

Rapid Detection of Dengue Virus Transovarial Transmission from Nature and Artificial Inoculation

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Abstract

Background: Dengue Hemorrhagic Fever (DHF) is still a major disease problem. Eradication of DHF through vector control has not been able to cut transmission from mosquito to mosquito because there is still empirical evidence of transovarial transmission. The purpose of this study is to rapid detection of dengue transovarial virus transmission from nature and artificial inoculation.

Method: Mosquito eggs obtained from ovi-traps installation in several cities are hatched and maintained until they become mosquitoes at a week old without blood feeding, then the presence of DENV-1,2,3,4 is detected based on the RT-PCR method. As a positive control, 2-3 days old *Aedes aegypti* mosquitoes were injected with DENV-1,2,3, and 4, intra-thoracically under a dissecting microscope. The injected mosquito is kept in a cylindrical cage. Furthermore, mosquito control was developed until progeny 1 (F1), after adult dengue virus was detected by PCR realtime

Results: The results showed the proportion of transovarial events from nature was 60%. The results of transovarial transmission by artificial inoculation showed differences in viral load variations in serotype

Conclusion: Transovarial transmission from nature, found various serotypes with DENV-4 serotype as the dominant serotype. Whereas transovarial transmission through artificial inoculation shows transovarial presence in progeny one (F1) with different viral load values for each serotype. Further research can be analyzed for viral load up to progeny with zero squen quantity, to see its role in maintaining the virus during the interepidemic period.

Keywords: Transovarial, Dengue, Serotype, Nature, Artificial, Aedes

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Introduction

Dengue hemorrhagic fever (DHF) as a vector-borne disease is still a major disease problem especially in the tropics and sub-tropics. The results of research with the cartographic approach estimate that there are 390 million / year infection due to DHF. This disease has infected 70-500 million people / year in more than 100 countries around the world (1). DHF is still a problem in Indonesia because of the presence of four DENV-1, 2, 3, 4 serotypes(2). In addition, the high rate of incidence and outbreak events still occur in some parts of Indonesia. The results of research by Hikmawati, I and Pattima, S showed a CFR of 8.69 and outbreak dengue fever with 10 people died(3). Eradication of DHF through vector control has been carried out in various ways, but has not been able to decide on transmission from mosquitoes to mosquitoes because there is still empirical evidence of transovarial transmission.

Transovarial is a vertical transmission of the dengue virus from female mosquitoes to their eggs. Transovarial is an important problem because there has been evidence of horizontal transmission by vertically infected mosquitoes(4). *Aedes aegypti* as the main vector has more proportion (92.9%) than the co-vector of *Aedes albopictus* (6.8%). All serotypes were detected in mosquitoes with the proportion of DENV-3 (24%), DENV-1 (24%), DENV-4 (20%), DENV-2 (8%) and DENV-1 plus DENV4 (4%), and 95% of human infections in this period were caused by DENV-4(5). The results of the RT-PCR analysis found the presence of dengue virus in *Aedes aegypti* mosquitoes and larvae in Khyber Pakhtunkhwa, Pakistan(6). The proportion of transovarial events in Kenya in 2013-2014 shows a proportion of 8-11%(7). The results of the Thongrungrat et al. Study showed that transovarial occurred in all four DENV-1, 2, 3, 4

serotypes with a proportion of 30.3% containing all four types of serotypes(8).

