

Transovarial transmission of dengue virus on *Aedes aegypti* at several endemic areas of dengue haemorrhagic fever in Indonesia

U K Hadi¹, S Soviana¹, and Sugiarto²

¹ Division of Parasitology and Medical Entomology, Faculty of Veterinary Medicine, Bogor Agricultural University, Bogor 16880 Indonesia

² Subdirector of Vector Control, Ministry of Health Republic of Indonesia, Jakarta, Indonesia

Email: upikke@ipb.ac.id

Abstract. Transovarial transmission of dengue virus (DENV) on *Aedes aegypti* is reported as other mechanisms that played a role in the transmission of dengue hemorrhagic fever (DHF). The aim of research to determine the prevalence of vertical transmission in *Ae. aegypti* from in several endemic areas of dengue in Indonesia. Sample of mosquitoes were collected from at least 25 locations in West Java and 29 locations outer of West Java (Palembang South Sumatra, Banda Aceh, Lombok West Nusa Tenggara, and Ternate Island) by using ovitraps. Then eggs obtained were hatched in the laboratory to be larvae and become adults. The virus detection from the adult specimens were done by using RT-PCR. The results showed the DENV transovarial transmission in *Ae. aegypti* in three endemic areas of dengue in West Java (25 isolates), for a while still negatives because the mosquitoes from the area Bogor, Sukabumi and Tasikmalaya examined were not found to contain DENV. Meanwhile, of the 29 isolates in the outer West Java (Palembang South Sumatra, Banda Aceh, Lombok West Nusa Tenggara, and Ternate Island), 8 of them were detected to have DENV-2. DENV transovarial transmission were not be detected in West Java because of many factors, such as the nature of DENV was very labile to temperature, humidity and chemical factors, as well as a short period of viremia that affect the success rate of DENV transmission to *Ae. aegypti*.

Keywords: *Aedes aegypti*, transovarial transmission, endemic area, dengue virus

1. Introduction

Dengue Hemorrhagic Fever (DHF) is a health problem that is a major concern in Indonesia. From 1968 until 2009, DHF cases in Indonesia were belong to the first ranks with the highest number of people affected in Southeast Asia.¹ The dengue viruses (DENV) are transmitted by *Ae. aegypti*(L.) and *Ae. albopictus* (Skuse).² *Aedes aegypti*, as the main vector is closely related to the human environment because its larval stages breed in containers such as bathtubs, drums, tires, gutter, flower vases, and other containers around the settlements.³ Although there are various vaccine tests against DHF, there is still no promising vaccine that has been found to be the most effective. Therefore, the main ways of preventing and controlling DHF are to attempt to break the life cycle of mosquitoes, to reduce the larval breeding places and mosquito, and to prevent human contact with mosquitoes.⁴ Human behavior is one of the important factors that can influence the epidemiology of dengue fever. Therefore, local vector profiles and control strategies will depend on certain



socioeconomic conditions, and behavioral characteristics of the population.⁵ Successful vector control requires a detailed locally specific vector bioecological knowledge that fits the conditions of society.⁶ Current environment-based DHF control program is still weak so that the incidence of dengue is always repeated throughout the year, even an extraordinary incident during the rainy season arrives.

Transmission of dengue occurs horizontally from mosquito to humans, and also occurs vertically from mosquitoes to mosquitoes (transovarial). Transovarial transmission of all serotypes of dengue virus (4 strains) is reported to occur in *Ae. aegypti* and *Ae. albopictus*.⁷ Dengue 2 virus was found in three groups of 123 groups of *Aedes* larvae naturally infected (6,200 insects) collected from water containers in Rangoon.⁸ In Thailand, DENV is detected on *Aedes* by RT-PCR technique. The mosquitoes were collected in four provinces of Krabi, Phuket, Phang-Nga and Surat Thani during the late dry season until the beginning of the rainy season in 2005. Three dengue serotypes (DEN-2, DEN-3, DEN-4) were detected on *Ae. aegypti* male and female, and 2 serotypes (DEN-2, DEN-3) were detected on *Ae. albopictus* female. Multiple infections with 2 serotypes of dengue virus (DEN-2 and DEN-3) were detected on *Ae. aegypti* male and female, and *Ae. albopictus* female. The DEN-2 and DEN-1 viruses were the most common serotypes found in patient sera in the area, followed by DEN-4 and DEN-3 viruses. The prevalence of dominant dengue serotypes varies from province to province.⁹

