of the significant village-specific posterior probability of the spatial random effect for Model 1. (C) Distribution of the significant village-specific posterior probability of the spatial random effect for Model 2. Villages in dark red in (B) and (C) show a posterior probability >0.8, indicating a relatively small level of associated uncertainty.

Based on Figure 4-3, there are several areas which cannot be explained by the tested variables. The area around Purwokerto City and the South-West of Regency showed different transmission processes than the rest of regency, which cannot be explained by the variables. This finding suggests that the models are good at explaining the global spatial structure of dengue in the regency but there are local epidemics in several areas that are possibly driven by local processes what cannot be explained by tested variables in the models.

4.4 Discussion

The development of spatial analysis provides many opportunities to analyse a large amount of data in a relatively short time without losing its accuracy. This analysis is widely used in public health to understand many aspects relating to infectious disease such as disease transmission, risk factors and outbreak prediction (Eisen & Eisen, 2011). Based on the several predictive models of DENV risk factors that have been carried out in previous

publications, there is still a lack of analysis that examines socioeconomic and environmental aspects at the local level (Racloz et al., 2012). The importance of conducting modeling at the local level has been proposed due to the diverse ecological setting and spatial scales (Lowe, R., 2015). In this study, several variables such as population, socio-economic and environmental factors were analysed as predictor variables of DENV risk factors in Banyumas Regency. Two models, spatial only and spatial-temporal were generated in order to determine the most influential factors on DENV cases in Banyumas Regency.

DENV cases in Banyumas Regency occured every year based on data available from Banyumas Regency Health Office (Figure 1-1). The incidence of disease has increased since 2000, with larger outbreaks noted in 2008 and 2010. This trend is in accordance with national DENV cases in Indonesia, with a significant increase from 2000 and incidence peaks in 2008, 2010 and 2013 (Karyanti et al., 2014). Based on the case distribution map in Figure 1-2, there is an obvious pattern that DENV cases clustered in several areas in Banyumas Regency. The areas with a high number of cases are mainly located in Purwokerto city, the capital of Banyumas regency, which has a high population, is an urban area and shows high mobility. Purwokerto is the busiest area in Banyumas Regency because this area is the centre of economy, public services and education. There are many companies, public administration offices and universities located in this area. This leads to the high mobility of people who travel from their home to their places of work in Purwokerto and also a number of immigrants who are mostly students who attend the University. These characteristics are in line with several publications which stated that endemic DENV areas are mostly related to high population density, urban areas and high mobility for instance through travel (Gubler, D., 2011; Murray et al., 2013; Wilder-Smith & Gubler, 2008). Previous studies also recorded evidence of obvious dengue clustering across the urban area (Almeida et al., 2009; Galli & Chiaravalloti Neto, 2008; Hu et al., 2011; Vazquez-Prokopec, Gonzalo M. et al., 2010).

In this study both models proposed that the socioeconomic variables, particularly the level of education and employment structure were the most important risk factors of DENV infection/report. This result in accordance with several studies indicates that socioeconomic factors are the important factors which drive DENV transmission (Hagenlocher et al., 2013; Khormi & Kumar, 2011). In both spatial and spatio-temporal models in this study, the level of education and employment structure showed a positive correlation with the village-level risk, where villages with the characteristic of more

educated people, for example people who have a job as a civil servant or in business and the services industry have an increased risk for DENV infection. This result could be explained by human mobility since more educated people or people who have a good job tend be more active in work activities through travel from one place to another and be in contact with more people, so then their risk of DENV infection is higher. As stated in many publications, human mobility has a strong correlation with the DENV transmission (Lee, S. & Castillo-Chavez, 2015; Nevai & Soewono, 2014; Stoddard et al., 2009; Vazquez-Prokopec, G. M. et al., 2009). High human mobility will increase the risk of exposure to *Ae. aegypti* bites, resulting in more DENV transmission and spread between co-workers or others. This is also in accordance with the behaviour of *Ae. aegypti* which bites primarily in day time (Higa, 2011), the same period when the workers are in the workplace. The limited flight range of the *Ae. aegypti* mosquito (maximum of 512 m) also stressed the importance of the human movement factor rather than the movement of the vector in the process of DENV transmission (Chao et al., 2013; Harrington et al., 2005). Stoddard et al (2009) also stated that the vector biting behaviour combined with an individual's daily human movements can influence the DENV transmission (Stoddard et al., 2009).

Besides the socioeconomic factors which are predicted to be the most influencial risk factor of DENV transmisison, distance to closest hospitals was also associated with an increase in the risk of dengue. This could be linked to DENV cases reporting and suggests underreporting which increased for every kilometre further away from the closest hospital. Healthcare for DENV infection treatment in Indonesia can only be dealt with at the level of hospitals, which are usually located in urban areas, located far from villages. Ibarra et al (2014) stated that the risk of dengue is increased where they were closer to hospital, indicating either bias reporting and/or true increase of DENV transmission near the city center (Ibarra et al., 2014). Another factor which is associated with DENV risk is the average minimum night-time temperature. The risk of DENV infection was increased following a decrease in minimum night-time temperature. No exact explanation behind this results, however Rogers et al (2014) stated that minimum night temperature noted as a key predictor for vector-borne diseases, affecting both the mosquito vectors and also the period of the extrinsic incubation period (EIP) (Rogers, David J. et al., 2014).

Both models also identify several areas such as Purwokerto and the South Western area of the regency, where the spatial DENV transmission process cannot be explained by the models. The characteristics of Purwokerto city explained above are proposed to be an

explanation of this phenomenon. For the South West area, it could be that DENV transmission is driven by human movement because this area is near the border with Cilacap Regency which is DENV endemic. In addition, infection of another virus such as chikungunya virus which as similar symptoms as DENV in these area could be another explanation since the DENV testing by ELISA is not always accurate.

The result of this study provides important information about local DENV risk factors and provides an understanding of the spatio-temporal dynamics of DENV transmission, particularly at the local level. The finding that more educated people and certain job categories (industry, civil servant, business) showed a higher risk for DENV infection is critical information for disease prevention and control, for example by targeting insecticide use or information of staff. Implementation of prevention in workplaces and public areas are still very limited and often neglegted in the current control strategies in Indonesia, and this study shows that this needs urgently changed. Further studies are important to be carried out, such as investigating the role of human movement on DENV transmission in local areas, or movement between homes, workplaces, schools or public areas. This kind of research also could identify the particular places where DENV transmission occurs. Further, studies to identify the risk factors for the areas where the DENV risk cannot be explained by the models in this study would also be important to be carried out.

Chapter 5

5 Effect of Temperature on antiviral RNA interference (RNAi) responses in Mosquito Cell Culture

5.1 Introduction

Temperature is one of the most important abiotic factors which is believed to correlate with the efficiency of DENV transmission (Morin et al., 2013). Temperature influences several aspects of the mosquito such as development rates, mortality and behaviour (Christophers, 1960; Rueda et al., 1990; Tun-Lin et al., 2000), viral replication (Watts et al., 1987) and vector competence (Xiao et al., 2014). Vector competence of a mosquito is related to the ability to acquire, maintain and transmit a virus or other pathogen (Beerntsen et al., 2000). It is associated with several infection barriers in the mosquito as described in Chapter 1 (Figure 1-8), namely the midgut infection barrier, the midgut escape barrier, the salivary gland infection barrier and the salivary gland escape barrier (Black et al., 2002). Vector competence also correlates with the duration of DENV infection from the midgut until the virus reaches the salivary glands called Extrinsic Incubation Period (EIP) of the mosquito (Carrington, Seifert, et al., 2013). Some studies reported that higher temperatures will shorten the EIP of the mosquito, accelerating the time it takes for the mosquito to become a competent vector for DENV transmission (Chamberlain & Sudia, 1955; Gould & Higgs, 2009; Rohani et al., 2009; Watts et al., 1987). Despite many studies describing the effect of temperature on EIP (Chan & Johansson, 2012; Lambrechts, L. et al., 2011), the mechanism of how temperature could effect the length of EIP remains unclear. Therefore, I sought to analyse the effect of temperature on the mosquito innate immune system, in particular the RNA interference (RNAi) response. It is based on the premise that the mosquito immune system influences replication of arboviruses such as DENV in the mosquito, thus affecting the period of EIP. We hypothesized that the different temperatures will effect the RNAi activity of mosquito.

RNAi is considered as the most important immune response of the mosquito in addition to the Toll, IMD and JAK/STAT signalling pathways (Donald, C. et al., 2012; Green et al., 2013; Merkling & van Rij, 2013). This innate antiviral response is evolutionarily conserved in many organisms, including plants, fungi and insects (Ding, 2010). The RNAi pathway has been characterized most comprehensively in the model organism *D.*

melanogaster. RNAi results in the degradation of dsRNA (double-stranded RNA) into smaller, usually 21 nucleotide siRNAs (or viRNAs if derived from virus) in the organism and is triggered by dsRNA (Ding, 2010; Merkling & van Rij, 2013). Long dsRNA is commonly associated with virus infection (Fragkoudis et al., 2009). The key components of the small RNA pathways are conserved between *D. melanogaster* and *Ae. aegypti*, importantly also within the exogenous siRNA pathway which targets viral infections (Donald, C. et al., 2012). DENV dsRNA replication intermediates are recognised and bound by key proteins of the exogenous siRNA pathway in particular the dsRNA binding proteins R2D2 and Dicer 2 (Dcr-2) (RNase III family member) which initiate exogenous RNAi responses. Dcr-2 will then cut the viral dsRNA into siRNA or viRNAs of 21 nucleotides in length (Sanchez-Vargas et al., 2004). These small interfering RNAs subsequently function in the silencing process as specificity factors and viRNAs are transferred to the multiprotein RNA-induced silencing complex (RISC) where they actively select and degrade mRNA targets through the function of the Argonaute protein (Ago-2) (Donald, C. et al., 2012). Degradation of mRNA or virus genomes results in the silencing of the target RNA or virus. The mechanism of the RNAi pathway can be seen in Figure 5-1. To date, in addition to the exogenous siRNA pathway, there are very similar endogenous siRNA pathway , microRNA pathway (miRNAs) (Moazed, 2009), and the most recently discovered PIWI-associated RNAs or piRNAs (Donald, C. et al., 2012; Merkling & van Rij, 2013).

