IJPHCS
Open Access: e-Journal

International Journal of Public Health and Clinical Sciences
e-ISSN : 2289-7577. Vol. 4:No. 3
May/June 2017

VERTICAL TRANSMISSION OF DENGUE VIRUS ON FIELD MOSQUITOES IN BANYUMAS REGENCY, CENTRAL JAVA, INDONESIA

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ABSTRACT

Background: The role of vertical transmission on the dengue virus (DENV) transmission is still under research. Some researcher believed that vertical transmission play an important role on DENV persistence in nature. However, still a few reports showed vertical transmission on the field mosquitoes and its importance. This study aims to detect the vertical transmision on field mosquitoes in Banyumas Regency, Central Java, Indonesia using immunohistochemistry (IHC) assay and Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR).

Materials and Methods: 100 houses were chosen in each of three dengue endemic villages, resulting in a total of 300 houses for ovitrap installations. Six days after initial installation, eggs were then collected from ovistrips and grown in a rearing room to adulthood. Caputs of Fillial 1 from adults mosquitoes were assesed using IHC assay, while thorax part were assesed using RT-PCR.

Result: Based on IHC assay, all of the studied villages showed the occurence of vertical transmission with percentage around 13-27%. *Ae. aegypti* showed higher percentage of vertical transmission than *Ae. albopictus.* There is a discrepancy results based on RT-PCR assay since there is no positive band detected on electrophoresis gel indicated negative of dengue virus.

Conclusion: The discrepancy results between IHC and RT-PCR is an interesting facts to be explored, due to the specificity and sensitivity of IHC. Even though there is no positive band on electrophoresis gel from RT PCR assay showed that negative result of DENV detection on the studied samples, with respect to positive results confirmation based on IHC assay, it should be an awareness to the health officer to prevent and control DENV tranmission.

Keywords: dengue virus, *Aedes sp*, vertical tranmission, RT-PCR, Indonesia

1.0 Introduction

Dengue Virus (DENV) infection is one of the important mosquito-borne diseases which is transmitted by *Aedes aegypti* (primary vector) and *Aedes Albopictus* (secondary vector)(Higa, 2011; Murray et al., 2013). DENV belongs to family Flaviviridae and genus Flavivirus, consisted of five serotypes DENV-1, DENV-2, DENV-3 ,DENV-4 and DENV-5 (Mustafa et al., 2015). This virus infections mostly endemic in tropical countries, however the spread of dengue disease also continues to occur in non-tropical regions (Bhatt et al., 2013). Most of this DENV infection is asymptomatic, however severe disease such as Dengue Haemmorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS) could be developed (Diamond & Pierson, 2015; Duong et al., 2015). In Indonesia, DENV infection has been spreading in most all of the provinces with various endemicity status since it was first detected in 1968 in Jakarta and Surabaya(Karyanti et al., 2014). Dengue cases in Indonesia have been shown to increase during the rainy season due the perfect condition for mosquito breeding (Setiati et al., 2006). Four DENV virus serotypes have been detected in Indonesia and DENV-3 infection has been reported to be associated with more severe cases (Ong et al., 2008). The identified risk factors of DENV tranmission are climate change, global warming, socioeconomic, human mobilization, uncontrolled urbanization and poor sanitation (Karyanti et al., 2014; Wesolowski et al., 2015; Wijayanti et al., 2016).

DENV virus infection could be transmitted through mosquito bites (horizontal), and also be transferred into the egg at the time of fertilization via the fallopian tubes during the process of embryogenesis (vertical transmission) (Khin & Than, 1983). These eggs will be infected by dengue virus and produce infected larvae which later be matured and produce adult mosquitoes containing DENV with infection rates of more than 80% (Beaty, 1996). This mode of transmission in mosquitoes was identified at the late 1970s and many researchers have hypothesized that this mode of transmission may allow maintenance of DENV during inter-epidemic periods (period between two epidemics)(Adam B & Boots, 2010). However, the importance of this mode of transmission in the epidemiology of this disease is still debated (Adams & Boots, 2010; Grunnill & Boots, 2016). Grunnill and Boots (2015) argue that the determinants of dengue persistence are more influenced by asymptomatic infection in human and movement of people than vertical transmission (Grunnill & Boots, 2016). Indeed, it is interesting to explore more about vertical transmission from field mosquitoes since it is poorly investigated.