In Indonesia, studies related to transovarial transmission in some locations have been conducted. However, in order to understand more about the epidemiology of DHF, studies of dengue virus types in vector bodies with natural conditions should be performed. The purpose of this research was to study the transovarial transmission of dengue virus in *Ae. aegypti* collected from dengue endemic areas in several regions of Indonesia. Information to be gained can be useful in efforts to control dengue diseases, especially regarding the circulation of dengue virus in vectors in the natural environment in endemic areas.

2. Material and methods

2.1. Location and time of study

A sample of *Ae. aegypti* eggs was done from February to April 2015. Transovarial detection of transmission from larvae was done after passing the rearing process into larvae. Hatching of eggs into larvae was done at the insectarium of Medical Entomology Laboratory, Faculty of Veterinary Medicine IPB Bogor. While detection of the presence of viruses (DENV) from the larvae *Aedes* was done at Eijkman Institute Jakarta. The research sites were Bogor (Baranangsiang and Bojonegara), Sukabumi (Cisarua), Tasikmalaya (Cikalang and Bungursari), Palembang South Sumatra, Banda Aceh, Lombok West Nusa Tenggara, and Ternate Island. Each area was randomly indoor and outdoor inspected of the house to collect *Ae. aegypti* mosquito larvae and laying the trap of a mosquito egg (ovitrap) for 5–7 days.

2.2. Mosquito egg collection

Methods of collecting eggs was done using ovitrap. The use of ovitrap by filling water as much as half of the container, then affixed filter paper around the walls in the ovitrap that serves as a medium for laying mosquito eggs. Ovitrap was placed indoor and outdoor especially in dark and humid places suspected as mosquito resting, such as under table, chair, bed, etc. After 5–7 days of examination of the presence or absence of mosquito eggs attached to filtered paper. Eggs contained on filter paper rearing to become mosquitoes. The larvae were reared in the laboratory according to the location sites. The mosquitoes that have been reared were then identified in the species, then tested by RT-PCR method to detect the presence of DENV transovarial transmission.

2.3. Detection of dengue virus in larva and mosquito

Detection of DEN virus, in mosquito larvae was performed using RT-PCR method which included viral RNA extraction and RT-PCR testing. Mosquito sample was inserted into eppendorf tube and crushed using pestle and then inserted BA1 medium. As many as 10–25 mosquitoes can be added 1 µl

BA1; 5–10 mosquitoes added 500 µl BA1. The scours were centrifuged at 10.000 rpm for 3 min, then 140 µl supernatant was taken for viral RNA extraction.¹⁰

The first step of 560 µl lysis mix consisting of 560 µl AVL and 5.6 µl RNA-carrier was inserted into 1.5 ml eppendorf tube and sample is inserted 140 µl and divortex for 10 sec, the mixture is then incubated for 5 min at temperature room. The next step was to add 560 µl of ethanol to the mixture and divortex for 15 seconds and centrifuged for 5 seconds. In the third step, 630 µl of the mixed solution was inserted into a spin column that has been attached to the collection tube and centrifuged for 30 seconds. After that, the spin column was removed from the centrifuge, the collection tube containing the filtrate was removed and the spin column was re-inserted into the new collection tube.

The next step was to add 600 µl AW1, then centrifuged for 30 seconds, collection tube was removed and replaced, then added 600 µl AW2, and centrifuged at maximum speed and then centrifuged for 30 seconds. The collection tube was again discarded and the spin column inserted into the eppendorf tube and added AVE 60 µl buffer right in the middle without touching the wall, incubated for 3 minutes and centrifuge at 10.000 rpm for 1 minute. After that the spin column was removed and the microcentrifuge tube containing the extracted RNA can be directly used as RT-PCR template or the extraction result can be stored at -80°C.

