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Detection of Transovarial Dengue Virus with *RT-PCR* in *Aedes albopictus* (Skuse) Larvae Inhabiting Phytotelmata in Endemic DHF Areas in West Sumatra, Indonesia

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Abstract Transovarial dengue virus detection by RT-PCR in *Ae.albopictus* larvae that inhabit Phytotelmata has been conducted in several dengue endemic areas in West Sumatra. The purpose of this study was to determine whether larval *Ae. albopictus* inhabiting Phytotelmata is a potential transmitter of Dengue Hemorrhagic Fever (DHF). The results concluded that *Ae.albopictus* larvae which inhabited Phytotelmata positively contained two serotypes of dengue virus (DEN-1) and (DEN-4) which has the potential to transmit and spread DHF.

Keywords: *transovarial, phyotelmata, DHF, Aedes albopictus*

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1. Introduction

Mosquitoes are one of the most important insects because of their role in the transmission of dengue virus. Virus transmission can occur horizontally within a cycle from mosquitoes as vectors to humans (human-mosquito-human). Additionally, vertical or transovarial transmission can also occur. Unlike horizontal transmission that depends on human-mosquito contact; transovarial transmission happens through viral entry into the body of female mosquitoes; infecting the ovary and then the produced eggs. Transovarial transmission is when mosquitoes born from infected eggs contain the same virus astheir parent(Akbar *et al*., 2008; Gunther *et al*., 2007).

West Sumatra is one of the dengue endemic areas in Indonesia, from the total of 19 cities and regencies in West Sumatra; 17 of them are dengue endemic areas (Provincial Health Office, 2010). Factors affecting the occurrence of dengue cases in an area are host factors such as people's behavior, pathogenic factors, and environmental factors such as vector breeding places around human settlements, both natural and artificial (Supartha, 2008). Phytotelmata plants found in these human settlements are one of the natural breeding places for the vectors.

Phytotelmata is a type of plant that can hold puddle of water within the plant or within the plant's parts (Kitching, 1971; Marguire, 1971, Fish, 1983). This plant, diverse in kind, can be found living anywhere. It is believed that it flourishes in damp places such as in tropical areas (Greeney, 2001). There are approximately 1500 plant species, categorized into 29 families and 60 genus, having the ability to hold puddle, that are classified into Phytotelmata. These plants are inhabited by various organisms including mosquitoes (Fish, 1983). More than 70 insect families that are classified into 11 orders inhabit Phytotelmata, and the most common of them found in this plant is the order of Diptera (Greeney, 2001). Previous observation of Phytotelmata reveals that *Aedes albopictus* is the most common mosquito found inhabiting Phytotelmata; usually in the egg or larval stage.

In Indonesia, the main vector of dengue fever is *Ae.aegypti* and the potential vector is *Ae.albopictus* (Fathi *et.al.*, 2005). Studies of transovarial transmission of the virus are still confined to mosquitoes that living artificial containerswithin or outside the house (Khin&Than in Myanmar (1983), Joshi *et al*., (1996) in India; Lee *et.al*., in Malaysia (1997); Kow *et.al*., in Singapore (2001), Akbar (2008) and Hasmiwati *et.al*., (2010)).

There is still no information available on the transovarial virus transmission in *Ae.albopictus* that inhabits the Phytotelmata, particularly in human settlements. Therefore, this study was conducted to determine the transovarial virus transmission in *Ae. albopictus* larvae that inhabit Phytotelmata through the detection of dengue virus using RT-PCR.

2. Materials and Methods

Sampling was conducted in the cities of Padang, Bukittinggi and Payakumbuh. Identification of mosquito larvae was performed in the Animal Taxonomy Research

Laboratory, Department of Biology, Andalas University and the detection of the virus was carried outin Biomedical Laboratory, Faculty of Medicine, Andalas University.

Larval sampling techniques used in this study were introduced by Derraik (2005). Samples were taken to the laboratory for sorting, separated mosquito larvae were identified using several identification key books. Larval samples analyzed for the purpose of dengue virus detection were *Aedes albopictus* larvae which were in the fourth larvae stage. Before examination, larvae were kept at a temperature of-80^{o}C. There were twenty larvae which were going to be detected, which referred to Gunther *et al.*, (2007) and Hasmiwati *et. al.*,(2010). Virus detection was done in several phases: virus isolation, cDNA *Reverse Transcriptase*, amplification and electrophoresis.