Detection of DENV vertical transmission on mosquitoes in Indonesia usually using Immunohistochemistry (IHC) assay based on the work of Umniyati (Umniyati, SR, 2009), and some of studies using RT-PCR (Mulyatno et al., 2012; Satoto et al., 2014). The IHC test was carried out based Streptavidin-Biotin-Peroxidase-Complex (SPBC) method which only needs a microscope light for analysis(Umniyati, SR, 2009). The sensitivity and specificity of IHC assay comparing to RT-PCR had been studied by Widiastuti et al (2011), and the results indicated IHC assay showed a high sensitivity, but lower specificity due to false positive (Widiastuti et al., 2011). This study aims to detect vertical transmission on Banyumas Regency in Central Java as area of study, using two methods which are IHC and RT-PCR. Banyumas Regency experienced long history of DENV infection. Moreover, dengue outbreak happened several times such as in 2008, 2010 and recently in the beginning of 2016. The annual high percentage of dengue cases which occur in Banyumas Regency provokes a questions about how the mechanism of DENV persist in this area. The hypothesis from several studies about the role of vertical transmission on DENV persistence in certain area

emphasized the importance of this study. We predicted that vertical transmission may take place in Banyumas Regency as DENV endemic areas are present. However, there is no studies performed in this area to determine this mode of transmission and maintenance.

2.0 Materials and Methods

2.1 Description of the study area

Banyumas Regency, Central Java located in the southwest of Central Java Province, Indonesia is used as the study site. The coordinates of this regency are as follows: 108" 39` 17`` - 109" 27` 15`` East longitude, and 7" 15` 05``- 7" 37` 10`` South latitude. This regency has total area of 132,760 km^2, population of 1.85 Million in 27 sub districts, number of community health centresof 39, and a total villages of 331. For this study, we selected three endemic villages in Purwokerto Timur which are Kranji, Purwokerto Lor and Sokanegara. The status of endemicity of DENV criteria are based on "The Technical Manual Eradication of Dengue Mosquito-borne Diseases, Indonesian Ministry of Health" (1992) (Indonesia, 1992).

2.2 Ethical statement

Studies conducted here were carried out with ethical approval from Faculty of Medicine, University of Jenderal Soedirman (Ref : 145/KEPK/VII/2016).

2.3 Immunohistochemistry assay

Two oviposition traps (ovitraps) were installed, one inside and one around the perimeter of 50 houses per village, giving a total of 100 ovitraps per villages. Ovitraps were placed both indoors (at places which are safe from the reach of children, usually near the bathrooms or bedrooms) and outdoors (usually 1-5 metres away from the house). Six days after initial installation, ovistrips (filter paper) were collected to calculate the number of eggs laid. The ovitrap index (OI) was measured by calculating the percentage of positive ovitraps from the total number recovered. In order to obtain F1 (Filial 1; first generation of mosquito hatched from the eggs in ovistrips) mosquitoes for head squashes, eggs were obtained from the ovistrips and then grown to larvae, pupae and adult mosquito stages in rearing rooms. Mosquitoes with a minimal age of 5 days were then processed for head squash samples. Species and sex identification of mosquitoes were carried out before the head squash analysis based on previously published taxonomic guidelines (Stojanovich, 1965). Caput part of both male and female mosquitoes were included in the IHC test. The IHC 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.

2.4 RT-PCR Assay

Thorax part of mosquitoes, which collected in a pool/group (10 thorax of mosquitoes = 1 pool), were used as the sample for RT-PCR analysis. Total of 30 pools of mosquitoes from

three villages were tested in this study. The extraction on mosquitoes' RNA was conducted following the procedure of High Pure Viral Nucleic Acid Kit (Roche). RT-PCR assay was conducted based on Yong et al (2007) using this following primer (Yong et al., 2007) in Table 1 :

Table 1: Primer used in this study

Virus serotype	Primer	Primer sequence	Primer position	Size of amplicon & primer combination
	Dcon	5'– AGT TGTTAGTCTACGTGGACCGACA	1–25	
DEN 1	D1	5'– CCCCGTAACACTTTGATCGCTCCATT	17–342	342 bp (Dcon and D1)
DEN 2	D2	5'– CGCCACAAGGGCCATGAACAG	231–251	251 bp (Dcon and D2)
DEN3	D3	5'– GCACATGTTGATTCCAGAGGCTGTC	14–538	538 bp (Dcon and D3)
DEN4	D4	5'– GTTTCCAATCCCATTCCTGAATGTGGTGT	26–754	754 bp (Dcon and D4)

The RT-PCR was conducted by one step RT-PCR using kit Thermo scientific Verso 1-Step RT-PCR Hot Start Kit. DENV-1, 2, 3 and 4 which grown on C636 medium were used as the positive control of this assay. The results of RT-PCR were confirmed using electrophoresis.