2.4. Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Techniques used to detect DEN virus using primary base pair. Detection of DEN virus using generic primary D1 virus as primary forward (5'TCAATATGCTGAAACGCGCGAGAAACCG3 ') and primary virus D2 as reverse primer (5' TTGCACCAAGAGTCAATGTCTTCAGGTTC3 ').

The first amplification was performed using a PCR kit (Invitrogen). The PCR stage was preceded by a reverse transcription stage performed within 60 minutes at 50 °C to produce cDNA then continued with 35 denaturation cycles at 94 °C for 30 seconds, then annealing at 55 °C for 1 minute and extending at 68 °C for 2 minutes and ends with a final extension at a temperature of 68 °C. At the first amplification prepared RNA targets to be amplified at 25 µl volumes with mixed material compositions that have been added Master Mix PCR. The mixture of Master mix PCR was prepared on cold block containing mixture with composition in dH₂O, buffer, Primer D1, Primer D2 and Enzyme. The prepared Master Mix was inserted as much as 20 µl into the PCR tube and then added 5 µl template for each sample. The tube containing the mixture of Master Mix PCR is centrifuged and inserted into PCR.

2.5. Electrophoresis

The amplification results were then electrophoresed to detect DEN virus. This process was done by making gelose 1%. At first 100 ml of TAE buffer was mixed with 1 gram agarose, then heated in microwave until homogeneous and cooled in running water. Then 5 µl of ethidium bromide was added and stirred until blended.

After the agarose gel solidifies, it was inserted into chamber electrophoresis that has been filled TAE buffer. Then 5µl of PCR product mixed with 6x dye loading on paraffin paper and put into well on agarose gel, then 5 µl DNA Ladder 1 kb; 5 µl positive control and 5 µl positive control. Positive control is the result of PCR amplification containing DNA corresponding to the DEN virus serotype obtained from the Ministry of Health of the Republic of Indonesia, whereas the negative control was the result of PCR amplification containing the mixed master mix RNA free water. Electrophoresis was done with power supply at position 120 V for 45 minutes, during this electrophoresis process DNA will move from negative pole to positive pole.

2.6. Visualization

Gel agarose was incorporated into the ultra violet transluminator to see the amplification result. The molecular bands visible on the agarose gel represent a DNA segment, then the DNA bands were compared to the bands on the positive controls and markers.

3. Results and discussion

DENV transovarial transmission data is very useful in dengue disease survey and to prevent and anticipate the outbreak of dengue hemorrhagic fever (DHF). In this paper, we report the result of examined DENV transovarial transmission of *Ae. aegypti* from dengue endemic areas in Indonesia namely Bogor City, Sukabumi, Tasikmalaya, Lombok, Banda Aceh, Palembang South Sumatra, and Ternate Island.

The result were presented in table 1 from West Java (Bogor City, Sukabumi, Tasikmalaya) and in table 2 from Ternate Island, and in table 3 from Lombok, Banda Aceh, and South Sumatra. Table 1 indicated that the DENV transovarial transmission of *Ae. aegypti* in three dengue endemic areas i.e. in Bogor (5 sites in Baranang Siang and 3 sites in Bojong Kerta), in Sukabumi (9 sites), and Tasikmalaya (8 sites) were all or not found (negative).

Table 1. Dengue virus detection result in *Ae. aegypti* mosquito by RT-PCR method in DHF endemic areas of West Java.