Virus isolation stage used a modified Qiagen Viral Preparation kit (*Qi Aamp ® Viral RNA Mini Kit*) referring to that stated by Hasmiwati *et.al*., (2010). The procedure is as follows:

Twenty *Ae.albopictus* larvae in stage IV which have been stored in the freezer at -80^{o}Cwere put into micro tube (Eppendorf).Samples were then soaked in 100 µl PBS buffer (*Phosphate Buffered Saline*) and then grinded with a plastic pistil. The next step was to add 560 µl AVL buffers which have been heated at temperature 80^{o}C and 5.6 AVE and RNA carrier into the grinded sample, put in vortex for fifteen seconds. Add 560 µL ethanol (96-100%) to the mixture then put the mixture in the vortex again for fifteen seconds. After that samples were centrifuged at 6000 rpm for oneminute. 630 µL of solution were put into a 2 ml micro tube cap, and then centrifuged at 8000 rpm for one minute. The solution was then moved to a new micro tube, added with 500 µL AW1 and centrifuged at 8000 rpm for 1 minute. Supernatant was also moved to a new microtube, added with 500 µL of AW2 and centrifugedat 14.000 rpm for three minutes. It was pipetted into a new micro tube,addedwith 60 µL buffer AVE and then incubated at room temperature for one minute. After incubation it was centrifuged at 8000 rpm for another one minute. RNA virus as the result from isolation was stored in a freezer at -80°C.

Reverse Stage - Transcriptase of cDNA was performed because the samples used werenot a segment of DNA but a segment of RNA. Therefore, before amplification, it must first be converted to cDNA (DNA complement) with the reverse transcriptase. *Reverse transcriptase* was performed using *iScript TM cDNA Synthesis Kit*cDNA(BIORAD Laboratories). After successfully obtaining the cDNA, it can first be stored at-20°C or amplification process could directly be performed.

Amplification Stage, cDNA was amplified with *multiplex* PCR using RTG (*Ready to Go*) PCR in which already contained the components of the PCR amplification: PCR buffer, MgCl2, and Tag DNA polymerase enzyme. Amplification of RNA dengue virus was performed by RT-PCR using specific primers with serotypes 1, 2, 3, and 4 (Harris *et.al.*, 1998; Johnson *et.al*., 2005. PCR profile was performed through several stages: one cycle of initial denaturation at 95°C for two minutes, denaturation at 95°C for thirty minutes, annealing at 55°C for thirty minutes, extension at 72°C for one minute, final extension at 72°C for ten seconds and cooling process at 20°C for fifteen seconds.

Primer sequences were as follows: DF, 5'TCA TGC TGA AAC ATA GAA ACC CGA GCG 3 '; DR1, 5' CGT GGG CCG CTC AGT GAT G 3 '; DR2, 5' GGC CGC CAC CAT GAA CAG AAG 3 '; DR3, 5 'CAT CATCAT TAA ACA GAT GAG C 3'; DR4, 5 'TGT TGT CTT AAA CAA GAG AGG TC 3'.

2.1. Electrophoresis and Documentation of PCR Results

DNA fragments, result of the amplification process, were separated using electrophoresis. The electrophoresis process was performed by putting DNA fragments, results of the amplification process, into a 2% agarose gel colored with ethidium bromide in 0.5 x TBE buffer (Tris-Borate EDTA) at 60 volts for 1.5 hours (Sambrook *et.al*., 1989).A 1% Agarose gel was prepared by dissolving 1 gram of agarose powder into 100 ml of TBE (Tris-Borate EDTA O, 5x). Solution was cooked in the microwave for 1.5 minutes at a high temperature, and then poured into molds that have been restricted with comb, until it reached a thickness of 0.5 cm. This process was done in order to produce wells.After the gel has hardened, the barrier was opened and the comb was removed. The hardened gel along with its mold was placed in anelectrophoresis chamber; 300ml of TBE 0.5 x solutions was added until the gel was covered. To see the formed DNA fragments on agarose gel, the gel electrophoresis results were captured using Doc^{TM}xR Imaging GEL Bio Rad system. The gel was placed over a UV Transluminator and was photographed for documentation of results. Data obtained from the extraction by RT - PCR method were based on the description of the fragment (band) in corresponding base pairs (bp), after electrophoresis was done. Expected sizes of bands from amplification were 482 bp for (DEN-1), 119 bp (DEN-2), 290 bp (DEN -3),and 389 bp (DEN-4) (Harris *etal*.,1998). If the DNA was clearand the band size is in accordance with DNA ladder (as a marker), itis consideredas a positive result. Variations in size of DNA show virus type.