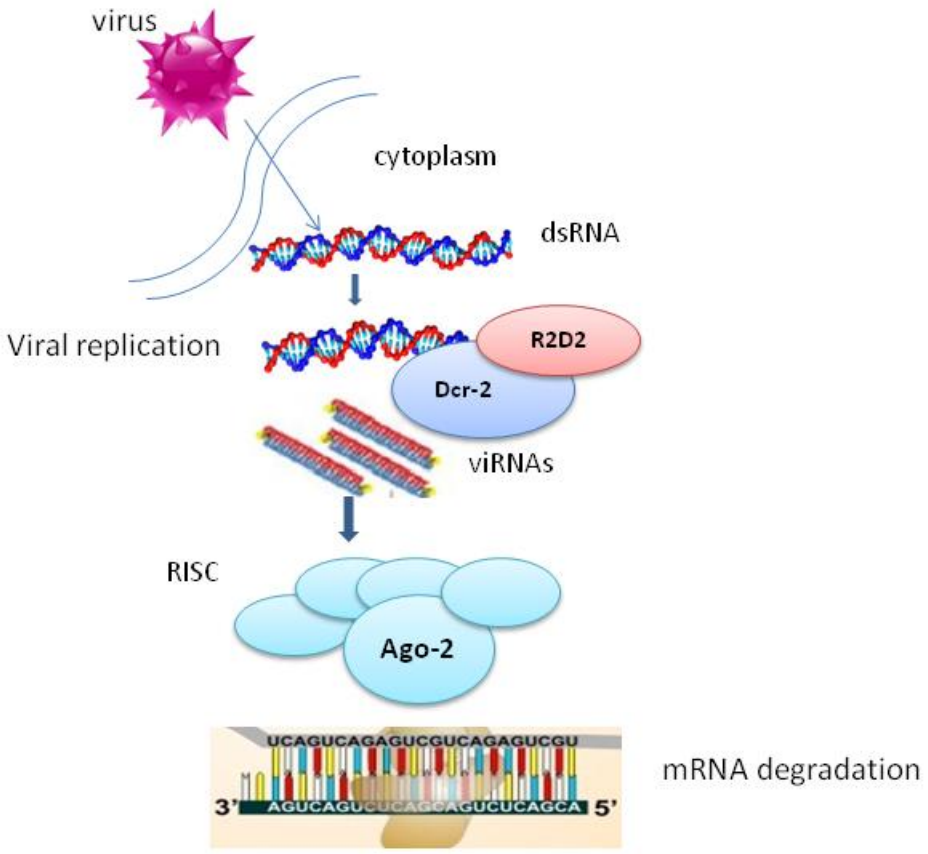


Figure 5-1 Schematic model of the antiviral exogenous RNAi pathway.
dsRNA is recognized by R2D2 and Dicer-2 (Dcr-2) and cleaved into 21nt dsRNA (called siRNAs or viRNAs) forming siRNA-protein complexes. Double-stranded viRNA molecules are unwound by the Dicer-associated helicase and incorporated into RISC. RISC is guided by the incorporated siRNAs to target RNA molecules of complementary sequence. RISC then cleaves target RNA. Dcr-2: Dicer-2; RISC: RNA-induced silencing complex; Ago-2: Argonaute-2. Modified from previous (Donald, C. L. et al., 2012)

A functional RNAi pathway is essential for the mosquito to fight against arbovirus infections including DENV; therefore interference with this system will influence the replication of DENV. The influence of temperature on the RNAi machinery is still unknown and poorly studied. There is a contradiction in results of previous research of the role of temperature on RNAi. Adelman et al (2013) found that rearing mosquitoes at a lower temperature (18°C) impaired the RNAi system and lead to increased susceptibility to chikungunya virus (CHIKV) and yellow fever virus (YFV). Szittya et al (2003) also observed that low temperature inhibits RNA silencing in plant species. These results imply that RNAi activity at lower temperature is impaired thus virus replication is increased and possibly causes more rapid virus dissemination. This is a contradictory with the previous general understanding that warmer temperatures usually lead to more cases. This phenomenon is indeed interesting to be explored. It is undoubtedly important to determine

the effect of temperature on the immune system of mosquitoes in particular the RNAi machinery. Therefore I sought to investigate the effect of temperature on RNAi activity in mosquito cell culture.

In order to address this, aedine Aag2 and U4.4 mosquito cells were transfected with two reporter plasmids pIZ-*FFluc* and pAcIE1-RLuc plasmids and *FFluc* and eGFP dsRNA and incubated at three different temperatures (24°C, 28°C and 32°C). Aag2 and U4.4 cells have been known to have a fully functioning exogenous RNAi pathway (Schnettler et al., 2013; Siu et al., 2011). In this experiment, the transfected dsRNA against *FFluc* should induce RNAi and silence the expression of FFluc expressed from pIZ-FFluc while the control RLuc plasmid should be unaffected. eGFP dsRNA serves as a control. In addition, Aag2 cells were infected with Semliki Forest virus (SFV), a positive-stranded RNA arbovirus genus *Alphavirus*/family Togaviridae (Ratnik et al., 2013) to asses the effect of temperature on virus infection. SFV is a well studied system for RNAi studies in mosquito cells in our laboratory and induces the production characteristic 21 nt viRNAs (Schnettler et al., 2013; Siu et al., 2011). Since experiments with DENV were not allowed in the laboratory when these experiments were planned, SFV was used because it grows well and is produced to high titres in cultured cells (Atkins et al., 1999), and many types of reporter viruses are available (Schnettler et al., 2013; Siu et al., 2011).

5.2 Material and Methods

5.2.1 Cell lines

Cell lines used in this study are Aag2 and U4.4 cells, since both mosquito cell lines are known to have functional RNAi responses (Morazzani et al., 2012; Siu et al., 2011). The cell lines used in this study are described in Table 5-1.

Table 5-1 Cell lines used in this study

Cell type	Cell name	Description	Media
Mosquito cell line	Aag2	*Aedes aegypti*-derived cells	L-15 medium with 10% foetal bovine serum (FBS) (Invitrogen) and 10% tryptose phosphate broth (Invitrogen) antibiotics : 1000U/ml Penicillin and 1mg/ml Streptomycin
	U4.4	*Aedes albopictus*-derived cells	L-15 medium with 10% foetal bovine serum (FBS) (Invitrogen) and 10% tryptose phosphate broth (Invitrogen) antibiotic : 1000U/ml Penicillin and 1mg/ml Streptomycin

5.2.2 Cell culture, maintenance and counting

Aag2 and U4.4 cells were grown in L-15 medium and maintained in culture by splitting the cells regularly before the experiments were carried out. For the experiments, Aag2 and U4.4 cells were plated in 24 well plates at a concentration of 2.3×10^5 cell/well and 1.7×10^5 cell/well respectively. The density of Aag2 and U4.4 cells was calculated using a haemocytometer. Co-transfection was performed after the cells grew well to approximately 70% density (confluency).

5.2.3 Bacterial culture

Plasmids used in the study were pAcIE1-RLuc (expressing *Renilla* luciferase and used as an internal transfection control) and pIZ-FFLuc (expressing *Firefly* luciferase) (Ongus et al., 2006) and also pIB-eGFP expressing eGFP (Green Flourescent Protein). These reporter genes are expressed under insect specific promoters. For plasmid transformations, the plasmids were transformed in DH5α sub-cloning efficiency chemically competent cells (Life Technologies) following the manufacturer's guidelines. To check the plasmid transformation efficiency, pUC19 control DNA was used as a transformation control.

Bacteria were grown on Lactose Broth (LB) agar plates containing the appropriate antibiotic. For antibiotics, both pAcIE1-RLuc and pIB-eGFP are resistant to ampicillin, whereas pIZ-FFLuc contains the zeomycin resistance gene. Bacteria were subsequently grown in LB broth containing the appropriate antibiotic (100 µg/ml for ampicilin and 20 µg/ml for zeomycin). A single colony was used to inoculate a small starter culture of 5ml LB broth and grown for 8 hours at 37°C. 100ml of LB broth was inoculated with 100µl of starter culture and incubated for 16 hours at 37°C.

5.2.4 Plasmid DNA extraction

Purification of plasmid DNA from large overnight bacterial cultures was carried out using the Qiagen Plasmid Midi and Maxi kit as described in the manufacturer's protocol.

5.2.5 PCR

PCR amplification was carried out using KOD Hot Start DNA Polymerase (Novagen). 50 µl of total PCR reaction volume was assembled as follows:
30 µl PCR Grade Water
5 µl 10X PCR Buffer for KOD Hot Start DNA Polymerase
5 µl dNTPs (final concentration 0.2 mM)
2 µl MgSO4 (final concentration 1 mM)
1 µl template DNA
1.5 µl 5' primer (5 pmol/µl, final concentration 0.3 µM)
1.5 µl 3' primer (5 pmol/µl, final concentration 0.3 µM)
1 µl KOD Hot Start DNA Polymerase (1 U/µl)

Primers used in these experiments are described in Table 5-2.