3.0 Result

3.1 Ovitrap Index

Following the installation of ovitrap in three endemic villages in Banyumas Regency, we calculated the number of eggs in each ovitrap. Ovitrap index (OI) is the number of positive ovitrap with eggs among all observed ovitraps. The detail of OI in all three endemic villages are shown in Table 2.

Table 2: Ovitrap Index in all three endemic villages

Villages	Position	Observed ovitrap	Ovitrap with positive eggs	Ovitrap index (%)	Total number of eggs	Average number of eggs
Kranji	*Outdoor*	41	40	97.56	1347	33.67
	Indoor	47	40	85.10	1011	25.27
Sokanegara	*Outdoor*	46	34	69.38	1002	29.47
	Indoor	49	25	51.02	769	30.76
Purwokerto Lor	*Outdoor*	43	36	83.72	1103	30.64
	Indoor	45	29	64.44	724	24.96

International Journal of Public Health and Clinical Sciences
e-ISSN : 2289-7577. Vol. 4:No. 3
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Based on Table 2, all three villages showed OI of more than 50% positive with eggs, and the highest percentage was identified in Kranji. The female gravid mosquitoes tend to lay her eggs outside the house since in all three villages showed higher percentage of OI in outdoor position than indoor position.

3.2. Immunohistochemistry assay results

Positive and negative results of the immunohistochemistry test can be distinguished from the image slide under a microscope light. An example of a positive and negative result is shown in the Figure 1.

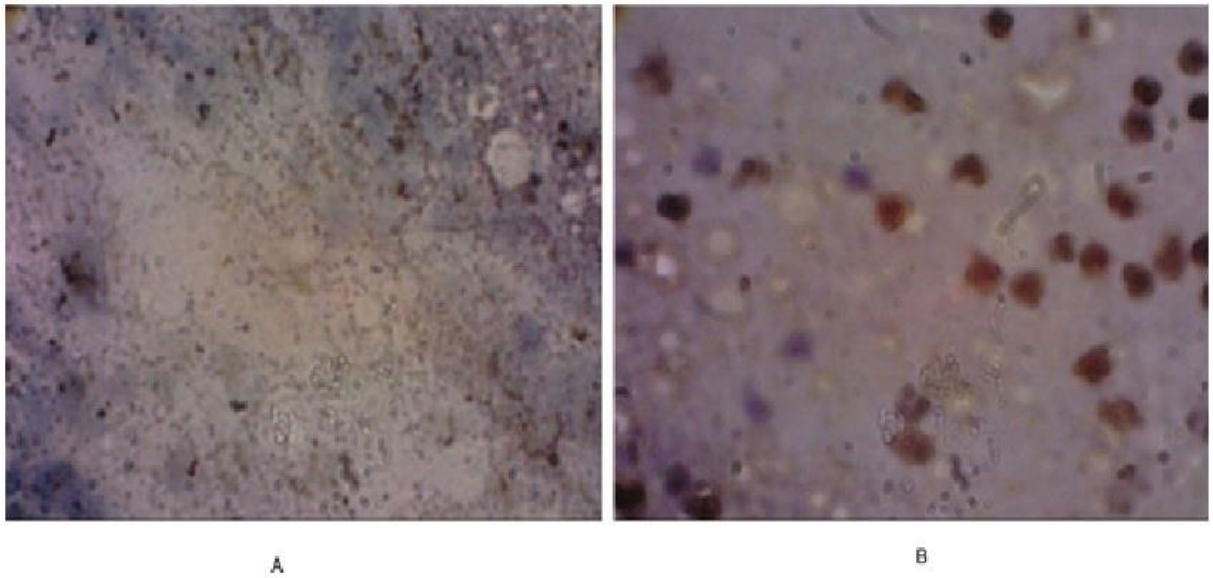

Figure 1: Negative (A) and Positive (B) result of the immunohistochemistry test of head squash samples. (A). Slide shows a positive result for DENV, the central nervous ganglion cells in the caput stains brown in colour in the cytoplasm, and brown granules are also surrounding the cell which indicates the mosquito is positive for antigen dengue (1000 x maginification). (B) Slide 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).

