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THE POTENCY OF POLYGAMY BEHAVIOR IN AEDES AEGYPTI MOSQUITOES BY VENEREAL TRANSMISSION DENGUE VIRUS

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Abstract

Male Aedes aegypti mosquito has been considered to not have any important role in transmitting dengue virus (DENV). The purpose of this study is to prove that male *Ae. aegypti* mosquito does have an important role in transmitting DENV 3 through venereal transmission with their potency of its polygamy behavior. The data collection was done using colonization method and intrathoracal injection. The presence of DENV 3 on male and female mosquitoes was proven by RT-PCR method (profile of DNA band specifically on 511 bp) and serotyping PCR (290 bp). The data were analyzed using univariate analysis followed by bivariate analysis with parametric test ANOVA. The result of the study demonstrated that male *Ae. aegypti* mosquitoes do have an important role in transmitting DENV 3 through venereal transmission with the potency of their polygamy behavior. There was no significant difference between the polygamy behaviors of *Ae. aegypti* male mosquito infected by DENV and the non-infectious *Ae. aegypti* male mosquito.

Introduction

Dengue fever is a mosquito-borne viral disease in human which is prevalent in the tropical and subtropical regions of the world. Dengue infection is caused by dengue (DEN) virus which has 4 serotypes (DENV 1, DENV 2, DENV 3, DENV 4), of the genus Flavivirus (Andriyoko, 2011). They are transmitted to human by Aedes (Stegomiyia) mosquitoes, with Aedes aegypti as the principal vector, and has been incriminated in major dengue outbreaks worldwide (Kraemer, 2015).

On November 2015 the number of dengue fever case was 77.633 cases with 778 deaths (CFR; 1 %). Prevention and eradication program had been going on for 43 years and it had succeeded in decreasing the mortality rate from 41.3% in 1968 to 0.87% in 2010, however it has not succeeded in decreasing the incidence rate of DBD. The number of patients tend to increase, the spread of disease is getting wider, not only infecting children but also elderlies (Ditjen PPPL, 2016).

The causative agent of dengue fever is dengue virus (DENV), which is an RNA single stranded virus and has 4 serotypes: DENV 1, DENV 2, DENV 3 and DENV 4. Those four virus types circulate in endemic areas of

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dengue fever in Southeast Asia countries such as Malaysia, Thailand and Indonesia, however only DENV 2 and DENV 3 are often connected with severe clinical manifestation. Previous studies found that DENV-3 was the primary cause of severe dengue cases in Indonesia (Andriyoko, 2011; Cucunawangsih, 2017).

Currently, the one effective way to prevent dengue fever is through vector control. Vertical transmission is the way the viruses are maintained in nature during the absence of viremic vertebrate host or when the climate condition are not favorable for mosquito population density. Several studies have confirmed vertical transmission of dengue virus in nature in infected *Ae. aegypti* larvae and from adult reared of wild-caught adult mosquitoes (Günther, 2007; Angel, 2008; Arunachalam, 2008; Rohani, 2008; Le Goff, 2011; Thongrungkiat, 2011; Martins, 2012; Martínez, 2014). It was also reported that in Singapore, 1.33% and 2.15% of *Ae. aegypti* and *Ae. albopictus* adult male mosquitoes, respectively, were positive for dengue viruses. The serotypes detected in male *Ae. aegypti* were DENV 1 (44%), followed by DENV 2 (22.2%), DENV 3 (22.2%), and DENV 4 (11.1%). For *Ae. albopictus* males, the serotypes were DENV 4 (38.9%), followed by DENV 2 (33.3%), DENV 3 (16.7%), and DENV 1 (11.1%).

The ability of *Ae. albopictus* to transmit DENV sexually (venereal transmission) from infectious male to non-infectious female was demonstrated in laboratory. The polygamous behavior of male *Ae. aegypti* (L.) and *Ae. albopictus* (Skuse) was confirmed by determining their insemination rates. The results indicated the polygamy behavior of male *Ae. aegypti* in venereal transmission could contribute to the maintenance of dengue virus in nature. The possibly significant role of their polygamy in relation to dengue virus transmission is to be discussed.

Based on that background, an understanding of the potency of polygamy behavior in male *Ae. aegypti* mosquito infected with DENV 3 in venereal transmission to non-infected female *Ae. aegypti* mosquitoes is important to be known so it can give more information in developing the strategy of dengue vector control.