The results of the transovarial study in Indonesia by Satoto at the Yogyakarta Adi Sucipto airport showed that the Transovarial Transmission Index (ITT) was higher in *Aedes aegypti* than *Aedes albopictus* (20% versus 16.7%), and serotypes found mostly DENV-2 and DENV -3(9). The results of the study in Manado City showed the Transovarial Transmission Index (ITT) ranged from 39.1% -70%(10). Transovarial studies in Pontianak City showed a transovarial transmission index of 54.5% and positive rates for *Aedes aegypti* were higher than those of *Aedes albopictus*(11). Spatial analysis is a geographic approach that is associated with various other disciplines. Spatial analysis allows a disease to be seen from various contexts so that better planning is expected to be able to eradicate and prevent an illness. Data on dengue virus distribution through transovarial can be mapped serotype types that are persistent through vector roles when interepidemic. The RT-PCR examination results showed that all serotypes detected in Nandro Aceh Darussalam (NAD) province with the most serotypes were DENV-4, followed by DENV-1, DENV-2 and 3(12). Transovarial DHF distribution shows that the infection rate of *Aedes aegypti* mosquito is higher (12.2%) than the infection rate of *Aedes albopictus* (8.3%) and the proportion of *Aedes aegypti* more in urban areas (88.9%), compared to *Aedes albopictus* which is more commonly found in rural areas (69.4%)(13). The purpose of this research is to detect transovarial transmission of dengue virus from nature and artificial inoculation through intratoracal methods

2 Materials and Methods

2.1 Subject and Research Design

The subjects in this study were female *Aedes aegypti* mosquitoes. The subjects in this study consisted of two populations namely natural populations and laboratory populations. The natural population is female *Aedes aegypti* mosquitoes which are captured by ovitrap in nature which are then rearing in the laboratory, while the laboratory population is female *Aedes aegypti* mosquitoes free of dengue virus which is rearing in the Parasitology Laboratory of the Gajah Mada University Medical Faculty. The research design uses two approaches namely observational and quasi-experimental designs. Observational design aims to describe transovarial maps of several regions in Indonesia and detect dengue virus in female *Aedes aegypti* mosquitoes from nature. While the quasi-experimental design aims to intervene the laboratory colonies of *Aedes aegypti* with artificial dengue virus (artificial inoculation) through the intratoracal method.

2.2 Sampling and epidemiological data collection

Mosquitoes

Mosquitoes consist of two types, natural mosquitoes are the result of catching eggs through ovitrap originating from five cities in Indonesia, namely: the cities of Medan, Palu, Magelang, Bengkulu and Timika. each sample from each city was taken by 10 female mosquitoes to detect dengue virus. While the laboratory mosquito is a F.115 laboratory colony that originally came from the city of Yogyakarta and has been maintained for more than five years.

Virus.

The virus to be injected through the piston was obtained from a C6 / 36 cell culture supernatant infected with DENV-1,2,3,4 and was a prototype originally from Namru 2 Jakarta.

Intrathoracal procedure

Total of 80 2-3-day-old *Aedes aegypti* mosquitoes, put in test tubes filled with ice cubes, after fainting, take one at a time, lay under a microscope and a set of mosquito intratoracal devices, then inject ± 200 µl DENV-1 virus supernatant tail, then do the same procedure for supernatant viruses DENV-2, DENV-3 and DENV-4. 3 days after inoculation, mosquitoes were put into a 20 cm³ cage and inside had been filled with male colony of laboratory colony and wet cotton mixed with sugar water. On the 5th day after intratoracal, mosquitoes were fed blood by the membrane feeding method. After being seen laying eggs. Mosquitoes are taken with an aspirator for dengue virus :

detection by PCR to see the success of intratoracal injection. Positive results according to the expected tape size from the amplification results were 482 bp (DENV-1), 119 bp DENV-2, 290 bp DENV-3, and 392 bp DENV-4. One month later, each serotype egg was hatched. As an adult, take 10 animals to do dengue virus detection with realtime PCR.