3. Result

The results indicated the presence of positively infected *Ae. albopictus* larvae by dengue virus. This is evident from the result picture of electro photogram using the GEL Doc^{TM}xR Imaging System, Bio Rad: a clear picture of the DNA and its bandwith the same size as the marker bands. This proves that within the larvae of *Ae.albopictus* that inhabited Phytotelmata, vertical transmission/transovarial transmissiom has occurred, such as shown in (Figure 1).

From examination of sample 1 and 4, the results of electro photogram indicatedthat sample 4 positively contained both serotypes of dengue virus. Both serotypes were believedas DEN-1 because the position of the band was at 482 bp(located in the range between 400 bp and 500bp), and as DEN-4 with band positioned at 389 bp(located in the range between 300 bp and 400 bp of DNA marker).

From examination of sample 1 to 9 (Figure 2), electro photogram results displayed that only sample-1 and sample-2 were positively detected carrying dengue virus serotype-1 (DEN-1), with position of DNA bands at 482 bp or between 400 bp and 500 bp

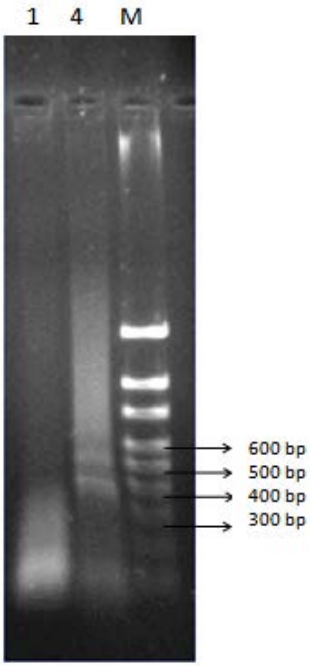

Figure 1. Results of electro photogram of dengue virus investigation by PCR on larvae of *Ae.albopictus* in Phytotelmata in Bukittinggi and Payakumbuh

Description:1. Sample larvae inhabiting taro Bukitinggi
4. Sample larvae inhabiting taro Payakumbuh
M.Marker

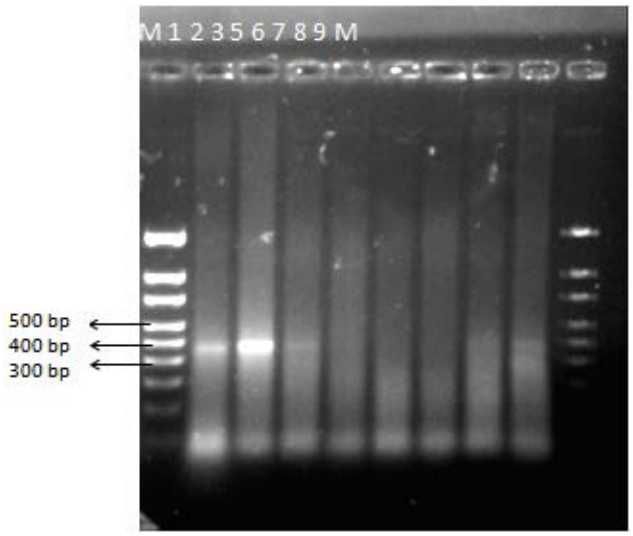

Figure 2. Results of Electro photogram examination of dengue virus by PCR on mosquito larvae of *Ae.albopictus* in phytotelmata in the city of Padang, Bukittinggi and Payakumbuh

Description: M. Marker
1. Larvae Sample inhabiting taro plant in Bukittinggi
2. Larvae Sample inhabiting bamboo stumps inPayakumbuh
3. Larvae Sample inhabiting bamboo stumps in Padang
5. Larvae Sample inhabiting taro plant in Padang
6. Larvae Sample inhabiting *pandan* plant in Padang
7. Larvae Sample inhabiting pineapple in Payakumbuh
8. Larvae Sample inhabiting *pandan* plant in Payakumbuh
9. Larvae Sample inhabiting *pandan* plant in Bukittinggi

Table 1. Base pair amplification result size from the larvae *Ae.albopictus* sample that inhabit Phytotelmata in dengue endemic areas in West Sumatra (using four specific primers)