No	Date of sampling	Name of Site (Isolate)	City	DENV Transovarial Transmission
1	25/04/2015	Baranang Siang #1	Bogor	Negative
2	25/04/2015	Baranang Siang #2	Bogor	Negative
3	25/4/2015	Baranang Siang #3	Bogor	Negative
4	25/4/2015	Baranang Siang #4	Bogor	Negative
5	25/4/2015	Baranang Siang #5	Bogor	Negative
6	27/4/2015	Bojong Kerta #1	Bogor	Negative
7	27/4/2015	Bojong Kerta #2	Bogor	Negative
8	27/4/2015	Bojong Kerta #3	Bogor	Negative
9	15/5/2015	Cisarua #1	Sukabumi	Negative
10	15/5/2015	Cisarua #2	Sukabumi	Negative
11	15/5/2015	Cisarua #3	Sukabumi	Negative
12	15/5/2015	Cisarua #4	Sukabumi	Negative
13	15/5/2015	Cisarua #5	Sukabumi	Negative
14	18/5/2015	Selabatu #1	Sukabumi	Negative
15	18/5/2015	Selabatu #2	Sukabumi	Negative
16	18/5/2015	Selabatu #3	Sukabumi	Negative
17	18/5/2015	Selabatu #4	Sukabumi	Negative
18	2/6/2015	Cikalang #1	Tasikmalaya	Negative
19	2/6/2015	Cikalang #2	Tasikmalaya	Negative
20	2/6/2015	Cikalang #3	Tasikmalaya	Negative
21	2/6/2015	Cikalang #4	Tasikmalaya	Negative
22	3/6/2015	Bungursari #1	Tasikmalaya	Negative
23	3/6/2015	Bungursari #2	Tasikmalaya	Negative
24	3/6/2015	Bungursari #3	Tasikmalaya	Negative
25	3/6/2015	Bungursari #4	Tasikmalaya	Negative

Table 2 showed the results of virus detection research on mosquitoes from Ternate Island. The result showed that among 21 sites *Ae. aegypti* collected, there were 5 sites positives DENV transovarial transmission serotype-2. Isolate *Ae. aegypti* positive was Sulamadaha (Ternate), Kulaba#1 (Ternate), Tafure (Ternate) (figur 1). Figure 2 showed that the positive *Ae aegypti* isolates from Dufadufa (Ternate), Bastion Karance (Ternate),

Table 2. Results of dengue virus detection on *Ae. aegypti* with RT-PCR method in Ternate.

No.	Name of Site (Isolate)	City	DENV Transovarial Transmission	Type of DENV
1	Akehuda	Ternate	Negative	
2	Sulamadaha	Ternate	Positive	DEN - 2
3	Kulaba #1	Ternate	Positive	DEN - 2
4	Takome	Ternate	Negative	
5	Jati	Ternate	Negative	
6	Kulaba #2	Ternate	Negative	
7	Mangga Dua	Ternate	Negative	
8	Tafure	Ternate	Positive	DEN - 2
9	Santiong	Ternate	Negative	
10	Kota Baru	Ternate	Negative	
11	Tanah tinggi	Ternate	Negative	
12	Jambula	Ternate	Negative	
13	Dufa – Dufa	Ternate	Positive	DEN - 2
14	Bastiong Karance	Ternate	Positive	DEN - 2
15	Salahudin	Ternate	Negative	
16	Salero	Ternate	Negative	
17	Gambesi	Ternate	Negative	
18	Tasik #1	Ternate	Negative	
19	Tasik #3	Ternate	Negative	
20	Tasik #2	Ternate	Negative	

The results of dengue virus detection on *Ae.aegypti* in Banda Aceh, Palembang South Sumatra and Lombok was presented in table 3. DENV transovarial transmission serotype 2 were found in 3 sites of *Ae. aegypti* collected from Palembang #1, 2 and 4 (figur 2).

Table 3. Results of dengue virus detection on *Ae. aegypti* with RT-PCR method in Banda Aceh, Palembang and Lombok.

No.	Name of Site (Isolate)	City	DENV Transovarial Transmission	Type of DENV
1	Sukaramai	Banda Aceh	Negative	
2	Palembang #1	Palembang	Positive	DEN - 2
3	Palembang #3	Palembang	Negative	
4	Palembang #5	Palembang	Negative	
5	Berare #2	Lombok	Negative	
6	Berare #4	Lombok	Negative	
7	Berare #5	Lombok	Negative	
8	Palembang#4	Palembang	Positive	DEN – 2
9	Palembang#2	Palembang	Positive	DEN – 2