Table 5-2 Primers used for this experiment.

The bold font in primer sequences indicating the T7 Polymerase binding sites

Primer name	Primer sequence (5'-3')	PCR Program
eGFP dsRNA F	**GTA ATA CGA CTC ACT ATA GGG** GGC GTG CAG TGC TTC AGC CGC	2 minutes at 95°C followed by 30 cycles of 20 seconds at 95°C, 10 seconds at 60 °C , and then 70°C for 10 seconds. This was followed by a final extension of 7 minutes at 70°C.
eGFP dsRNA R	**GTA ATA CGA CTC ACT ATA GGG** GTG GTT GTC GGG CAG CAG CAC	
FFLuc dsRNA F	**GTA ATA CGA CTC ACT ATA GGG** ACT TAC GCT GAG TAC TTC	
FFLuc dsRNA R	**GTA ATA CGA CTC ACT ATA GGG** GAA ATC CCT GGT AAT CCG	

5.2.6 Gel Electrophoresis

To confirm the amplification process, samples were electrophoresed on a 1 % agarose gel (0.5g agarose powder was added to 50 ml TAE buffer). Gels were run in 1XTAE buffer with the addition of ethidium bromid for visualisation of DNA, and viewed under UV.

5.2.7 Purification of DNA

Amplified DNA was purified using the Roche High Pure PCR purification kit, following the manufacturer's guidelines. This purification process intends to purify PCR products by removing substances which may possibly inhibit further downstream enzymatic reactions such as mineral oils, salts, unincorporated nucleotides and the thermostable DNA polymerase.

5.2.8 dsRNA synthesis

For synthesis of dsRNA from the purified PCR products, the Megascript RNAi kit (Life Technologies) was used following the manufacturer's instructions. One slight change was carried out by increasing the incubation time from 4 to 8 hours, to increase the yield of dsRNA. The concentration of dsRNA was measured by nanodrop and stored at -20°C.

5.2.9 Co-transfection of mosquito cells with plasmids and dsRNA

The plasmid concentrations used for transfection were 30 ng pIZ-FFLuc plasmid and 5 ng pAcIE1-RLuc plasmid.,while for dsRNA 0.5 ng was transfected. This concentration was applied after optimisation. After the co- transfection, the cells were incubated at three different temperatures (24°C, 28°C and 32°C) for 24 hours, then lysed using 1 x passive lysis buffer (Promega). Passive lysis buffer is a concentrated lysis buffer designed for use with the Renilla Luciferase Assay Kit or Firefly and Renilla Dual Luciferase Assay Kit. The readings of the luciferase expression were determined using the dual luciferase kit (Promega) on a GloMax Luminometer. The entire co-transfection process was carried out using the following scheme (Figure 5-2).

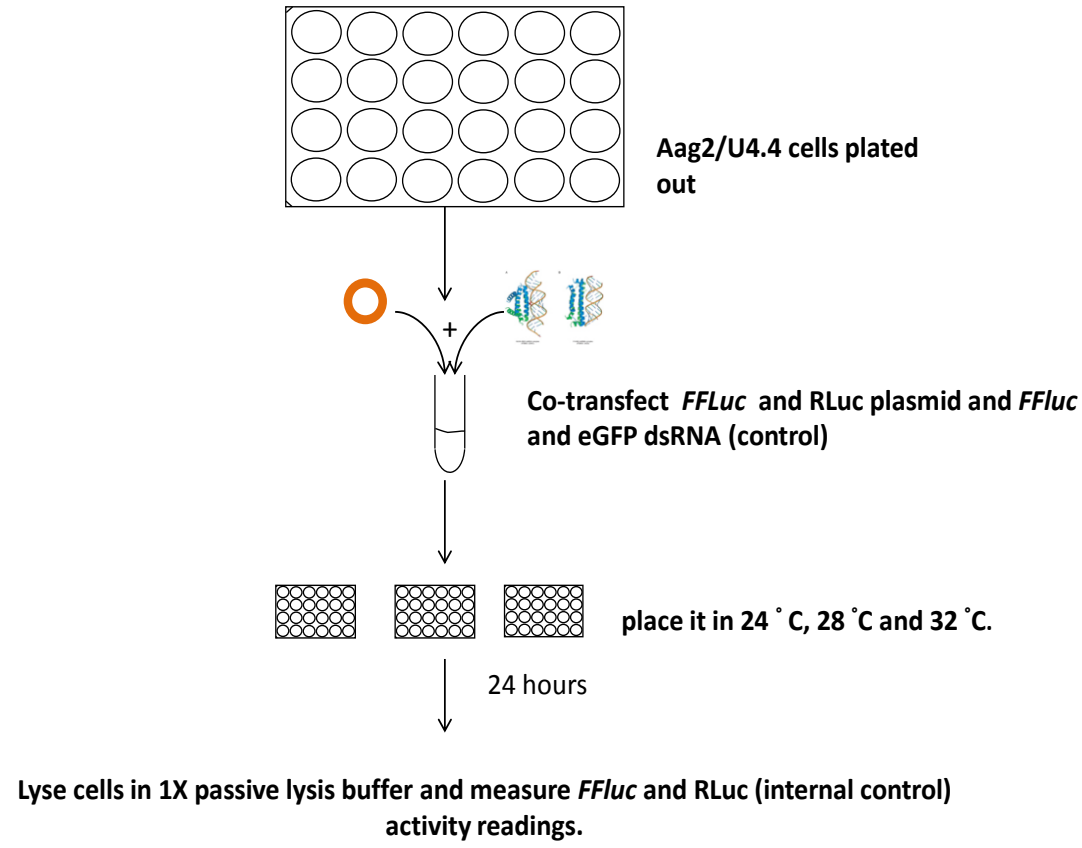


Figure 5-2 The schematic process of co-transfection Aag2 and U4.4 cells..
The schematic flow above described the steps of experiment. Aag2 or U4.4 cells were plated out and co-transfected with *FFLuc* and *RLuc* expressing plasmids and dsRNA then shifted to 24°C, 28°C and 32°C. After 24 hours,the cells were lysed in 1X passive lysis buffer and luciferase activities measured.

5.2.10 Viability Assay

To check the viability of the cell lines after incubation at different temperatures, a cell viability assay was performed using The CellTiter-Glo Luminescent Cell Viability Assay (Promega) following the manufacturer's guidelines.

5.2.11 Infection of Aag2 cells with SFV

The effect of temperature on virus replication was also tested by infecting Aag2 cells with SFV 4 (3H)-*FFLuc*. A lab stock of SFV4 expressing Firefly luciferase from duplicated nsp2 protease cleavage sites inserted between nsp3 and nsp4 was used for this experiment (Rodriguez-Andres et al., 2012). The schematic figure of SFV 4 (3H)-Ffluc can be seen in the figure below,

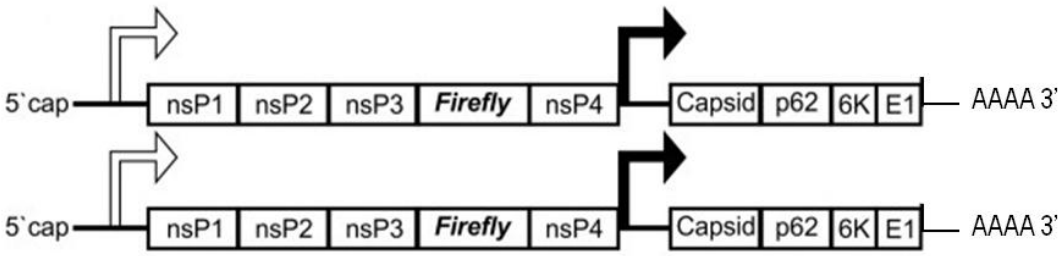


Figure 5-3 Schematic figure of SFV 4 (3H)-FFLuc virus used in this study
SFV4 expressing Firefly luciferase which inserted between nsp3 and nsp4 (Rodriguez-Andres et al., 2012).

Multiplicity of Infection (MOI) 0.1 and MOI 10 was calculated and then used to infect Aag2 cells at a density of 2.3 x 10^5. Calculation of MOI was performed using the following formula:

Number of cells x number of wells x desired MOI = $\frac{x}{\text{titer of virus.}}$

The virus stock was diluted in Phospat buffer saline (PBSA) following the calculation results and after removal of the media from the wells, 200 µl of diluted virus stock was added to the cells and incubated at 28°C, with occasional gentle shaking. After incubation for 1 hour, the virus inoculum was replaced with fresh media and the cells shifted to 24, 28 or 32°C. After 48 hours, the cells were lysed with 1x passive lysis buffer and the luciferase activity measured using the dual luciferase assay kit on the Glomax Luminometer.

5.2.12 Statistical analysis

Data were analysed using a General Linear Mixed model using Minitab version 17. Prior to analysis data were checked for normality. Replicate was added as a random effect to allow for variation due to replication. Analysis of RNAi activity of Aag2 cell and U4.4 cells were carried out separately.