Detection of Dengue Virus

Samples used to detect the presence of dengue virus from nature and artificial inoculation through intrathoracal were 10 each. The first step is carried out Virus isolation: as many as 10 female mosquitoes per group carried out dengue virus RNA isolation. For Isolation of dengue virus RNA was carried out using the Viral Qiagen RNA Isolation kit (QiAmp Viral RNA mini Kit)(15).Viral RNA isolation used a centrifuge method, with the procedure: 560 ul pipette AVL buffer containing carrier RNA was inserted into a 1.5 ml tube, then add 140ul dengue virus 2 supernatant, vortex for 15 seconds and leave room temperature for 10 minutes. Spin down to lower the mixture from the lid. Absolute ethanol of 560 ul was added to the mixture and vortex for 15 seconds. Spin down to lower the mixture attached to the tube cap. Transfer 630 ul to mix carefully into QIAamp Mini column in the collection tube. Close the QIAamp column and centrifuge 8000 rpm for 1 minute. Discard the collection tube filtrate move the remaining sample into the QIAamp Mini column. Close the QIAamp column and centrifuge 8000 rpm for 1 minute. The filtrate collection tube is removed and replace the new collection tube. 500 ul buffer AW1, close the QIAamp column and centrifuge 8000 rpm for 1 minute. The filtrate collection tube is removed and replace the new collection tube. 500 ul of AW2 buffer and centrifuge 14,000 rpm for 3 minutes. The filtrate collection tube is removed and replace the new collection tube and centrifug the full speed for 1 minute to remove the remaining residual filtrate. The QIAamp column is placed in a 1.5 ml tube and add 60 ul buffer AVE. Incubate at room temperature for 1 minute and centrifuge 8000 rpm for 1 minute. Insulated RNA is stable at temperatures above -30 for storage above 1 year. if there is a sample that has not been isolated from RNA, samples stored in the freezer of -80°C. Examination of dengue virus serotypes in the sample was carried out by experts from the Parasitology Laboratory, Faculty of Medicine, Gajah Mada University using RT-PCR. The expected tape size from the amplification results is 482base-pair (bp) DENV-1, 119 bp DENV-2, 290 bp DENV-3, and 392 bp DENV-4. The measurement bpis compared with the marker bp and is documented(16). Detection of dengue virus in F1 using realtime PCR. The Primary sequences are used as in Table 1 below

Tabel .1 Primer Sequences

No	Assay	Primer	Sequences
1	DENV-1	DENV1_F DENV1_R	CAA TGG ATG ACA ACA GAA GAY ATG TCC ATC CAT GGG TTT TCC TCT AT
2	DENV-2	DENV2_F DENV2_R	GCA GAA ACA CAA CAT GGA ACR ATA GT TGA TGT AGC TGT CTC CRA ATG G
3	DENV-3	DENV3_F DENV3_R	ATG GAA TGT GTG GGA GGT GG GGC TTT CTA TCC ART AGC CCA TG
4	DENV-4	DENV4_F DENV4_R	GCA GAT CTC TGG AAA AAT GAA CCA GAG AAT CTC TTC ACC AAC CCY TG

Data Analysis

The data obtained were analyzed using proportion values in transovarial transmission from nature, whereas artificial inoculation transovarial transmission uses squen quantity (SQ) results from real time PCR results

Results

Transovarial Transmission From Nature

The results of the Reserve Transcription-Polymerase Chain Reaction (RT-PCR) test showed that three cities, Medan, Timika, Magelang, showed positive results of transovarial

dengue virus, whereas Palu and Bengkulu did not detect transovarial transmission (Figure 1). Distribution of transovarial serotypes in *Aedes aegypti* mosquitoes in the three cities showed DENV 1 and -4 from Medan, DENV-3 and 4 from Timika, DENV-4 from Magelang (Figure 2): Detection of

mosquito dengue virus from Bengkulu, carried out using real time tests PCR, and the results of the analysis show zero sequential quantity (SQ)

