Transovarial infection in *Aedes Aegypti*: correlational study on incidence of dengue hemorrhagic fever (DHF) at endemic area of Semarang city, Indonesia

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Abstract. Indonesia is an endemic area of DHF. The incidence in 2019 reached 70.03 / 100,000 population. The one mechanism of the dengue virus survive in nature is through transovarial infections. This mecahnism can cause the virus to persist, leading to the endemicity of dengue in a region. The aim of this study was to analyze the correlation of larval density and transovarial in *Aedes aegypti* with the occurrence of DHF using a spatial analysis approach. The study was correlational design, located in 11 sub villages in Meteseh village of Semarang City. The sample was 33 houses per sub village, so total sample was 363 houses. Survey of entomological method was done by examination the houses to measure indexes of larvae density and collection the eggs using ovitrap. Female Aedes aegypti from rearing eggs or lavae was used to identify transovarial infection by immunohistochemical assay. The result showed that transovarial transmission of dengue virus was found in 8 sub villages, (ITT was around 5-20%. There was a relationship between transovarial infection with the incidence of DHF (p= 0.043). This study provides information that Aedes aegypti transmit virus by transovarial infections, and the distribution of DHF has spatially related to transovarial infections.

1 Introduction

Dengue Hemorrhagic Fever (DHF) is caused by dengue virus transmitted through the bite of *Aedes aegypti* mosquito[1]. *Aedes aegypti* mosquitoes are known as the most common spread of dengue virus in transmission of dengue disease. World Health Organization (WHO) reported that the number of dengue cases increased each year from 0.4 to 1.3 million in the 1996-2005, reaching 2.2 million in 2010 and 3.2 million in 2015[2].

Central Java Province is one of the dengue endemic areas in Indonesia with a total of 14,376 cases, an incidence rate (IR) of 42.26 / 100,000 and a case fatality rate (CFR) of 1.48% above the Indonesian CFR standard of 0.78% [3] Meanwhile, according to Semarang City

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Health Office report, Semarang City, a city in Central Java Province, is an endemic city with the highest number of dengue cases to be found in Tembalang District with 12 villages. In the last three years, Meteseh village had an IR of 60.47 / 100,000 population in 2017 although in 2018 there was a drastic decline of IR to be 14.01 / 100,000 population, but in 2019 the IR increased again to be 70.03 / 100,000 population.

The vast distribution of the virus changes the behavior of the vector to spread the virus. Viral infections in the mosquito's body cause mosquitoes to be less capable in sucking blood, repeatedly thrusting the probabilities, but unable to suck the blood, so mosquitoes move from one person to another. As a result, the risk of transmission of the virus becomes even greater [4].

More evidence for transovarial dengue haemorrhagic fever transmission is likely to be a potentially contagious factor for dengue outbreaks in endemic areas. The occurrence of transovarial transmission of dengue virus in *Aedes aegypti* was demonstrated in a study conducted by Lee et al[5]. who found infected larvae from 16 different locations in Malaysia, with higher rates of virus infection in *Aedes aegypti* compared to *Aedes albopictus.*

Subsequent research using the same method, in Semarang City, Kendal Regency, Sukoharjo Regency, and Salatiga City found transovarial Dengue Fever transmission by transovarial with infection rates ranging from 0.66-8.77%[6]. Rapid dengue transmission can occur due to population growth and the increasing number of mosquito vectors. The density of the population facilitates the transmission of dengue virus because it is (multiple biting) of the vector[7].

Density of the number of mosquito vectors increases due to of the large number of potential sites for mosquito breeding, causing high larval densities. The high density of larvae increases the transmission of dengue virus from human vectors so that it causes high DHF cases.[7] In a research conducted by Martini et al in relation to larvae density, Tembalang has been identified to have high risk in DHF infection and have *Aedes albopictus* existed. However, one-third of all larvae found are *Aedes aegypti*, as Tembalang has bad sanitation infrastructure[8].