5.3 Results

5.3.1 RNAi activity of Aag2 and U4.4 cells at different temperatures

To assess the effect of temperature on RNAi activity an RNAi sensor assay was performed. To this end, plasmids expressing reporter genes were transiently transfected into cultured cells in addition to dsRNA targeting one of the reporter genes, and the knockdown capability of the cells assessed by reporter gene expression. Aag2 and U4.4 cells were co-transfected with Firefly luciferase (*FFluc*) and Renilla luciferase (RLuc) expressing plasmids and double-stranded RNA (dsRNA) against *FFLuc* or eGFP (enhanced green fluorescent protein) (dsRNA control) at 28°C, and the cultures then placed at 24°C, 28°C and 32°C. After 24 hours incubation, the cells were lysed in 1x passive lysis buffer and *FFLuc* and RLuc activity was measured on a GloMax luminometer using the Dual luciferase assay kit. Transfected dsRNA will trigger the exogenous RNAi pathway in mosquito cells, so the hypothesis is that if RNAi works efficiently the expression of *FFLuc* from the plasmid will be decreased. Renilla luciferase was used as an internal control as the *FFLuc* and eGFP dsRNAs do not target RLuc therefore RLuc levels should act as an internal transfection control to confirm that any reduction in *FFLuc* levels is due to the RNAi pathway activity and not to a reduced transfection level at the different temperatures. The result of the experiment in Aag2 cells can be seen in Figure 5-4.

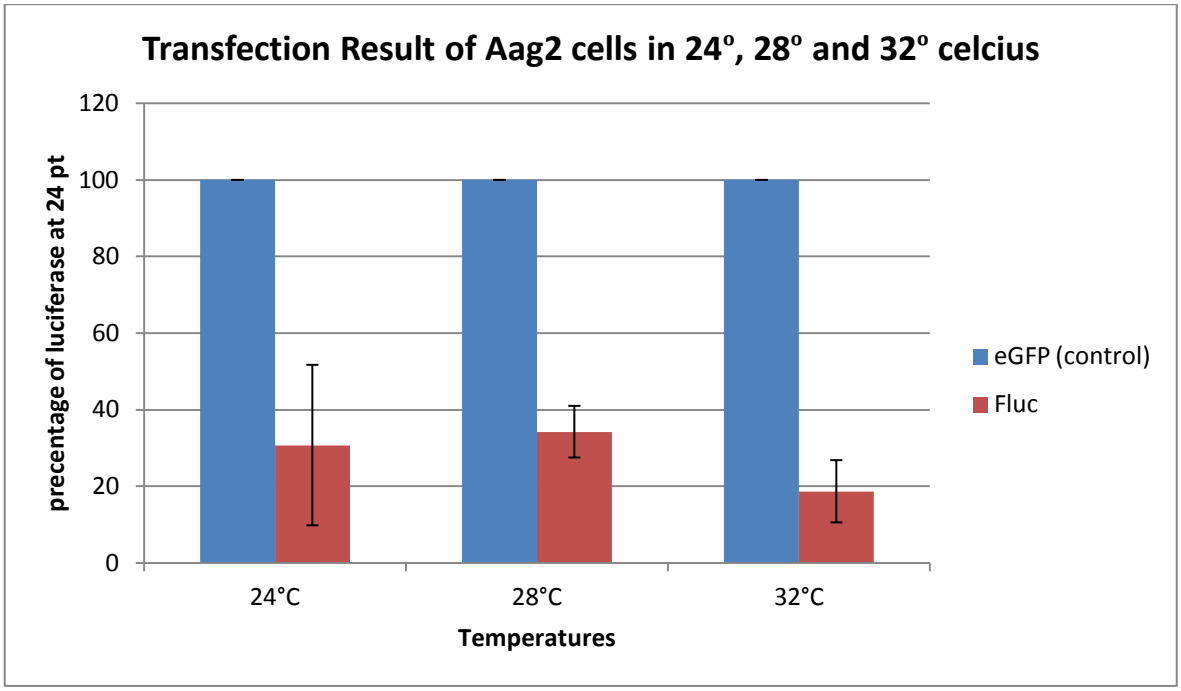


Figure 5-4 RNAi activity in Aag2 cells at 24°C, 28°C and 32°C.
The graph shows the mean of three independent experiments carried out in triplicate. Blue bar shows eGFP dsRNA as a control, and red bar is *FFLuc* expression at the three different temperatures. *FFLuc* knockdown has been calculated relative to the eGFP dsRNA control and normalised to RLuc expression. Error bars show standard error.

Based on data analysis using a General Linear Mixed model, there was no significant difference in the response across all temperatures examined (p=0.469). The summary of result analysis can be seen in Table 5-3.

Table 5-3 Result analysis of RNAi activity in Aag2 cells

Factor Information

Factor	Type	Levels	Values
temp2	Fixed	3	1, 2, 3
rep2	Random	3	1, 2, 3

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
temp2	2	400.5	200.3	0.92	0.469
rep2	2	231.9	116.0	0.53	0.624
Error	4	871.6	217.9		
Total	8	1504.0			

RNAi activity was also tested in U4.4 cells, and the result shown in figure below,

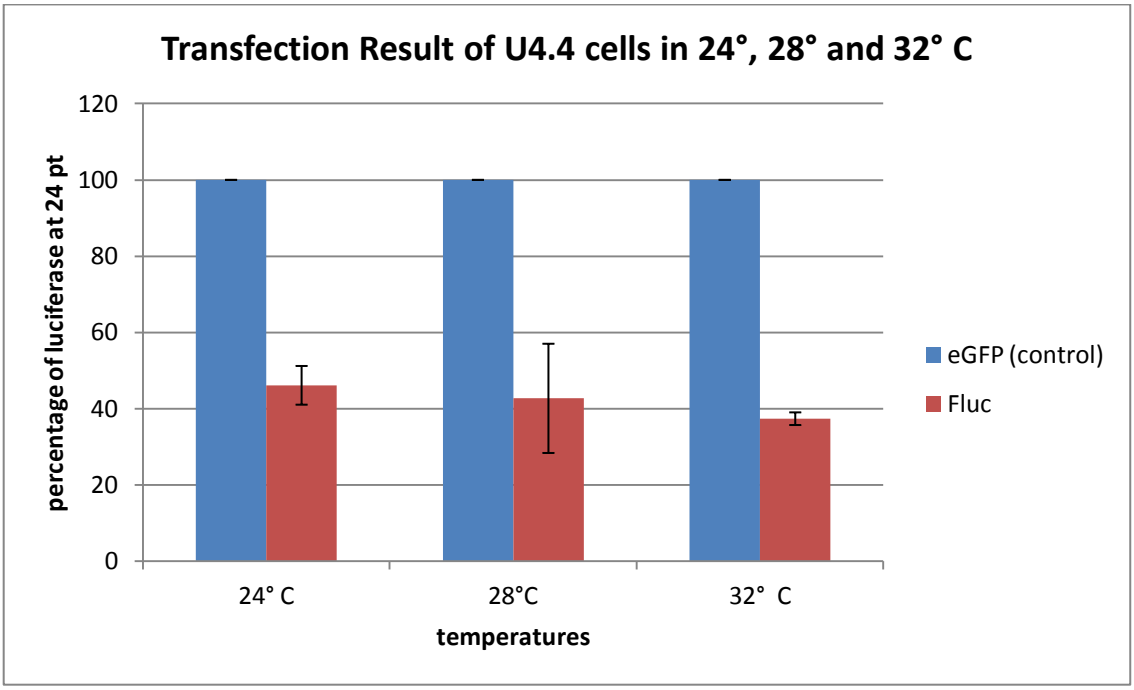


Figure 5-5 RNAi activity in U4.4 cells at 24°C, 28°C and 32°C.
The graph shows mean of three times experiments with triplicate measures in each experiment. Blue bar shows eGFP dsRNA as a control, and red bar is *FFLuc* expression in three different temperatures. *FFLuc* knockdown has been calculated relative to the eGFP dsRNA control and normalised to RLuc expression. Error bars show standard error.

Data analysis of the effect of temperature on RNAi activity in U4.4 cells was conducted using a General Linear Mixed model. Similar to the RNAi activity in Aag2 cells, there was no significant difference in the response across all temperatures examined ($p=0.433$). A summary of the analysis is shown in Table 5-4.

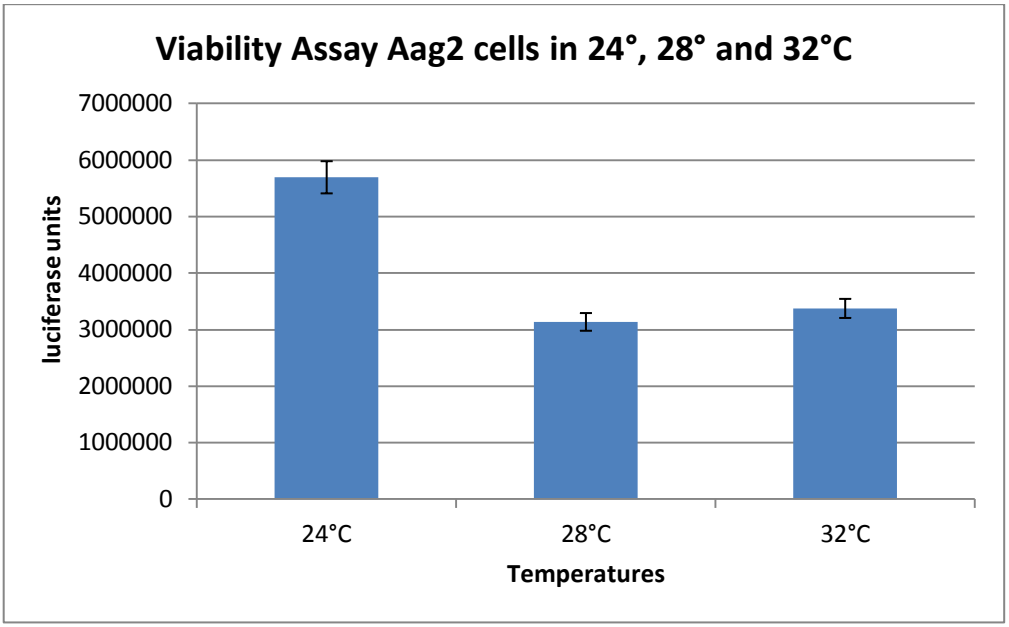
Table 5-4 Summary of result analysis RNAi activity in U4.4 cells

```
Factor Information

Factor  Type   Levels  Values
temp3   Fixed       3  1, 2, 3
rep3    Fixed       3  1, 2, 3


Analysis of Variance

Source   DF  Adj SS  Adj MS  F-Value  P-Value
  temp3   2   116.7   58.34     1.04    0.433
  rep3    2   242.8  121.42     2.17    0.230
Error     4   224.1   56.03
Total     8   583.7
```