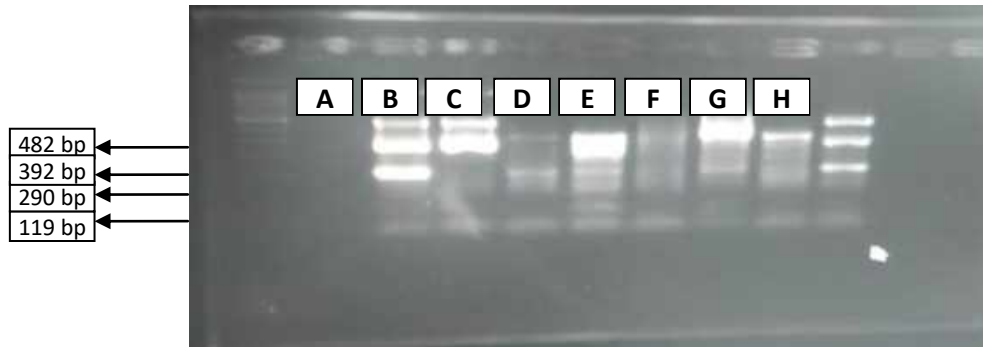


Figure 1. Detection of dengue virus from nature by PCR shows Code B (Transovarial DENV-1 and 4 from Medan), Code D (Transovarial DEN3 and 4 from Timika), Negative transovarial E Code from Palu City, Code G (Transovarial DEN 4 from Medan Magelang). Detection of dengue virus from artificial inoculation by PCR shows Code A (intratoracal positive Den-2). Code C

(positive intratoracal Den-3) Code F (positive intratoracal Den-4) and Code H (positive intratoracal Den-1).

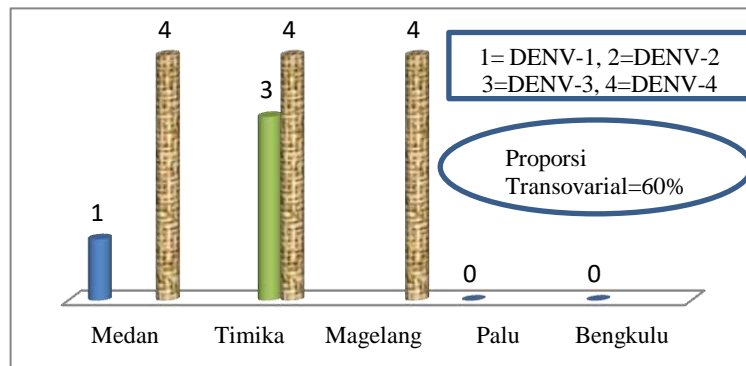


Figure 2: Distribution of Transovarial Serotypes From Nature

Artificial Inoculation Transovarial Transmission

Artificial inoculation transovarial transmission begins by interfering with the *Aedes aegypti* mosquito by administering the dengue virus through the intratoracal method. PCR test results showed an intratoracal success in infecting the dengue

serotype DENV-1,2,3,4 virus (Figure 1). Results of DENV viral load 1,2,3,4 based on the *Aedes Aegypti* (F1) real-time PCR vector transovarial transmission test using the intrathoracic method, as shown in Table 2:

Table. 2Real-time PCR Vector Results of *Aedes Aegypti* (F1) Transovarial by Intrathoracic Method

Serotype	Melting (CQ)	Viral Load (SQ)	Detection *
DENV-1	23.78	892000000	+
DENV-2	39.77	5172000	+
DENV-3	28.88	22890000	+
DENV-4	38.41	13770000	+