Spatial analysis is a technique used to analyze spatial data. The result of the analysis rely on the location being examined that need access to it and its attributes. The function of analysis is to provide specific information regarding incidences occurring in an area, geographical components and its changes, or trend at a specific interval time[9].

As Meteseh Village is in endemic status, the existence of *Ae aegypti* as the main vector of dengue virus need to be further examined. There have been limited studies in this area regarding vector density, viral infection in vectors, and the incidence of dengue hemorrhagic fever (DHF). Endemicity is significantly influenced by the persistent presence of the dengue virus in nature. The possibility of virus transmission within the vector through transovarial mechanisms is crucial to be proven through entomological surveys in the area, as dengue virus transmission is likely ongoing. Epidemiologically, the findings of such studies will serve as a basis for implementing dengue control measures.

Given the explanation, the aim of the study was analyze the correlation of larvae density and transovarial transmission on *Ae aegypti* mosquitos on DHF incidence using spatial analysis approach.

2 Material and Method

This analytically observational research using correlational study design was conducted in August – October 2019.

The independent variables were larvae density and transovarial transmission, while the dependent variable was the incidence of DHF. The unit of analysis in this study was the hamlet in Meteseh Village. A survey was conducted in 33 houses within each hamlet to collect data on mosquito larvae density. The larvae were then used for transovarial testing. Meanwhile, case data was obtained from reports provided by the local health center (Puskesmas), and the distribution of cases was mapped by hamlet.

2.1 Mosquitoes Samples Collection

Larvae sample of *Ae. aegypti* as research subject were collected from *ovitrap* that was used to collect *Aedes sp* eggs. The eggs were reared in parasitology laboratory of Faculty of Medicine, Public Health and Nursing Gajah Mada University.

2.2 Larva Ae. Aegypti Survey in Residential Area

Entomological survey in residential area was conducted in Meteseh village consisting of 30 RW with 6,741 houses. The location of the study was in Meteseh village consisting of 11 RW. After calculating using *cross sectional Lemeshow,* every RW was represented by 33 samples; thus, the total samples were 363 houses.

2.3 Determination of Entomological Status

To determine the entomological index, the WHO formulation used is as follows:

$$HI = \frac{\textit{Total house with positive larvae}}{\textit{Total house observed}} \; X \;\; 100\,\% \qquad (1)$$

$$CI = \frac{\textit{Total countainer with positive larvae}}{\textit{Total house observed}} \; X \;\; 100\,\% \qquad (20$$

$$BI = \frac{\textit{Total countainer with positive larvae}}{\textit{Total house observed}} \; X \;\; \textit{100 house} \qquad (3)$$

2.4 Immunohistochemical Examination

Stages of observational procedure of immunohistochemical squash tssues:

a. Preparing squash preparat from tissues (adult mosquito head).
b. Fixation the squash using cold methanol for 10 minutes, then washing with running water, and dried for 2 minutes
c. Add *peroxidase blocking solution;* a mix of H_2O_2 and methanol with ratio of 1:9, for 10 minutes and rinsed with distilled water.
d. Continue with pre blocking solution by adding 2 drops on every preparat after that roll out for 10 minutes, do not rinse.
e. Add primary antibody (*monoklonal* antibody 1:50). Preparat was incubated for 1 hour at room temperature.
f. Next, wash with PBS 3 times for 2 minutes each. minutes.
g. Add Biotinylated secondary broad spectrum antibody 2 drops for each preparate after that roll out for 10 minutes in 10 minutes

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h. Next, wash with PBS 3 kali for 2 minutes each.
i. Followed by the administration of conjugate streptavidin peroxidase, add 2 drops for each preparation after 10 minutes.
j. Washing followed by giving PBS 3 times each of 2 minutes.
k. Preparation of DAB chromogenic solution with a composition of 2 drops plus 6 drops DAB Substrate if 6 preparations are done after it is mixed then each preparation is given 100 μL for 5 minutes.
l. After drying, wash it with running water after that add Mayer haematoxylin for 1-2 minutes, then wash it with running water.
m. Washing is continued by giving PBS for 1 minute and rinsing with distilled water.
n. If the preparation is dried in drops with softening as mounting media.
o. Then close the preparation with a cover slip and leave it for 2-3 hours
p. The examination is performed under a microscope with weak, moderate and strong magnification light.