Based on the test result, there were no significant differences in the RNAi activity across all temperatures examined both in Aag2 and U4.4 cells

5.3.2 Viability assay for Aag2 and U4.4 cells

The results of the co-transfection could be influenced by the viability of the cells at the different temperatures used in the experiment. In this experiment, cells were incubated at different temperatures either lower or higher than normal temperature for mosquito cell lines (24°C and 32°C). Indeed temperature may affect cell metabolism and other processes. Therefore, the CellTiter-Glo Luminescent Cell Viability Assay (Promega) was used to check the viability of Aag2 and U4.4 cells. This method can assess the number of viable cells in culture based on quantitation of ATP, an indicator of metabolically active cells. Cells were plated out and incubated at 28°C overnight then shifted to the different temperatures for 24 hours. Cell viability was assessed by luciferase expression. The result of the viability assay in Aag2 cells and U4.4 cells can be seen in Figure 5-6.



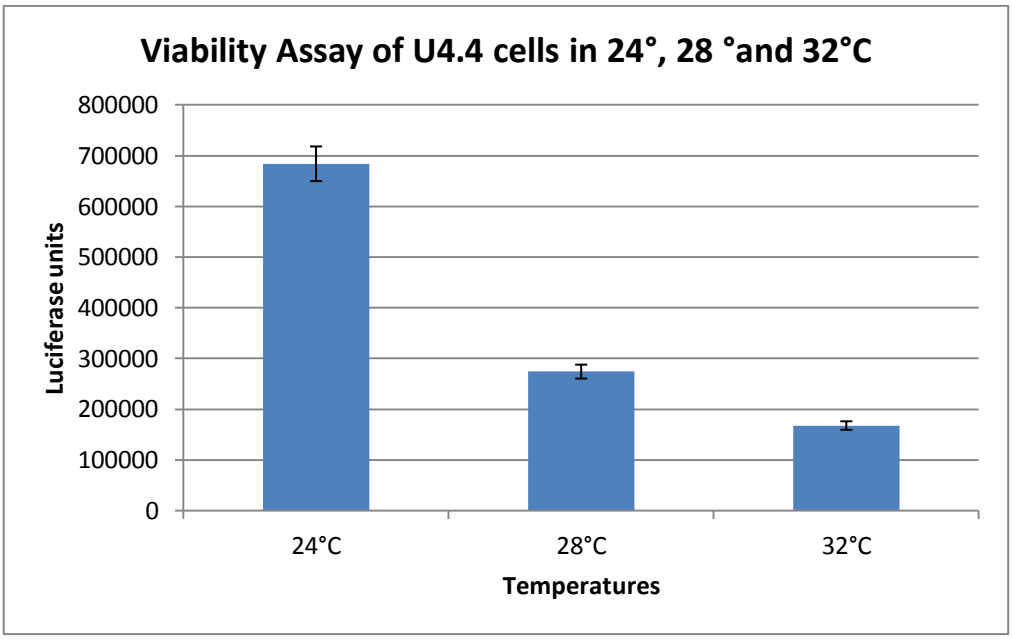


Figure 5-6 The Graph of the viability Assay of Aag2 (upper panel) and U4.4 cells (lower panel) at 24, 28 and 32°C.
The bars show the cell viability at each of the different temperatures measured in luciferase units.

From the graph of the viability assay, the viability of Aag2 and U4.4 cells was highest when cells were incubated at 24°C. When Aag2 cells were incubated in 28° C and 32°C their viability was similar, though they appeared to be less viable compared to the cells incubated at 24 °C. The results of the transfection assay are therefore best compared between 28°C and 32°C degrees as viability is similar. In U4.4 cells, the viability showed a decreased trend along with the increase of temperature. At 32°C, viability of U4.4 cells looked very low compared with 24°C, therefore it is likely to affect the co-transfection.

results. It should also be pointed out that the normal growth conditions for these cells are at 28°C therefore cells incubated at this temperature should be considered to be the base level of viability.

5.3.3 Infection of Aag2 cells with SFV: effect of temperature

An opposing hypothesis for the shortening of the EIP with temperature changes is that temperature affects the virus replicative cycle. To this end, Aag2 cells were infected with SFV4(3H)*FFLuc* at MOI 0.1 and MOI 10 then incubated at 24°C, 28°C and 32°C for 48 hours. The result of infection of Aag2 cells with SFV is shown in Figure 5-7.

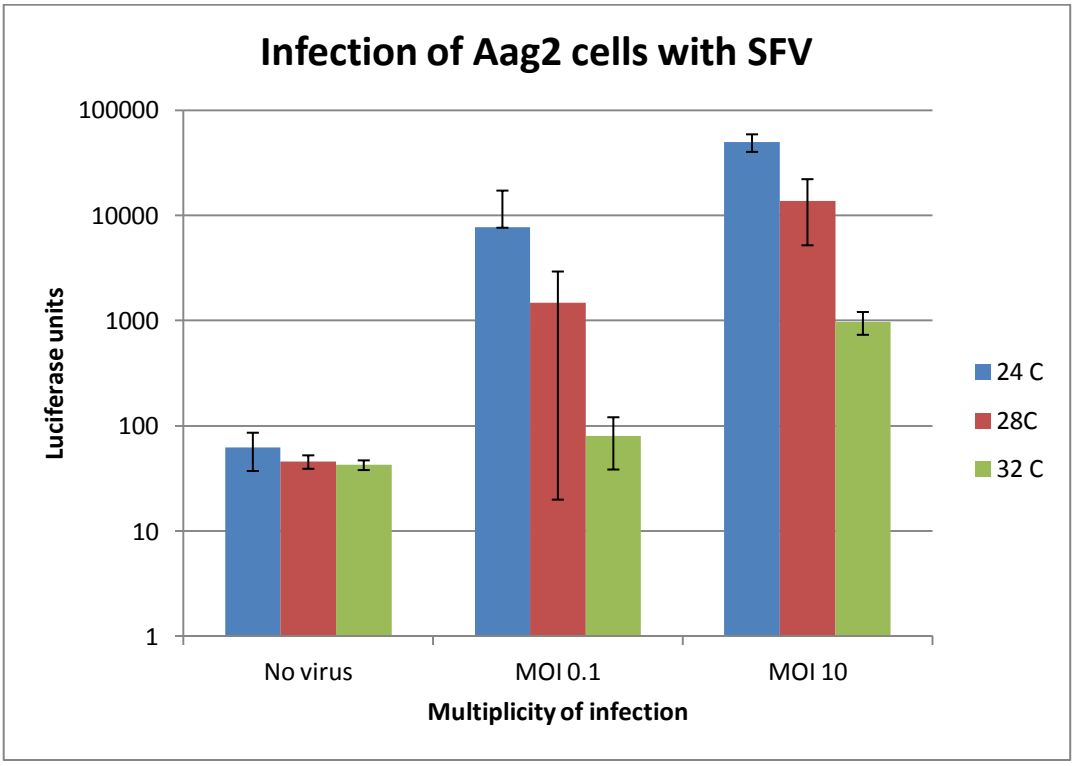


Figure 5-7 Infection of Aag2 cells with SFV at three different temperatures (24, 28 and 32°C). Blue, red and green bars shows virus replication in 24°C, 28°C and 32°C respectively. Error bars show standard error is shown in each bar.

Based on these results, SFV replication in Aag2 cells is higher at 24°C compared to 28°C and 32°C in both MOI 0.1 and MOI 10. Thus virus replication decreases in response to an increase in temperature.

5.4 Discussion

Several reports have stated that RNAi activity is temperature dependent. Kameda et al (2004) found a suppressive effect on the efficiency of RNAi in cultured mammalian cells

at hypothermic temperatures when investigating the influence of different temperatures (Kameda et al., 2004). This result re-emphasizes temperature sensitive RNAi effects as discovered in plants (Szittya et al., 2003) and insects (*Drosophila)* (Fortier & Belote, 2000) and mosquitoes (Adelman et al., 2013). The dependency of the RNAi system on temperature might be highly conserved between eukaryotes. The RNAi response differs with diverse conditions and this may allow an organism to control a response to certain environmental signals (Habig et al., 2008).

In order to test the affect of temperature on RNAi activity, mosquito cell lines were co-transfected with plasmids and dsRNA and then incubated at three different temperatures, 24, 28 and 32°C. A decrease in *FFluc* expression in U4.4 and Aag2 cells indicated that RNAi works effectively at temperatures of 24, 28 and 32°C. From the data shown in the results, silencing of the reporter plasmid (*FFluc* reporter) occurs to varying degrees in Aag2 and U4.4 cells depending on the temperature the cells are grown at. From the trend of *FFluc* activity in both Aag2 and U4.4 cells, *FFluc* activity at the lower temperature (24°C) is slightly higher than at 28°C (standard growing temperature) and 32°C (higher temperature). In contrast, *FFluc* expression at 32°C is lower compared to 24°C and 28°C, this suggests that the RNAi pathway works more efficiently at higher temperatures, although from the statistical analysis, the difference between RNAi activity at 24°C, 28°C, and 32°C is not significant. This finding supports the previous research by Adelman et al (2013) where the RNAi activity impaired in the lower temperature, caused the more effective of virus replication (Adelman et al., 2013).