Discussion

Transovarial Transmission From Nature

The results of this study indicate that transovarial events are still quite large in Indonesia, out of 5 egg sampling cities, three cities show positive results, this means the proportion of transovarial events is 60%. The existence of dengue virus through transovarial shows the role of *Aedes* sp vector in maintaining the virus during interepidemic. This finding becomes a consideration in vector control strategies through virological surveys. DENV-4 as the most dominant serotype found in the field / nature of several cities in Indonesia shows that transovarial transmission is a maintenance mechanism of DENV-4 in several regions in Indonesia. The results of this study are in line with research conducted by Edillo, et al who discovered dengue virus infection via Transovarial transmission (TOT) occurring in four serotypes with serotypes with the most DENV-4, followed by DENV-3, DENV-1, and DENV-2. (17) The same results were concluded from a study at Jardim Industriário Cuiabá, Mato Grosso found DENV-4 as the only flavivirus detected, and was found in 8/50 ponds (16.0%). This study provides the first evidence of a natural transovarial infection by DENV-4 in *Aedes aegypti* in Mato Grosso, suggesting that this type of infection might function as a mechanism of virus maintenance during the interepidemic period in Cuiabá, a city where dengue epidemics are reported annually. These results emphasize the need for efficient vector population control measures to prevent arbovirus outbreaks in the state. (18) However, the results of this study differed from studies conducted by Ayu Rahayu, et al who did not find DENV-4 in transovarial transmissions in Yogyakarta, but three other serotypes (DENV-1, DENV-2, and DENV-3) were detected transovarial. (19) Transovarial findings and the distribution of serotypes from various regions in this study, are important to be used by health program holders, especially epidemiologists, regarding syndrome surveillance problems, where these data can be used to anticipate the high spikes or wide spread of DENV to prevent various negative things such as the occurrence of Extraordinary Events (KLB). The success of the *Aedes aegypti* mosquito vector to maintain the dengue virus during interepidemic possible influence of the mosquito resistance. This is as research conducted in these cities shows that in Medan and Magelang there has been a resistance to malathion (20). (21) The distribution of dengue viruses in Figure 2 shows that not all regions of the virus defense mechanism occur during interepidemic via transovarial. This shows that not all regions, transovarial as a mechanism of transmission of dengue virus. Serotype differences in transovarial occurrences in this study were estimated due to differences in sampling time, this is as the findings of previous studies that different seasonal patterns between interepidemic and epidemic years, affect the rate of transovarial incidents, and persistent transmission occurred after June in interepidemic years. (22) In addition, there are differences in rainfall in the sampling area with three differences, namely: Palu, Bengkulu and Medan including quatorial rainfall areas, Magelang city including the Musoon rainfall area and Timika City including local rainfall areas. This difference certainly affects the climate and weather which can affect bionomics and mosquito breeding places. This is as the study conducted by Wu et al, that the number of rainy days in a month has a large influence on the DF events the following month, the research concludes that temperature and rainfall have a nonlinear effect on DF events in Guangzhou.(23) The results of previous research concluded that the presence of dengue virus through a spatial approach is influenced by among others: urban village, population density, road density. (24)

More transovarial transmission (TOT) comes from inside the house compared to outside the house / yard and more in the summer than the rainy season. This shows that TOT increased

gradually in the summer, reaching its peak in April to June, while the DHF cases peaked in September, a month after entering the rainy season. Therefore, mosquito infections due to TOT are prevalent four months before the high incidence in humans. (17) Transovarial transmission, rapid urbanization and industrialization have led to an increase in vector populations in these Northeast Indian states. This study shows the transovarial transmission of dengue virus in this state and is useful information in the epidemiological concept to take appropriate vector control measures. (25) The DENV-3 serotype, although not as a dominant serotype, still has an important role in maintaining dengue virus during interepidemic. The results of this study provide important information related to the correlation of vertical transmission and horizontal transmission. The alleged correlation between the two transmissions is seen from the findings of the vertical transmission of the DENV-3 serotype in Timika, and previous research shows the detection of DENV-3 serotypes horizontally in a patient's blood. This phenomenon indicates the persistence / maintenance of virus serotypes through the intermediate host before being transmitted to humans as horizontal transmission. However, the results of this study differ from previous studies conducted by Sival et al instead found the DENV-3 serotype as the most dominant serotype during an extraordinary event at Havelock Island. (26) The detection of DENV-2 serotypes from field colony mosquitoes in this study shows transovarial transmission is not the main mechanism for maintaining DENV-2 during interepidemic conditions in the environment. This is in line with research by Edillo, DENV of all serotypes except the DENV-2 serotype detected in larvae, pupae, both male and female adult mosquitoes. In the prevalence ranking, DENV-1 was coinfecting with DENV-3 or -4 or both in April 2012; DENV-3 and -4 are present in both seasons. More mosquitoes that were DENV positive were collected from households than in the field location ($p < 0.001$) and in the dry season than in the rainy season ($p < 0.05$), with significant interactions ($p < 0.05$) between locations and buildings but there is no interaction between location and season ($p > 0.05$). With the Generalized Linear Mixed model, monthly rainfall is a significant predictor of monthly dengue cases ($p < 0.05$). MIR, season, temperature, and relative humidity are not significant predictors. Surveillance of DENV prevalence in *Aedes* sp and detecting their natural focus in the dry season provides an early warning signal for dengue outbreaks. (17)