3 Result

Meteseh village was a DHF endemic. Data were taken from houses suspected to be DHF case in 11 RW. The following is the distribution of DHF cases in Meteseh village from January – June 2019. There were 13 cases of DHF spread out in 11 RW of Meteseh village, Tembalang sub-district. The highest cases were found in RW 02 and RW 06, while others were detected only 1 case. The description of the DHF distribution is presented in picture 1. Data collection on DHF cases was carried out based on data from the Health Office of Semarang City to 11 hamlets (RW) in Meteseh village, Tembalang District. Picture 2 describing *Incidence Rate* (IR) at the level of RW based on national standard of 100.000 population shows that the highest IR is found in RW 20, which is 25.19 per 100.000 population. Meanwhile, the lowest IR of DHF cases is RW 05, which is 8.69. The average IR of DHF cases in Meteseh village of Tembalang sub-district is 135.7 per 100.000 population.

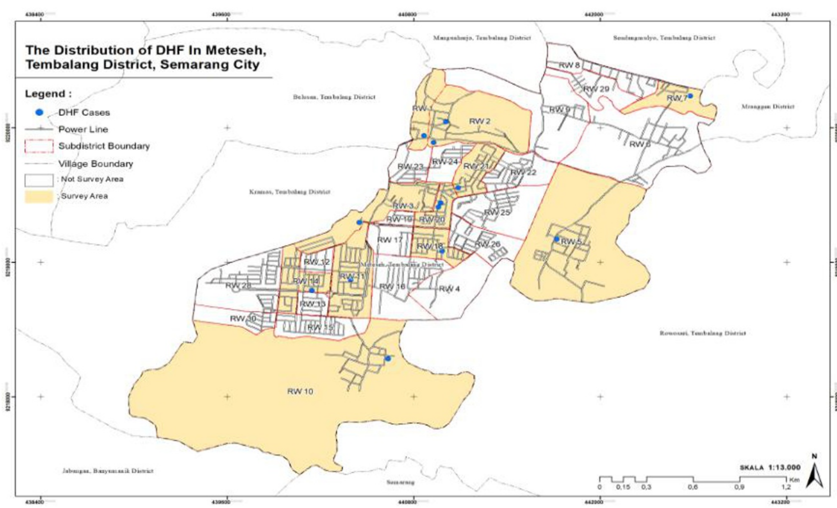


Fig. 1. Map of DHF distribution cases in 11 RW in Meteseh Village of Tembalang sub-district in 2019

3.1 Entomology Status of Larvae Density

House index (HI) is the number of positive houses divided by all houses examined multiplied by 100%. Figure 7 describes 11 hamlets (RW) having House index (HI). The highest HI was RW 05 (27.27%), the lowest one, no larvae found, was RW 18 (8.82%). The RW border line above HI in Meteseh village are RW 01, RW 02, RW 05, RW 07, and RW 11.

Countainer index (CI) is the number of positive containers divided by the number of containers inspected multiplied by 100%. Table 4 shows that of 11 hamlets (RW) having *Container Index* (CI), the highest was RW 02 (10.13%). In RW 02, larvae was found to be inside bathroom containers, water scuttle, and dispenser, while the lowest one was in RW 18 (0%), located in housing area with an average of 3.89%. The hamlet (RW) boundaries that passed the Meteseh Village *Container Index* were hamlet (RW) 02, hamlet (RW) 05, and hamlet (RW) 07.