The more effective RNAi effects were seen at 32°C, and this is contradictory to previous publications about the correlation between temperature and EIP where it has been reported that warmer temperatures could shorten the EIP in mosquito. An effective RNAi system is supposed to be able to inhibit viral replication in the midgut and slow the spread of arboviruses to the salivary glands. From these experiments, the enhanced RNAi activity at higher temperatures would be expected to prolong the EIP which contradicts with previous general understanding about temperature and EIP. However, it is possible that RNAi alone cannot be the only relating factor that influences the length of EIP in the mosquito. Lindenbach and Rice (2007) believed that once the arbovirus has established the early step of infection, the RNAi response is not able to inhibit the infection entirely. Viral amplification begins in the midgut epithelial cells, escapes and spreads to secondary targets such as the salivary glands and continues to replicate (Lindenbach & Rice, 2003). Sanchez et al (2004) also proposed that arboviruses could replicate faster than the ability of the

RNAi system to prevent the virus infection. It is possible that arboviruses such as DENV replicate in and escape from the midgut before the RNAi response begins to be active. Certain threshold levels of dsRNA could also be required to trigger RNAi (Sanchez-Vargas et al., 2004). In addition, Carrissimo et al (2015) stated that exogenous siRNA pathway plays role mainly in the later stages after viral infection to control viral load, not in the initial stage of infection in the midgut although the Anopheles/o'nyong-nyong alphavirus system they used may not representantive for the more common aedine arbovirus vectors (Carissimo et al., 2015) .

The more effective virus replication at lower temperature as observed in (Adelman et al., 2013) and (Szittya et al., 2003) was also shown in this study. The infection results of Aag2 cells with SFV showed with both MOI 0.1 and 10 that the increasing temperature resulted in lower virus replication. The higher SFV replication in lower temperatures (24°C) compared to 28°C and 32°C is in accordance with several previous studies. The first report of an inverse relationship between temperature and EIP within the range of 25°C to 32°C was reported by Kramer et al in 1983. They revealed that the infection level of Western Equine encephalitus virus (WEEV; genus Alphavirus) in *Culex tarsalis* decreased along with the increase of temperature (Kramer et al., 1983). Turell (1993) also stated that the infection rate of Venezuelan equine encephalitis (VEE) and Rift Valley fever (RVF) viruses in *Ae. taeniorhynchus* at lower temperature is at higher levels than compared to higher temperature (Turell, 1993). In addition, Westbrook et al (2010) found that the infection rate of CHIKV is higher when mosquitoes were reared in lower temperatures (Westbrook et al., 2010), and the most recent Adelman et al (2013) also stated that rearing mosquitoes at cooler temperatures resulted in higher infection rates of CHIKV and yellow fever virus. Some studies have shown that several viruses bind better at lower temperature which could be an explanation behind these results (Keilian & Helenius, 1986; Nunes-Correia et al., 1999).

The result of Aag2 infection by SFV also supports the finding of RNAi silencing. The activity of RNAi seems better in higher temperature (32°C) and the replication of SFV in Aag2 cells at 32°C was lower than other lower temperatures (24 and 28°C). If RNAi works more efficiently, then virus replication should indeed be inhibited. However, our experimental results do not support the fact that generally dengue cases increase following a period of high temperature (Hii et al., 2012; Pinto et al., 2011) so other factors such as behaviour may come into play. These results corroborate the important effect of temperature on DENV transmission. The reasons for the observation that the higher

temperatures will reduce the replication of virus remain not well understood. The decreased viability of the mosquito cells at higher temperatures is one possible explanation of these results, however 28 and 32°C compare in terms of cell viability.

To conclude, we can assume that the lower or higher temperature only contributes a minor effect on RNAi machinery *in vitro*. We are unable to expand the temperature range tested to find out the effect of temperature on RNAi because the viability of cell lines is impaired when they are incubated at relatively extreme temperatures. Another limitation of this laboratory research is that the results cannot be associated directly with the real conditions in the field. Adult mosquitoes are exposed to constant temperature fluctuations depending on conditions in the surrounding environment (Alto & Bettinardi, 2013; Lambrechts et al., 2011). Influence of temperature on RNAi work with mosquito cells incubated at constant temperatures less well represents the natural state because mosquitoes are exposed to naturally diurnal temperature changes. More in-depth studies on the effect of temperature on the RNAi machinery for example, by comparing this effect on *Ae. aegypti* and *Ae. Albopictus,* is still needed for a better understanding of the relationship between temperature and RNAi system.

Chapter 6

6 Overall Conclusions and Future Directions

The overall aim of my research was to conduct an integrated epidemiological study of DENV transmission in Banyumas Regency, Java Indonesia. An infectious disease, in this case DENV, is influenced by three essential variables as set forth in the triangle of epidemiology i.e. the agent, the host and the environment. In addition, the mosquito vector also plays an important part in DENV transmission, adding to the complexity of the transmission process. All of these factors combined make it difficult to understand the transmission of DENV in a given area. It is also interesting to note that each area has different characteristic of variables that can influence DENV tranmission. Based on previous publications, there are several variables which are believed to affect DENV tranmission such as DENV genetics, human population density, human movement, mosquito population, demography factors, socio-economic and environmental factors. The results of many studies relating to DENV tranmission are different to each other, due to the unique characteristics of each area. This indicates the importance of carrying out a study based on the local area of interest, and that covers factors which are believed to play important roles on DENV tranmission locally. Indeed, this emphasizes the importance of my study which was to carry out an integrated epidemiological study of DENV transmission in my local area.

In my study, I attempted to identify entomological factors affecting DENV transmission risk by conducting entomology surveys in several villages in the study area, to identify virus factors by carrying out serotyping and genotyping from patient serum samples and to identify socio-economic, demographic and environmental factors by generating spatio and spatio-temporal models to determine what are the most important factors influencing DENV infection. In addition, I aimed to assess the effect of temperature on mosquito innate immunity, in particular RNAi activity. Here is the summary of important findings of this study.

6.1 Traditional survey/Larvae surveys are not a relevant determinants for mosquito vector populations.

Entomology surveys as a part of routine surveillance programs, particularly in endemic areas, are important in order to detemine the mosquito population in certain areas. The mosquito population information can be used as a prediction of the risk of DENV transmission and become a basis for prevention and control strategies. Unfortunately, to

date there is still some difficulty in obtaining reliable measurements to determine the mosquito populations in the correlation with DENV risk tranmission. Larvae surveys, for many decades, were applied as the routine entomology survey in most endemic countries. However, limitations of this technique have been observed in several studies, mainly because there were little evidence of quantifiable correlation between larvae indices and dengue incidence, thus it is is not useful for DENV transmission risk prediction. The results of my study, which found that the traditional larvae survey is not a relevant measurement of mosquito population, and cannot predict the DENV incidence in an area, re-emphasises previous studies which came to similar conclusions (Bowman et al., 2014; de Melo et al., 2012; Focks, D. & Alexander, 2006; Focks, D. A., 2003; Romero-Vivas & Falconar, 2005). This issue must be addressed in the near future since entomology surveys cost a lot to perform and take a lot of energy and time, therefore the resulting unrealiable measurements will result in a waste of resources. In my study, I observed that adult mosquito populations and the high incidence of vertical transmission provides a more relevant measurement of DENV risk transmission. Adult mosquitoes are potential vectors of DENV, compared to immature stages which must first go through another metamorphosis stage to become competent vectors. This makes an adult mosquito survey a more relevant measure, however this requires trained people to conduct adult mosquito collection. In my study, the high percentage of vertical transmission observed in the study area also provides one possible explanation why DENV is continually circulating in the area endemically. How important the transvorial transmission is to DENV tranmission or outbreak still remains an important question.

6.1.1 Future directions

To further determine the importance of adult mosquito collection as a relevant measurement of mosquito population density, it is required to carry out the study in more sampling areas. Because of funding restrictions and time considerations, I only surveyed four villages (400 houses) in Banyumas Regency in the dry and rainy seasons. More details measurement of container such as pH, O_2, sun exposure, temperature, level of organic matter and water condition are important component to be added in further studies. For vertical transmission, it is crucial to conduct PCR and sequencing on F1 mosquito samples to confirm the presence of DENV in these mosquitoes and also the salivary glands to asses wether the mosquitoes can transmit DENV. These more realiable techniques will give more accurate results to ensure the vertical rates in the study area.

6.2 The awareness of age group shift and high numbers of secondary infections

From the serum sample collection, adult patients in the 30-44 age range are the majority of suspected dengue patients during the period of sample collection. This age group shift is in agreement with the general age shifting pattern of dengue reported cases which is observed in Indonesia and other DENV endemic countries in Southeast Asia. Information about age group and gender must be considered by public health authorities for their planning on DENV prevention strategies. The high percentage of the suspected patients who had secondary infections (89.4%) by serological tests, indicates the possibility of developing severe clinical manifestations as a result of secondary infections. As in many publications, secondary infections are believed to have strong associations with more severe DENV disease manisfestations. Unfortunately, I cannot correlate the DENV serotype with the disease severity in the patient samples because serotyping of DENV from the samples was unable to be completed due to time constraints and delays in obtaining an MTA for shipment of the samples to Glasgow. Previous studies argue that some serotypes or genotypes will result in more severe DENV infection.