The sum up of immunohistochemisty assay results could be seen in Table 2.

Table 2: Immunohistochemistry assay results

Villages	IHC results								Total VIR (%)
	Ae. aegypti				*Ae. albopictus*				
	Positive	Negative	Total	VIR (%)	Positive	Negative	Total	VIR (%)	
Kranji	14	41	55	25.45	8	37	45	17.78	22
Purwokerto Lor	26	62	88	29.54	1	11	12	8.33	27
Sokanegara	13	80	93	13.97	0	7	7	0	13

All of the three villages had adults, either *Ae. aegypti* or *Ae. albopictus* or both, testing positive for DENV by the immunohistochemistry test. This indicates that vertical transmission occurs in both *Ae. aegypti* and *Ae. albopictus*. Purwokerto Lor showed the highest virus infection rate (VIR): 27%, followed by Kranji (22%), and the lowest infection detected in Sokanegara village.

3.3 RT-PCR results

In addition to the IHC assay, we also conducted RT-PCR using the thorax part of mosquitoes. The result of RT PCR test based on electrophoresis gel assay can be seen in Figure 2.

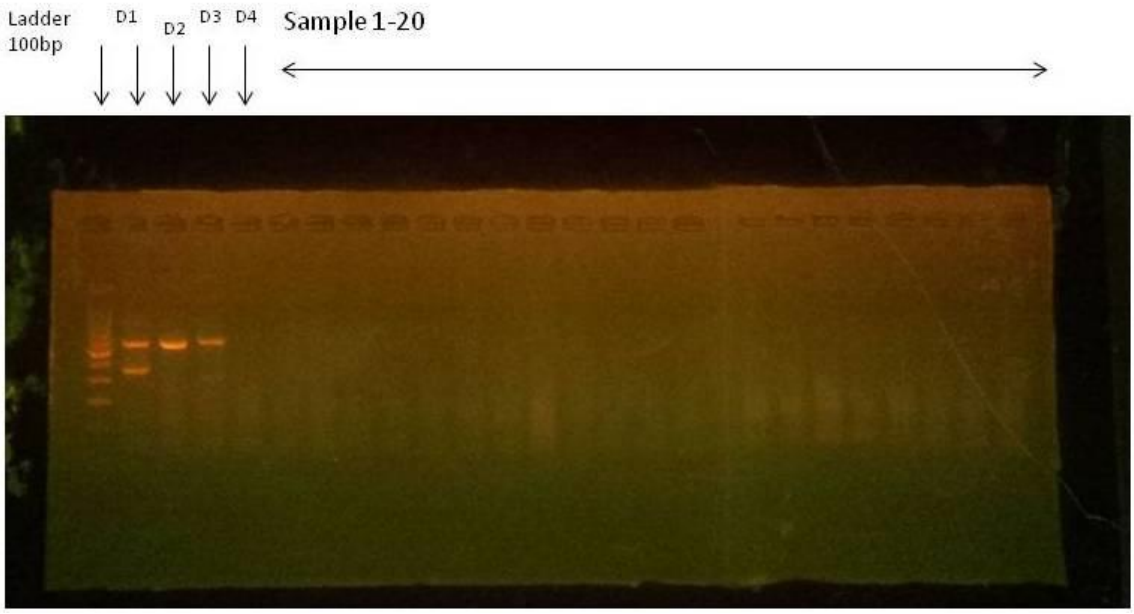

Figure 2: RT PCR assay of thorax mosquitoes samples. No positive band detected on electrophoresis gel on the studied sampel, while positive controls (D1, D2, D3) showed positive band, confirming that the RT-PCR assay work properly.