Breteau index (BI) is the number of positive countainers divided by the number of houses inspected multiplied by 100 houses. Figure 9 shows that one of the villages having a high *Breteau Index* (BI) was hamlet (RW) 05 with 30.30. Many containers were found to be having positive larvae and several houses were found to be having more than one containers larvae positive. The lowest BI is RW 18 (9.37%). The RW border line above BI in Meteseh village, Tembalang Sub-District were RW 02, RW 05 dan RW 07. The results of HI, BI and CI obtained larval density we got DF score. The data showed that the highest larva of DF 1 (low) was 6 RW with 54.54%, while 5 other RW were categorized as DF 2-5 (medium) with 4545%. Therefore, it could be concluded that RW in Meteseh village mostly had larvae density categorized as low and medium.

3.2 Transovarial Infection of 11 Hamlets (RW) in Meteseh Village, Tembalang Subdistrict

The distribution of transovarial infection of DHF incidence was a collection of data observed by researchers in a radius of 100-200 meters in every RW. The result showed that in RW dengue virus spreading out in a radius of 100-200 meters caused DHF cases. The z score was -0,360 and the ANN *(Average Nearest Neighbor)* score was 0.94 < 1, meaning that the spreading pattern of the dengue events that occur was clustered. More details can be seen Figure 2.

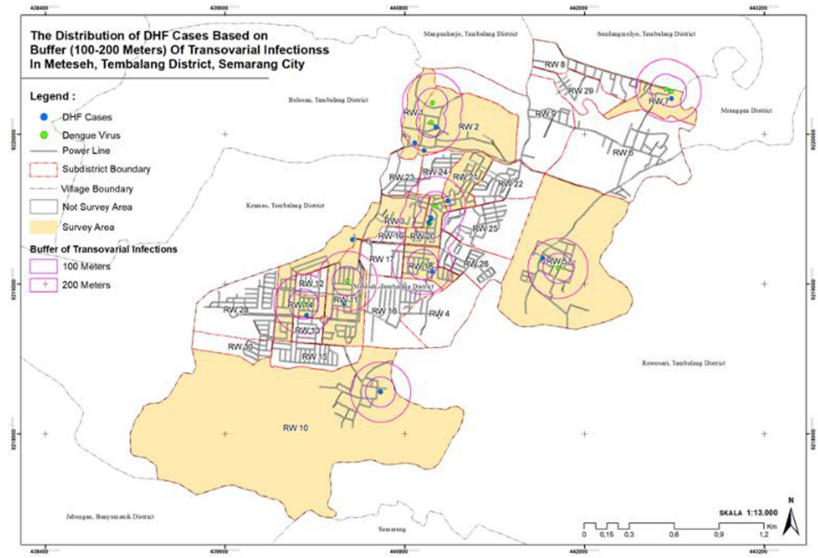
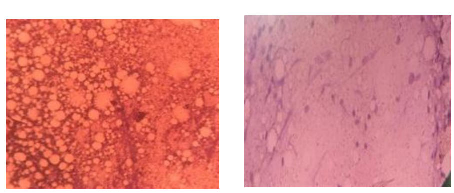


Fig. 2. DHF Larvae Densit

3.3 Immunohistochemical Examination

Eggs collected from research location were reared until adult; then, female *Aedes aegypti* mosquitos aged ≥ 5 days were selected. The test used 220 mosquitos or 20 mosquitos per RW. Next, dengue virus was identified using imunohistochemical method on the availability of *head squash* of female *Aedes aegypti* mosquitos reared from eggs. Details can be seen in Figure 3.

The results of the analysis of the relationship of transovarial infections indicated that the correlation coefficient (r) of 0.618 had the direction of a positive correlation (+) or unidirectional where the greater the value of one variable, the greater the value of other variables. The strength of the correlation was in the range of 0.4-0.6 (moderate) and the value of p = 0.043, which meant that there was a significant correlation between the two variables tested.