Besides DENV-3 and 4, DENV-1 was also found in this study. DENV-1 findings in Medan City, show the role of DENV-1 in maintaining dengue virus during interepidemic and the role of *Aedes aegypti* as intermediate host. This is supported by previous research conducted in Medan to find the serotype DENV-1 as a serotype detected in patients also detected in mosquitoes. (27)(28) The results of this study are in line with research conducted by Velandia et al who discovered DENV-1 through transovarial transmission. The study was conducted by taking live larvae and pupae from 53 houses and 54.7% of the houses found *Aedes aegypti* larvae and pupae, and analysis with RT-PCR showed DENV-1 was the most dominant serotype. However, the simultaneous presence of DENV-1 and DENV-2, DENV-1 and DENV-3, DENV-1 and DENV-4, and DENV-1, 2 and 3 were detected in several houses. This situation increases the risk of DENV infection in Colombia and consideration of prevention and control programs at all stages of mosquitoes. (29) Further research with the discovery of the DENV-1 serotype was carried out in rural Colombia, namely in the Anapoima and La Mesa regions. DENV in mosquitoes was detected in 74% of locations with the estimated result of individual mosquito infections being 4.12% and the minimum infection rate was 33.3 o / oo mosquitoes. Four serotypes were detected, with the most dominant serotypes being DENV-2

(50%) and DENV-1 (35%). The presence of infectious mosquitoes in rural areas can be a potential increase in urban cases because of the migration of people from villages to cities. (30) In contrast to a study conducted by Moraes, et al who conducted a study by taking larvae in three cities that had a high incidence of dengue fever, namely in Taubaté, São Paulo and Brazil. The analysis showed there was no detection of DENV-1 in the study with the Real-Time Polymerase Chain Reaction test. (31) Research results at Amazon show a transovarial infection rate of 46%, an average MIR (Minimum Infection Rate) of 17.7 with the lowest MIR of 11.4 and the highest of 24.1 and transovarial detection with RT-PCR showing the presence of the DENV-serotype 1 and DENV-4 in larvae. The conclusion of the study was the DENV xenomonitoring model in larvae, contributing to the development of systems for early detection of viral circulation and predictive models for outbreaks and epidemics of dengue fever. (32)

The high transovarial proportion in this study shows the role of *Aedes aegypti* as a host of dengue virus intermediates. This is an important problem in vector control efforts because *Aedes aegypti* can together transmit the chikungunya virus (CHIKV). Research on Reunion Island shows that *Aedes Aegypti* can be an efficient vector for dengue and chikungunya viruses. (33) For this reason, efforts can be made through vector control at all stages of the mosquito stage. Research on larvasides carried out by Joshi et al. Showed that more than 50% of the total city districts / research areas, larvicidal intervention in the right place for mosquito breeding that was positive for dengue virus was able to reduce 90-100% incidence of dengue fever. (34)

Transovarial Transmission From Artificial Inoculation

The results showed a DENV-1,2,3,4 serotype viral load in progeny one (F1), this indicates a transovarial transmission through artificial inoculation. The results of this study indicate that dengue virus is an organism that is able to attack cells in the body of mosquitoes. The entry of the virus into the mosquito's body, shows the success of the virus overcoming the various barriers that exist. The success is influenced by many factors including: anatomical, physiological, and molecular characteristics of the mosquito itself. During extrinsic incubation, viruses entering the mosquito midgut and entering the hemolymph can infect fat body cells, trachea, hemocytes, ovaries, nerve tissue, and reach the salivary glands. (35) The mechanism for virus entry begins with infecting the midgut epithelium and replicates before it passes through the basal lamina into the hemolymph and spreads throughout the mosquito's body. In order to be transmitted to the next host, the virus must infect the salivary glands so that it can be transmitted to the next host. Viral genetic diversity is reduced if there are anatomical barriers such as midgut infection, salivary gland infection. (36) The results of previous studies found differences in the level of virus replication in various dengue virus vectors, DEN2-FJ10 strain replication was greater in *Aedes aegypti* than in *Aedes albopictus* 5 days after infection while DEN2-FJ11 replication was greater in *Aedes albopictus* than in *Aedes aegypti* than in *Aedes albopictus* 5 days after infection while DEN2-FJ11 replication was greater in *Aedes albopictus* than in *Aedes aegypti* 7 days after infection. (37)