4.0 Discussion

Our results confirmed that the vertical tranmission occured in all three villages in the area of study based on IHC assay results while no positive DENV detected using RT-PCR assay. This different result could be an interesting facts to be explored. Many of study of vertical tranmission in Indonesia are still using IHC due to the budjet consideration, since the PCR assay is more expensive. Widiastuti et al (2011) claim that IHC assay has good sensivity to detect DENV antigen but less specific than RT-PCR. Again, the false positive is the issue of IHC assay since the results from IHC were influenced by the objectivity of the reseacher, because brown/blue colours on the cell granule under microscope could create bias of diagnostic(Widiastuti et al., 2011). However, negative DENV on RT-PCR assay could be also caused by the RNA degragation during the storage before the assay was performed. The application of real time PCR assay should be a better method since it is more sensitive, faster and able to quantify the viral load from the samples (Alm et al., 2015; Gurukumar et al., 2009).

Despite negative DENV detected in samples based RT-PCR, positive results on IHC assay should be still an important finding with implications for local risk assessments which must be considered by the health authorities. Vertical transmission may explain why DENV transmission regularly occurs in endemic areas. Further, these findings support the hypothesis that the virus can persist in communities and studies of vertical transmission may correlate with endemicity status in the area of study. Satoto et al (2014) believed that vertical tranmission could play an important role of maintaning mosquito population in the archipelago structure of Indonesia (Satoto et al., 2014). We found positive DENV in both female and male mosquitoes based IHC, as it is believed that both sexes could be an important reservoir allowing the virus to persist. Sexual transmission of DENV in *Ae. albopictus* has previously been described and similarly CHIKV can be transmitted in this manner (venereal transmission)(Mavale et al., 2010; Thongrungkiat et al., 2012).

Ae. aegypti in this study showed higher percentage on vertical tranmission than *Ae. albopictus*. Previous studies showed conflicting results about the comparison between *Ae. aegypti* and *Ae. albopictus* in vertical transmission, both laboratory and field work. Lee at al (1997) confirmed that *Ae aegypti* is more susceptible for vertical transmission (Lee et al., 1997), however several studies showed in contrary (Bina et al., 2008; Castro et al., 2004). The higher percentage of vertical transmission of *Ae. aegypti* in our study may reaffirm the role of this species as the primary vector of DENV transmission (Lambrechts et al., 2010).

The importance of vertical tranmission in the context of DENV infection epidemiology is still controversial. Moreover, while several studies argue that vertical transmission does not have a significant influence on the incidence of dengue infections (Adams & Boots, 2010; Grunnill & Boots, 2016), Lee and Rohani (2005) investigated the occurrence of dengue disease and vertical transmission of DENV in *Ae. albopictus* in Malaysia and found that vertical transmission of DENV happened prior to the reporting of human cases. The interval between vertical DENV detection and the first clinical human case was between 7 and 41 days. Thongrungkiat et al (2011) reported that vertical transmission rates in Bangkok gradually increased at the beginning of the rainy season and reached a peak four months before reporting of human cases, towards the end of the rainy season (Thongrungkiat et al., 2011). However, Grunnil and Boots (2015) argue that combination of asymtomatic infection

and movement of people are more important factors which determined the persistence of DENV.

5.0 Conclusion and recommendation

The presence of DENV confirms vertical transmission but more work will be required to establish its role in maintenance of the virus in the local environment. The mixed mode of transmission (horizontal-vertical) which happened in nature leads the difficulties to clarify the role of vertical tranmission in the epidemiology of DENV infection (Lequime et al., 2016). However, it will be interesting to include several variabels such as climate, viral genus, gonotropic cycle their association to the occurence of vertical transmission in further studies. The confirmation of vertical transmission in our study should be an alarm ring to the local health officer to increase their awareness in order to prevent and control DENV tranmission. An outbreak in the beginning 2016 in Banyumas Regency, should be an alert sign to the health authorities and community to fight DENV in this area.

Acknowledgement

We gratefully acknowledge the people in Kranji, Purwokerto Lor and Sokanegara villages for their cooperation during the ovitrap installations in and around their homes. We thanks the students of Public Health Department, University of Jenderal Soedirman who participated in the field work. We would also like to thank to Ulfah Farida, the staff of the Vector Borne Disease Research and Development Unit Banjarnegara for her help on rearing of mosquitoes, and Suprihatin, the technician in Parasitology Department for her guidance during IHC and RT-PCR work. We also thank for University of Jenderal Soedirman for the funding.

Declaration

Authors declare that we have no conflict of interest

Authors contribution

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