(a) (b)

Fig. 3. (a) H*ead squash tissue* positive *Den Virus* and (b) Negative *Den Virus,* in female *Aedes aegypti* mosquitoes from 11 Hamlets (RW) of Meteseh Village Tembalang Subdistrict

Cytoplasm cells in brown and spread out in brain tissue. Negative reaction was in the form of blue cytoplasm cell and there was no brown sand grain around brain tissue cells. The results of the examination of Dengue virus in adult female *Aedes aegypti* in 11 hamlets (RW) in Meteseh Village Tembalang District showed that there was a transovarial transmission in female *Aedes aegypti* in 11 hamlets (RW) in Meteseh village Tembalang District. Transovarial transmission takes place at 11 RW in Meteseh Village of Tembalang Sub-District. There are 14 *Aedes aegypti* mosquitos positively infected from??? AMONG? 220 mosquitos examined with TTI ranging from 5% to 20% with average of 6.36%.

The results of statistical analysis of the relationship between larvae density and the incidence of DHF in Meteseh village, Tembalang District showed that the correlation test between the density of larvae and the incidence of DHF in the Pearson Test (contained in the attachment) was p = 0.845, meaning that there was no significant correlation between the two variables tested.

4 Discussion

The analysis of *House Index* (HI), *Countainer Index* (CI), and *Breteau Index* (BI) in 11 RW of Meteseh Village, Tembalang Sub-District showed that the average value of *House Index* (HI) was 8.82%, *Countainer Index* (CI) was 3.89%, and *Breteau Index* (BI) was 9.37 to yield average Density Figure (DF) of 2.3. The result suggested that the larvae density in 11 RW of Meteseh Village, Tembalang Sub-District was categorized as moderate and therefore it potentially transmitted DHF.

The examination in 11 RW of Meteseh Village, Tembalang Sub-District showed that the average value of *House Index* (HI) was 8.82%. WHO entomology parameter to identify whether a region is vulnerable or impervious to DHF categorizes suggested that if the value of *House Index* (HI) is ≥5%, the infection risk is high, and if the value of HI is <5%, the infection risk is low

The average score of Density Figure (DF) in 11 RW of Meteseh Village, Tembalang Sub-District was 2.3, among which 5 RW belonged to medium category (2-5) and 6 RW belonged to low category (1). This figure indicated that Meteseh Village, Tembalang Sub-District might possibly transmitted DHF disease in low category; the average DF was 2.3.

The correlation test between larvae density and DHF incidence in 11 RW of Meteseh Village, Tembalang Sub-District was p = 0.854 > 0.05, meaning that there was no significant correlation between larvae density and DHF incidence. This findings were in line with the one stated that there is no correlation between the existence of larvae density and DHF cases in Bandung City[10]. There were other factors affecting this correlation, such as vector capacity, dengue virus virulence, host status and the environment. The finding of Sallata also revealed that there was no correlation between the presence of Aedes aegypti larvae and endemic DHF areas in Makassar City[11].

In some area, not all *Aedes spp* mosquitoes were DHF vectors based on an assumption that less than 5% population at the infection season will become vector [12]. The larvae density index is generally not related to the dynamics of the spread of disease by DHF vectors[13]. The accurate disturbance vector as part of the "risk" level of distribution of dengue was influenced by many factors, especially duration of mosquito live span and human immunological status.

In Singapore, the spread of dengue occurs even though the HI is <2%[14]. Larval density index does not represent the true distribution of DHF vectors. As a result, the relationship between larvae density and DHF incidence was difficult to be analyzed. According to Scoot and Marison, in general, the correlation between entomology index and DHF incidence was sometime not consistent, difficult to study, and difficult to be defined, all of which was caused by larvae index is very sensitive [15].

In the immunohistochemical test was conducted with a total of 220 consisting of 20 female *Aedes aegypti* F0 mosquitoes each hamlet (RW) with reference to the method of Purnama et al[16]. This was because female mosquitoes actively suck blood, so they were active in transmitting the dengue virus. In the body of a mosquito, the virus replicated in the salivary glands for 8-10 days.