Primer	Sequences	bp	1	2	3	4	5	6	7	8	9
DR1	5'CGTCTCAGTGAT CCG GGG G3'	535	-	-	-	+	-	-	-	-	-
		482	-	-	-	-	-	-	-	-	-
		476	-	+	-	-	-	-	-	-	-
		466	+	-	-	-	-	-	-	-	-
DR2	5'CGCCACAAGGGCCATGAACAG 3'	119	-	-	-	-	-	-	-	-	-
DR3	5'TAA CAT CAT CAT GAT ACA GAGC 3´	290	-	-	-	-	-	-	-	-	-
DR4	5'TGT TGT CTT AAA CAA GAG AGG TC 3´	416	-	-	-	+	-	-	-	-	-
		389	-	-	-	-	-	-	-	-	-

Description of samples:
1. Larvae inhabited caladium in Bukittinggi
2. Larvae inhabited bamboo in Payakumbuh
3. Larvae inhabited bamboo stumps in Padang
4. Larvae inhabited caladium in Payakumbuh
5. Larvae inhabited caladium in Padang
6. Larvae inhabited pandanus in Padang
7. Larvae inhabited pineapple in Payakumbuh
8. Larvae inhabited pandanus in Payakumbuh
0. Larvae inhabited pandanus in Bukittinggi
+ Amplified samples
- Non-amplified samples

4. Discussion

We had performed an investigation with twenty larvae of *Ae.albopictus* in several areas in West Sumatra. Result have shown that from sample-1 to sample-9 (Figure 1 and Figure 2), sample-1, 2 and 4 were positively detected carrying dengue virus, which was possibly obtained from a mother infected by transovarial transmission. Transovarial transmission had been reported to occur in urban and suburban areas and within *Ae. aegypti* but not in *Ae. albopictus*. (Lee and Rohani, 2005; Lee *et.al*., 1997; Akbar, 2008; Hasmiwati, 2010; Joshi *et.al*., 1996). Transmission of dengue virus through transovarial process in mosquitoes is one way to maintain or preserve the lives of virus in the nature at a given time such as in dry and winter season or when there was no suitable vertebrate host (Rosen *et al*., 1983). Mosquitoes do not only play an important role in the transmission of dengue virus but also acts as a biological agent in maintaining the existence of dengue virus in nature. The presence of virus infected by transovarial in the wild can be a reservoir for dengue virus (Fouque *et.al*., 2004). Transovarial transmission of the virus is one of the ways to preserve the virus in nature (Gunther *et al*., 2007). The presence of dengue virus in nature can be caused by the differences in susceptibility of mosquito vector species originating from different geographical strains. The difference of susceptibility is thought to be related to the genetic control on the mosquito midgut, so thatthe infection can pass the barrier

of midgut and spread to various tissues that allow for transovarial transmission (Akbar *et.al*, 2008). Another factor that causes the presence of dengue virus in nature may be due to lack of vulnerable vertebrate hosts and climate conditions that do not support the transvorial transmission from mother to offspring (Lee *et al.*, 1997).

The results of this study also found two virus serotypes that were both discovered in one larvae sample which was larvaesample-4 (Figure 1). It is believed that the parent has been infected with two serotypes of virus and these viruses were then also transmitted to the offspring, so the larvae also inherited two virus serotypes. Mosquitoes which carry two serotypes will also infectthe host that it bit. According to Hariadhi and Soegiyanto (2004) double infection of two serotypes of virus will increase the virulence of disease on host.

The illustration result of electro photogram sample for sample3, 5, 6, 7, 8, 9 (Figure 2.) showed a negative result because there was no amplified DNA. This might be due to unfavorable conditions such as genetic factors of mosquito or disruption of mosquito activity, so that transovarial transmission did not occur. Precious research has also obtained the same results as described above (Ramalinggam, 1986; Watts *et.al*., 1985).

5. Conclusion

From the result of transovarial dengue virus detection in *Ae.albopictus* larvae that inhabits Phytotelmata in dengue endemic areas in West Sumatra by RT-PCR method, it could be concluded that *Aedes albopictus* larvae inhabiting Phytotelmata was positively detected for dengue virus; (DEN -1) and (DEN -4). Larvae which positively contained dengue virus had the potential to a source of dengue transmission if the larvae continue to grow and develop into adult females.

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