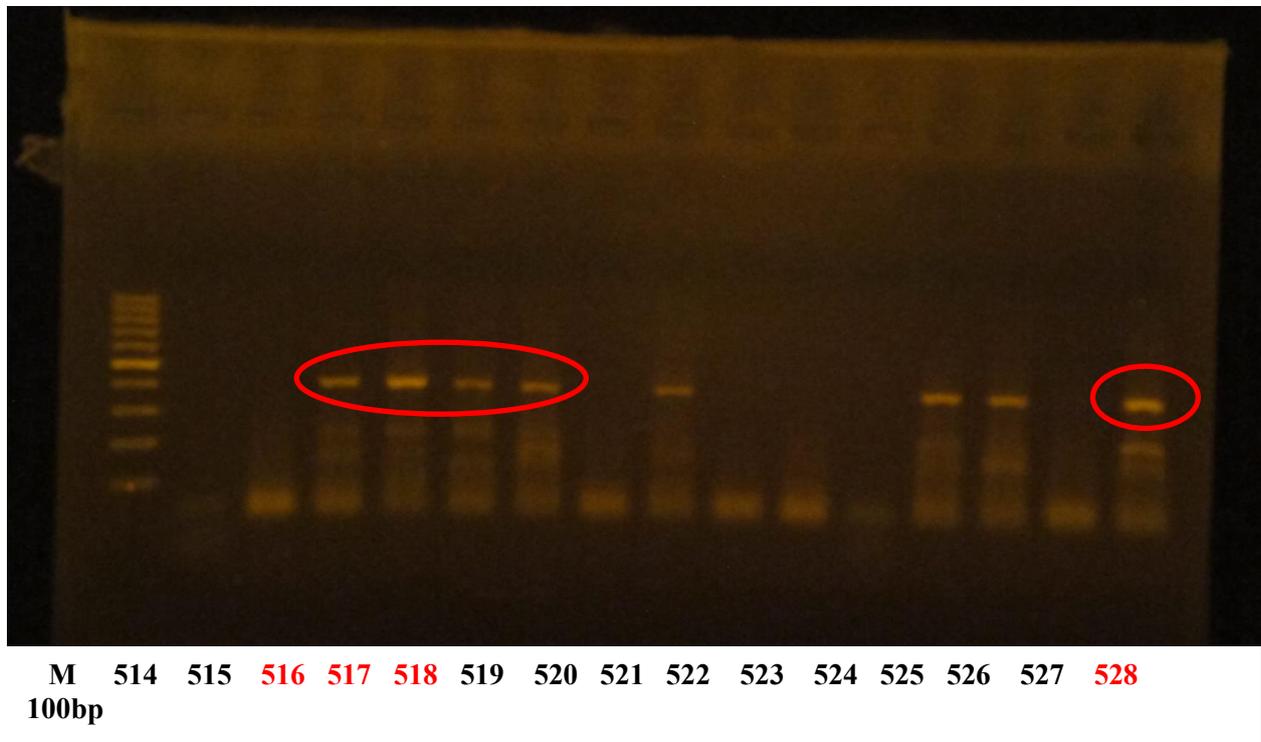


Figure 1. The number marked red shows the codes of isolates *Ae. aegypti* containing DEN-2 virus.