The results of the analysis using the Pearson test was p value = 0.043 <0.05. This suggested that there was a significant relationship between transovarial infections in *Aedes aegypti* mosquitoes and the incidence of dengue fever. The results of head squash smear showed positive results on transovarial infections with 14 positive mosquitoes from the dengue virus out of 220 mosquitoes examined. Dengue virus ITT in female *Aedes aegypti* mosquitoes in 11 hamlets (RW) of Meteseh village, Tembalang District ranged from 5% - 20% with a total of 6.36%. This research finding was higher than that of in Brontokusuman village, which showed that the highest ITT score in hamlet (RW) 7 was 10% and hamlet (RW) 13 was 5%. In three other locations, hamlet (RW) 4, hamlet (RW) 9 and hamlet (RW) 18, there were no mosquitoes that were positive for dengue virus, so the ITT value was 0%. Thus, the value of ITT in Brontokusuman Village is 3%[17]. This research was in line with Purnama et al., that there is evidence of transovarial transmission from female mosquitoes to eggs using Immunocytochemical Techniques known as transovarial indices in Denpasar (11%), Gianyar (7.14%) and Tabanan (7.14%).

This study was also in line with Deni's that there is a significant correlation between the trannsovarial transmission index (ITT) of Dengue virus in *Aedes aegypti* mosquitoes and the incidence (IR) of DHF in 10 villages in Pontianak City DHF case location, while the value of the close relationship with r = 0.645.

This finding suggested that the existence of dengue virus in the nature was vertically protected. There are three transovarial mechanisms, among which is vertical transmission of dengue virus in the female mosquitos' body that is infected by female mosquitos on their eggs (transovarial). This might happen when the virus is transferred through oviduct during embryogenesis period. Consequently, the infected eggs produced infected larvae that later become mosquitos with 80% infection rate[18].

The multiplication of viruses in different organs during the embryogenesis process or in the final stages of mosquito life can vary due to tissue tropism, hereditary viruses and genetic host production[19]. According to Mardhihusodo et al, gravid female of infected *Aedes aegypti* mosquitos by dengue virus to ovum in the mosquitos' uterus will active in the embryo that lastly they will propagate in the embryo. Next, the dengue virus use larvae to imago as life support medium so human might be infected by dengue virus when the newborn mosquitos bite and suck human blood[20]

The higher the mosquitos carried the virus was, the higher the risk of being infected by DHF through transovarial that tended to be potentially become factor affecting DHF. Of 11 RW of Meteseh village, the larvae density was relatively low, but they carried virus. As Meteseh village was likely to be infected by DHF, the status of this area was epidemic proven by the increased incidence rate in 2019, which was 70.03/100.000 population.

A study in Kerala, India, found natural transovarial transmission at the level of Den 3[21] In Malaysia, infected larvae are found in 16 different location in which virus infection level of *Aedes agypti* is higher than that of *Aedes albopitus*[5].

The phenomenon of dengue virus transovarial transmission occured in several neighboring countries, including Malaysia and India while in Indonesia transovarial transmission occured in Semarang, Yogyakarta, Manado and Bali. The transovarial transmission found in the *Aedes aegypti* 11 hamlets (RW) mosquito in the Meteseh village of Tembalang District deserved special attention because the Meteseh village was a dengue endemic area.

5 Conclusion

The result of the research shows that the larvae density has no correlation to DHF incidence. However, it has been proven that transovarial infection correlates with DHF incidence; thus, it is likely that potentially female *Aedes aegypti* mosquitos transmit the virus through transovarial transmission in Meteseh Village of Tembalang Sub-District. The z score of spatial analysis on l transovarial infection is -0,360 and ANN *(Average Nearest Neighbor)* score is 0.94 < 1, meaning that the distribution pattern of DHF was clustered.

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