6.2.1 Future directions

It is important to continue the DENV serotyping and genotyping analysis in patient samples to identify any potential links between DENV serotype or genotype and severity of disease in the study area. PCR methods also should be applied as DENV diagnosis since most hospitals still rely on serological test (ELISA) which could leading to false negative or false positive, thus caused less realibility of DENV surveillance. Serotyping information will also give important information what DENV serotypes are circulating in Banyumas Regency. Phylogenetic analysis based on genotyping results could show the geographical movement and divergence of DENV. For that, obtaining GPS coordinates of the patient's address would be essential. With this information, we can build a map based on the area where the patient comes from and then show which DENV serotypes are circulating in each area. Full virus genome sequencing on the patient samples would also provide important information with regards to the patient's infection history.

6.3 Socioeconomic factors, particularly the level of education and employment structure were the most important risk factors of DENV infection.

The main finding from the spatial analysis using both spatio and spatio-temporal models was that the socioeconomic variables, particularly the level of education and employment structure were the most important risk factors of DENV transmission. This finding showed that socio-economic factors play a more important role than environmental factors for DENV infection risk in Banyumas Regency. Human movement is predicted to be the main explanation of this result, because more educated people or people who have a good job tend to have an increased risk of DENV infection since they are more active in work activities through travel from one place to another and are in contact with more people. This result re-emphasises the importance of human mobility and movement in the process of DENV transmission. Based on Louis et al (2014), the influence of human movement and mobility on DENV transmission seems greater than previously thought (Louis et al., 2014). However, predictors dealing with human mobility on DENV transmission are still very poorly researched. The finding of this study also triggers a question as to where DENV transmission mostly takes place? Findings that the higher level of education and people with certain job types have higher risk to exposure to DENV infection, could raise the assumption that the interaction between the infected vector and the host possibly occurs in work places and public areas. This could be interesting and further research is required to explore this observation. The findings of this study should be used to advise public health authorities on those areas where prevention and control strategies should be focused.

6.3.1 Future directions

In order to obtain a more complete story about DENV transmission in Banyumas Regency, research on the effect of human movement on virus transmission is important to be carried out. Investigating the role of human movement on DENV transmission in local areas, such as movement between home, workplaces, school or public areas, along with vector movement will give more clear perspective of DENV transmission dynamics. Tracking human movement using mobile phone data could be applied (Schneider et al., 2013). Identifying the risk factors for the areas where the DENV risk cannot be explained by the models in this study would also be important to be carried out. Adding host serological profiles and DENV genetic diversity into the model is also essential since the models do not include these factors. Additionally, information on what other factors may be influencing DENV transmission in those areas, for example whether are there other viruses

or infectious agents circulating which may be interfering with the analysis, is going to be important.

6.3.2 The RNAi pathway works more efficiently at higher temperatures

The finding of the effect of increased temperature on RNAi activity was contradictory to our previous general understanding about temperature and EIP where it has been shown that higher temperatures will shorten the EIP (Rohani et al., 2009; Watts et al., 1987). In this study, we observed that RNAi pathway works more efficiently at higher temperatures both in Aag2 and U4.4 cells although from statistical tests, there were no significant differences in the RNAi activity across all temperatures examined. More efficient RNAi systems at higher temperatures should to be able to inhibit viral replication in the midgut and slow the spread of DENV to the salivary glands, and therefore prolong the EIP not shorten it. However, my findings are in accordance with work by Adelman et al (2013) who observed that mosquito exposure to cooler temperatures after virus infection would reduce RNAi activity, and virus production decreases with increasing temperatures (and with more efficient RNAi) (Adelman et al., 2013). Several studies also observed that virus replicate better at lower temperature (Carrington, Armijos, et al., 2013; Kramer et al., 1983; Turell, 1993; Westbrook et al., 2010). This is also observed in my study where SFV replication decreases in response to an increase in temperature. This evidence re-emphasizes earlier findings about a temperature-dependent effect on RNAi activity as found in in some plant species (Szittya et al., 2003) and on virus replication of other viruses such as Western equine encephalomyelitis virus (WEEV) and Ross River virus (RRV) (Kay & Jennings, 2002; Kramer et al., 1998; Szittya et al., 2003). An interesting paper from Carissimo et al (2015) stated that siRNAi activity in Anopheles is not a component of antiviral defense in midgut infection, but only protects later stage of infection (Carissimo et al., 2015). Thus any relationship between RNAi and EIP requires further exploration.

6.3.3 Future Directions

For future studies a more in-depth analysis on the effect of temperature on the RNAi machinery should be carried out, for example by comparing the temperature dependent effect on *Ae. aegypti* and *Ae. albopictus* mosquitoes. One of the challenges, however, to analyse the effect of temperature on RNAi activity is how to set up the experiment with experimental settings similar to mosquito environmental conditions in a natural setting.

Mosquitoes are naturally exposed to fluctuating temperatures during the day and night, as reflected by diurnal temperature range (DTR). Several studies stated that DTR fluctutation possibly plays more important role in pathogen amplification than previously thought (Lambrechts, L. et al., 2011; Paaijmans et al., 2010). This issue must be addressed in order to obtain a better understanding of the relationship between temperature and RNAi system.

In conclusion the results of this study have outlined that current surveillance programs are not the most effective way of determining DENV risk in this part of Indonesia and these findings could be used by local authorities to provide more reliable information and inform control strategies. This study has identified that adult mosquito population are a better predictor of DENV transmission risk than standard larvae and pupae indices. In addition, the modelling data suggests that instead of spending time and money spraying insecticides indiscriminately, perhaps a more useful strategy would be to target the areas defined as being most at risk and therefore reduce the cost of extensive spraying programs and also reduce the burden of insecticide resistance. A higher secondary infection and age-group shift observed in patient samples should be become an awaraness for clinical DENV managament and also in the preventive strategies. Furthermore, although the effect on temperature on RNAi to EIP remains to be defined, ongoing global warming and environmental changes must be considered in the relation to DENV transmission.

Appendices

Appendix 1. Questionnaire for KAP Survey

QUESTIONNAIRE

INTEGRATED EPIDEMIOLOGICAL STUDY OF DENGUE VIRUS TRANSMISSION IN JAVA, INDONESIA

(Knowledge, Awareness and Practices of Respondents to dengue disease and prevention)

Name of interviewer :

Date of Interview :

Name of Village :

Sub District :

I. CHARACTERISTICS OF RESPONDENT

1. ID Number : ……..
2. Age : …….years old
3. Gender : a. Male
   b. Female
4. Education level : a. Uneducated
   b. Unfinished from Elementary school
   c. Graduated from Elementary school
   d. Graduated from Junior High School
   e. Graduated from Senior High School
   f. Bachelor degree
5. Job : a. Unemployed b. Farmer
   c. Seller d. Private employees
   e. Civil servant f. Labourer
   g. Others
6. Economy status/income

a. Under Standard Salary of Banyumas Regency (<795.000 IDR)

b. Above Standard Salary of Banyumas Regency (>795.000 IDR)

7. Source of Drinking/Consumable water

a. Wells

b. Drinking Water Supplier

c. Others

8. House Construction

a. Good

b. Bad

II. **KNOWLEDGE**

No	**STATEMENTS**	**TRUE**	**FALSE**
1	Dengue Fever (DF) /Dengue hemorhagic fever (DHF) is a communicable disease		
2	DF/DHF is caused by dengue virus		
3	DF/DHF is not a dangerous disease		
4	DF/DHF is transmitted by mosquito (*Aedes sp*) bite		
5	*Aedes sp* mosquitoes prefer to rest in places which are exposed to light		
6	Sign and symptoms of this disease are high fever for 2-7 days, red rash (skin) and nosebleed		
7	This disease only affects children		
8	*Aedes sp*. are night biting mosquitoes		
9	Eradication of mosquito breeding places can be done by draining of bath-tubs , closing water containers and burying unused potential water reservoirs around the house.		
10	Water which contained abate powder (commercial larvacide) is dangerous to drink.		

III. AWARENESS

Code information (Based on Likert Scale) :
AS = Agree Strongly;
A= Agree;
LA = Less Agree,
DA =Disagree;
VDA = Very Disagree

No	**STATEMENTS**	AS	A	LA	DA	VDA
11	Efforts to prevent dengue disease must be supported by all, including community participation					
12	Eradication of mosquito breeding sites and avoiding mosquito bites helps to prevent dengue disease effectively.					
13	Draining bath-tubs and closing water containers areactivities which require extra efforts in your daily life.					
14	Unused things surrounding the house which have the potential to be breeding sites of mosquitoes do not need to be buried or covered because they do not increase risk.					
15	Spreading of Abate powder (larvacide) in water containers is an extra effort which is also costly.					
16	Dengue bites must be prevented in order to minimise number of dengue cases.					
17	If family members show the signs or symptoms of dengue, it is important to take them to a doctor, hospital or other health services.					
18	Dengue is not dangerous disease, but a kind of self limiting disease which patients recover from after several days even without any treatment.					
19	To eradicate mosquito breeding sites requirescommunity participation in government programs to prevent and control dengue disease.					
20	We do not need to pay much attention to preventing dengue because it already a common disease.					

IV PRACTICES

No	STATEMENT	YES	NO
21	Do you drain your bath-tub at least once a week?		
22	Do you brush the inside surface of your bath-tub when you drain it?		
23	Do you close your water container properly?		
24	Do you bury or put into waste storage any unused potential water container surrounding your house?		
25	Do you often hang up your clothes within the house?		
26	Do you spread abate powder (larvacide) in your water container?		
27	Is there any regular communal work to clean your environment?		
28	Do you use mosquito repellent to avoid mosquito biting?		
29	Do you use long clothes to protect your body from mosquito biting?		
30	Will you go to a doctor or hospital if any of your family members present signs and symptoms of dengue disease?		

Banyumas,……

Interviewer

(.....)