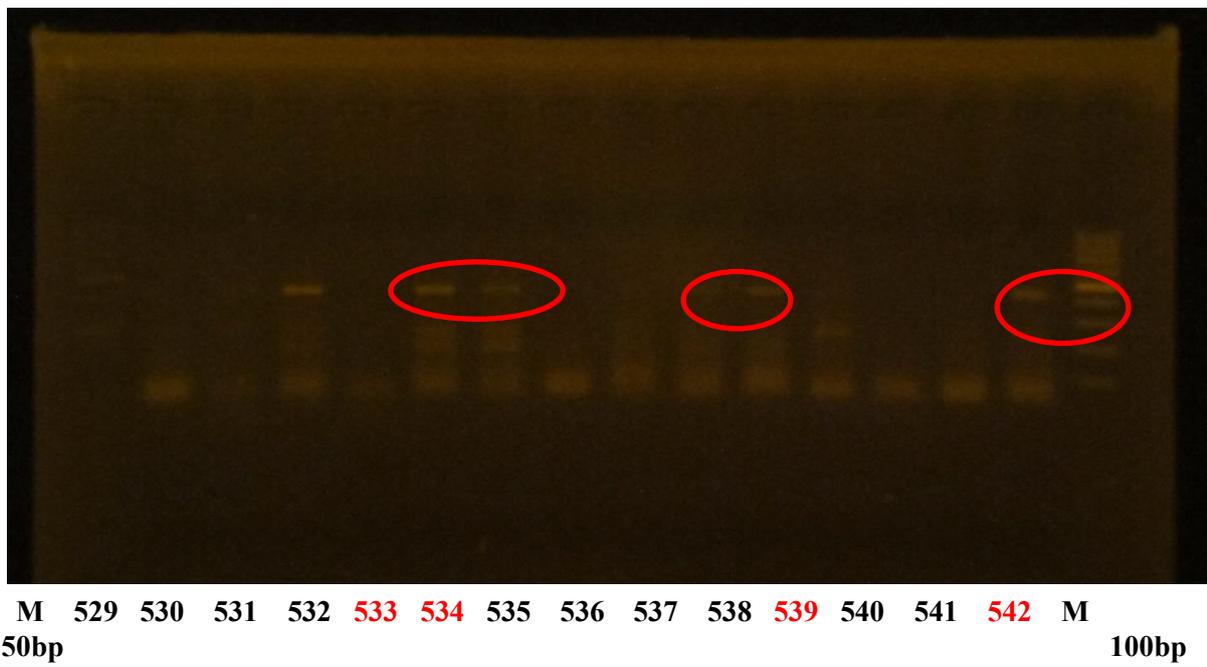


Figure 2. The number marked red shows the codes of isolates *Ae. aegypti* containing DEN-2 virus.

Detection results on *Ae. aegypti* collected from several areas in West Java showed that DENV transovarial transmission in three dengue endemic areas (25 isolates / point locations) were negative. *Ae. aegypti* F1-F2 culture results from 5 isolates in Baranang Siang and 3 isolates in Bojong Kerta (Bogor city area) did not contain DENV, the same result was also shown on mosquito isolates from Sukabumi (9 isolates) and Tasikmalaya (8 isolates).

The result of PCR analysis from 29 isolates from outside of West Java showed positive result of Den-2 virus from 5 isolates of Ternate Island mosquitoes (Sulamadaha, Kulaba, Tafure, Dufa-dufa, Karance Bastion) and 2 isolates from South Sumatera (Palembang). The findings of the study indicated that virus strains were circulated in nature among *Ae. aegypti* mosquitoes in Palembang South Sumatera and Ternate Island. This is one of the reasons why dengue fever occurs throughout the year in that regions.

No evidence of transovarial transmission among *Ae. aegypti* was found in three endemic cities in West Java. The absence of DENV transovarial transmission can be caused by many factors, namely the highly labile nature of DENV to temperature, humidity and chemical factors, and a shortened viremia period affecting the success rate of DENV transmission to *Ae. aegypti*.¹¹WHO¹ states that DENV takes 8-10 days to complete the extrinsic incubation period from the stomach to the salivary glands of the mosquito. The correct time interval for detecting DENV in *Aedes* sp. ranged from 7 to 41 days after the appearance of the first clinical symptoms in humans.¹²The period of DENV viremia is short so that the success rate of DENV isolation from *Ae. aegypti* is highly dependent on the speed and accuracy of the sampling at the endemic location of the DHF. Relatively higher or lower humidity and humidity are thought to reduce the viability of DENV to live within the body of the mosquito itself.^{11,14}

4. Conclusions

DENV detection on *Ae. aegypti* in three dengue endemic areas in West Java gave negative result because mosquitoes from Bogor area (8 isolates), Sukabumi (9 isolates), and Tasikmalaya (8 isolates) examined were not found to contain DENV. Meanwhile, out of 29 isolates outside West Java (Palembang South Sumatera, Banda Aceh, Lombok West Nusa Tenggara, and Ternate Island), 8 isolates were positive DENV-2 transovarial transmission.

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