The results showed different viral load values among various serotypes. This indicates the presence of different strains of the virus in the ability to bind and infect target cells. In this case the ability to produce progenic viruses with the results of different gene products and provide different aspects. The results of previous studies concluded the severity of dengue infection correlated with high viremia viral load, secondary infection and serotype type. (38) The results of other studies conclude that the entry of antibody mediated viruses increases

host dependency factors to increase viral infections, these factors include RNA splicing, respiratory mitochondria, vesicle trafficking and ribosomal genes. The findings of the study provide insight into how differences in entry can affect host responses and specific therapies can inhibit the increase in antibodies that are dependent on dengue virus infection. (39) Reactive and nonneutralizing or subneutralizing antibodies that occur after primary DENV or flaviviral infection can bind to heterologous DENV to increase infection, resulting in viral load and virulence of the disease. penyakit. (40) This concept is known as antibody-dependent enhancement (ADE) from DENV infection. In a prospective study in Bangkok, Thailand, by taking serum specimens from school children suffering from secondary dengue, the ability to increase replication of DENV type 2 (DENV-2) in human monocytes in vitro was tested. The results of the study showed that the specific neutralizing dengue virus antibody specific titer could increase and be associated with an increased risk of severe dengue disease. A prospective study with a cohort design concluded that ADE of dengue occurred in human subjects with pre-condensation anti-DENV antibody infection at a certain concentration range. (41) Other studies conclude that viral load is significantly higher in patients negative for IgM antibodies. In contrast, circulating NS1 levels were found to be unaffected by the presence of IgM. Thus, IgM antibodies in serum can be a modulator that affects the level of viremia of the patient. (42) The results of this research show the important role of *Aedes aegypti* as a host of dengue virus intermediates. This has become an important problem in controlling the vector because *Aedes aegyptus* can together transmit the chikungunya virus (CHIKV). Research results on Reunion Island show *Aedes Aegypti* can be an efficient vector of dengue and chikungunya viruses (33). For this reason, efforts can be made through vector control at all stages of the mosquito stage. Research on larvasides carried out by Joshi et al, showed that more than 50% of the total city of the regency / research area, larvicidal intervention at the right place for mosquito breeding that was positive for dengue virus was able to reduce 90-100% incidence of dengue fever. (34) The limitations of this study are the results of detection of transovarial transmission of dengue virus through artificial inoculation with PCR (Figure 1) showing the presence of another type of serotype detected, in one intervention action. This is because the virus taken through RNA isolation, was originally a dengue virus in DHF patients. The virus was cultured in C636 cells, when in c636 cells the small amount of dengue virus serotype was not detected through PCR, but when it entered mosquitoes, as intermediate hosts, the virus replicated very quickly so that the type of virus serotype was not initially detected in c636 cells, when the mosquito becomes positive.

Conclusion

The conclusion of this study, transovarial transmission from nature, found various serotypes with DENV-4 serotype as the dominant serotype. Whereas transovarial transmission through artificial inoculation shows transovarial presence in progeny one (F1) with different viral load values for each serotype. Recommendations in future studies can be analyzed for viral load up to progeny with zero squen quantity, to see its role in maintaining the virus during the interepidemic period.

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Competing Interests

The authors declare that there is no conflict of interest exists in the submission of this paper.

Ethical approval

The study was approved by the Medical and Health Research Ethics Committee (MHREC) Faculty of Medicine Gadjah Mada University and Dr. Sardjito General Hospital by Number : Ref: KE/FK/0176/EC/2018 and data were confidentially preserved according to the revised Helsinki declarations of biomedical ethics.

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