Appendix 2. Consent Form

Study Number :

Subject Identification Number for this trial :

CONSENT FORM

Title of Project: Integrated Epidemiological Study of Dengue Virus Transmission in Java, Indonesia

Name of Researcher: Siwi Pramatama Mars Wijayanti

Please initial box

1. I confirm that I have read and understand or the information sheet dated..... (version.....) for the above study or had the study explained to me and have had the opportunity to ask questions. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my legal rights being affected. ☐

3. I agree to take part in the above study. ☐

6.3.3.1 Name of subject	**Date**	**Signature**
6.3.3.2 Name of Person taking consent (if different from researcher)	**Date**	**Signature**
Siwi Pramatama Mars Wijayanti **6.3.3.3 Researcher**	**Date**	**Signature**

1 for subject; 1 for researcher

Note : If the patient are children, the consent form must be read and signed by their parent/guardian.

Appendix 3. Ethical Clearance from MVLS College Ethics Committee

University of Glasgow | College of Medical, Veterinary & Life Sciences

28 March 2014

Dear

MVLS College Ethics Committee

Project Title: INTEGRATED EPIDEMIOLOGICAL STUDY OF DENGUE CASES IN BANYUMAS REGENCY
Project No: 2012082

The College Ethics Committee has reviewed your application and has agreed that there is no objection on ethical grounds to the proposed study. They are happy therefore to approve the project, subject to the following conditions

- The research should be carried out only on the sites, and/or with the groups defined in the application.
- Any proposed changes in the protocol should be submitted for reassessment, except when it is necessary to change the protocol to eliminate hazard to the subjects or where the change involves only the administrative aspects of the project. The Ethics Committee should be informed of any such changes.
- If the study does not start within three years of the date of this letter, the project should be resubmitted.
- You should submit a short end of study report to the Ethics Committee within 3 months of completion.

Yours sincerely

Dorothy McKeegan

Dr Dorothy McKeegan
College Ethics Officer

Dr Dorothy McKeegan

Senior Lecturer

R303 Level 3
Institute of Biodiversity Animal Health and Comparative Medicine
Jarrett Building
Glasgow G61 1QH Tel: 0141 330 5712
E-mail: Dorothy.McKeegan@glasgow.ac.uk

Appendix 4. Ethical Clearance from Medical and Health Research Ethics Committee, Faculty of Medicine Gadjah Mada University

MINISTRY OF NATIONAL EDUCATION
FACULTY OF MEDICINE GADJAH MADA UNIVERSITY
MEDICAL AND HEALTH RESEARCH ETHICS COMMITTEE (MHREC)

ETHICS COMMITTEE APPROVAL

Ref : KE/FK/ 323 /EC

Title of the Research Protocol	:	Integrated Epidemiological Study of Dengue Cases in Banyumas Regency, Central Java
Documents Approved	:	1. Study Protocol 2. Information for Students 3. Informed Consent Form 4. Case Report Forms (CRF)
Principle Investigator	:	Siwi Pramatama Mars Wijayanti
Name of medically Responsible Physician(s)	:	-
Date of Approval	:	16 May 2012 (Valid for one year beginning from the date of approval)
Institution(s) / place(s) of research	:	Banyumas Regency, Central Java

The Medical and Health Research Ethics Committee (MHREC) states that the above protocol meets the ethical principle outlined in the Declaration of Helsinki 1975 and therefore can be carried out.

The Medical and Health Research Ethics Committee (MHREC) has the right to monitor the research activities at any time. The investigator(s) is/are obliged to submit final report upon the completion of the study or a progress report in case a continuing review is needed.

(signature)

Prof. dr. Mohammad Hakimi, Sp OG(K), Ph.D
Chairman

(signature)

dr. Madarina Julia, Sp.A(K), MPH, Ph.D
Secretary

AUTHORIZED & SWORN TRANSLATOR
S.K. GUB. KDKI. JKT.
Dra. ESTIA MAHYUDIN
No. 6[illegible]
PENERJEMAH RESMI & DISUMPH

MINISTRY OF EDUCATION AND CULTURE
FACULTY OF MEDICINE GADJAH MADA UNIVERSITY
MEDICAL AND HEALTH RESEARCH ETHICS COMMITTEE (MHREC)

CONTINUING REVIEW APPROVAL OF APPROVAL

Ref: KE/FK/323/EC Year 2012

Ref : KE/FK/ 466 /EC

Title of the Research Protocol	:	Studi Epidemiologi Terintegrasi Kasus Demam Berdarah di Kabupaten Banyumas Jawa Tengah
Documents Approved	:	1. Study Protocol continuing review 2013 2. Information for Subjects Approved May 2012 3. Informed consent form Approved May 2012
Principle Investigator	:	Siwi Pramatama Mars Wijayanti
Date of Approval	:	10 MAY 2013 (Valid for one year beginning from the date of approval)
Institution(s)/place(s) of research	:	Kabupaten Banyumas Jawa Tengah

The Medical and Health Research Ethics Committee (MHREC) states that the above protocol meets the ethical principle outlined in the Declaration of Helsinki 2008 and therefore can be carried out.

The Medical and Health Research Ethics Committee (MHREC) has the right to monitor the research activities at any time.

The investigator(s) is/are obliged to submit:
- ☐ Progress report as a continuing review : Annually
- ☐ Report of any serious adverse events (SAE)
- ☑ Final report upon the completion of the study

Prof.dr.Mohammad Hakimi, Sp.OG (K),Ph.D
Chairman

Dr. dr. Eti Nurwening Sholikhah, M.Kes
Secretary

Attachments:
- ☐ Continuing review submission form (AF 4.3.01-014.2012-02)
- ☐ Serious adverse events (SAE) report form (AF 6.1.01- 019.2012-02)

MEDICAL AND HEALTH RESEARCH ETHICS COMMITTEE (MHREC)
FACULTY OF MEDICINE GADJAH MADA UNIVERSITY
– DR. SARDJITO GENERAL HOSPITAL

CONTINUING REVIEW APPROVAL OF APPROVAL

Ref: KE/FK/323/EC Year 2012

Ref : KE/FK/ 729 /EC

Title of the Research Protocol	:	Studi Epidemiologi Terintegrasi Kasus Demam Berdarah di Kabupaten Banyumas Jawa Tengah
Documents Approved	:	1. Study Protocol continuing review 2015 2. Information for Subjects Approved May 2012 3. Informed consent form Approved May 2012
Principle Investigator	:	Siwi Pramatama Mars Wijayanti
Date of Approval	:	18 JUN 2015 (Valid for one year beginning from the date of approval)
Institution(s)/place(s) of research	:	Kabupaten Banyumas Jawa Tengah

The Medical and Health Research Ethics Committee (MHREC) states that the above protocol meets the ethical principle outlined in the Declaration of Helsinki 2008 and therefore can be carried out.

The Medical and Health Research Ethics Committee (MHREC) has the right to monitor the research activities at any time.

The investigator(s) is/are obliged to submit:
□ Progress report as a continuing review : Annually
□ Report of any serious adverse events (SAE)
☒ Final report upon the completion of the study

Prof. dr. Mohammad Hakimi, Sp.OG (K), Ph.D
Chairman

Dr. dr. Eti Nurwening Sholikhah, M.Kes
Secretary

Attachments:
□ Continuing review submission form (AF 4.3.01-014.2012-02)
□ Serious adverse events (SAE) report form (AF 6.1.01- 019.2012-02)

Appendix 5. Memorandum of Understanding

University of Glasgow

MEMORANDUM OF UNDERSTANDING
BETWEEN
THE UNIVERSITY COURT OF THE UNIVERSITY OF GLASGOW, UNITED KINGDOM
AND
FACULTY OF MEDICINE AND HEALTH SCIENCES, JENDERAL SOEDIRMAN UNIVERSITY, INDONESIA

The University Court of the University of Glasgow's MRC Centre for Virus Research ("CVR") and Faculty of Medicine and Health Sciences, Jenderal Soedirman University, Indonesia have come to this agreement to facilitate academic exchange between the two universities with a view to promoting academic studies in various fields, as described below :

1. CVR and Faculty of Medicine and Health Sciences, Jenderal Soedirman University, Indonesia shall facilitate the following activities regarding academic exchange.
   a. Exchange of faculty members and research scholars
   b. Joint research activities
   c. Research collaboration, academic publication, and other information
2. Any specific activity, related to those listed above, will be carried out after consultation between the two universities.
3. This agreement shall not bind either university to any financial commitment.
4. Both Parties agree that prior written approval is required before using the other Party's name, logo, or other Intellectual Property rights in any advertising or associated publicity.

5. This agreement shall become effective when the appropriate representative of the two universities affix their signature below.
6. This agreement shall remain effective for five years (September 2013-2017)
7. Extension or amendment of this agreement, or termination prior to the end of the 5-years period, is to come into effect after deliberation by both universities, and after formal written notice is given by one or both universities to the other, six (6) months prior to the extension, amendment or termination of this agreement.
8. The Parties may disclose certain confidential information to the other in relation to any future proposal made under this agreement. Each Party therefore agrees that the contents of this agreement and the negotiations in relation to any future proposal remain strictly confidential and each Party hereby undertakes not to disclose the same to any third Party, save for its professional advisers, without the prior written consent of the other Party except where such disclosure is required by law (including, without limitation, under applicable freedom of information legislation).

5 September 2013

For and on behalf of the University Court of the University of Glasgow

Director of Centre of Virus Research
University of Glasgow, United Kingdom

Dean of Faculty of Medicine and Health Sciences
Jenderal Soedirman University, Indonesia

PAUL GRIFFIN
SENIOR CONTRACTS MGR

Prof. Massimo Palmarini

dr. Hj. Retno Widiastuti, MS

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