Methods

The *Ae. aegypti* mosquitoes were obtained from laboratory colony maintained at the Department of Parasitology Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia. The *Ae. aegypti* mosquito eggs were generated from non-infectious mosquitoes. The mosquitoes come from Yogyakarta, which parental line has been proven non-infectious by laboratory examination. The DENV-3 source was infected C6/36 cell culture supernatant provided by the Department of Parasitology Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta. The virus was a prototype of DENV 3 strain H87obtained from Laboratory of Namru 2 Jakarta in 1996.

While the colonization process of *Ae. aegypti* includes transforming into pupa, male pupa and female pupa were separated. The separation was based on the size of pupa with male pupa being smaller than female pupa. Then, every 10 male pupas and female pupas were placed into different cages. The pupas were maintained to be imago or adult mosquitoes. Confirming the sex of the pupa was done by observing the developing stadium of adult mosquitoes. Male mosquito has a long and bushy antenna (plumose) meanwhile female mosquito has less bushy antenna (pilose) (Arthur, 2014). If any one male mosquito was found within a female mosquito cage or vice versa, the cage which contains them must be dropped out. For the preparation of intrathoracal injection method, the age of both the male and female mosquitoes were one day old.

After emerging, one day old *Ae. aegypti* male mosquitoes were inoculated intrathoracally with 1,5-0,2 µl DENV 3 suspension per mosquito. The infected mosquitoes were held in cages and maintained on a 10% glucose solution. Mosquitoes were kept in accordance with the incubation period of the virus, i.e. 5 days, and 14 days. Then, infectious *Ae. aegypti* male mosquitoes will be chosen to be mated in polygamous way with non-infectious *Ae. aegypti*. The criteria of male mosquitoes to be mated with female mosquitoes: alive male mosquitoes during the accordance with the incubation period, still able to fly and can survive at least 24 hours

after being placed into the cages because at that current time the mating can happen naturally (Ponlawat, 2009).

Infectious male *Ae. aegypti* mosquito (male mosquito which was infected with DENV 3 through intrathoracal) at incubation period of 5 days, and 14 days were placed into cage containing non-infectious one-day old female *Ae. aegypti* mosquitoes (female mosquitoes which were not infected by DENV) with ratio 1 male : 10 female to mate. As the control, 5 days old and 14 days old *Ae.aegypti* non-infectious male mosquitoes would be mated in polygamous way with one-day old non-infectious *Ae.aegypti* with ratio 1 male : 10 female. All male and female mosquitoes were kept in one cage for 7 days. They would be fed glucose solution 10% by absorbing glucose through cotton and being changed daily. On the seventh day, male mosquitoes were collected and stored in a freezer with the temperature of -80^{O}C, meanwhile female mosquitoes were given blood feeding by using the blood of mice. Afterwards the female mosquitoes would be kept separately in paper cup covered with gauze for laying eggs process (individually rearing). Cotton wetted with glucose solutions 10% was put on the gauze and replaced daily. Paper cups for individual rearing were placed into closed Styrofoam container. Female mosquitoes were maintained for 7 days until they laid eggs then the eggs were labeled. On the fourteenth day the female mosquitoes were collected and stored in a freezer with the temperature of -80^{o}C. The eggs were dried then stored for 7 days within room temperature.

The intrathoracal injection of the stored male mosquitoes that had mated with non-infectious female mosquitoes in polygamous way was detected using the One-Step RT-PCR and serotyping PCR and the results proved that the DENV 3 injected was replicated during defined incubation period thus allowing the sexual transmission (transvenereal transmission) of DENV 3 to the non-infectious female mosquitoes. The male mosquitoes with positive DENV 3 were traced through the female mosquitoes. The 14 day-old female mosquitoes collected after polygamous mating with DENV 3 positive infectious male mosquitoes were tested using One-Step RT-PCR and serotyping PCR to see the proof of any venereal transmission from infectious male mosquito. The eggs of DENV 3 positive female mosquitoes were hatched to prove the mosquitos' fertility.

An analysis was made to compare the polygamy behavior between the infectious male mosquitoes and non-infectious male mosquitoes using univariate analysis method. We carried out a normality test to determine the data distribution, if the distribution was normal, the analysis would be continued with parametric bivariate test using ANOVA. However, if the data distribution was not normal, the non-parametric bivariate test (Kruskall Wallis) should be used instead.

Result and Discussion

The result of this study indicates that male mosquitoes infected with DENV 3 through intrathoracal injection within 5 to 14 days of incubation period showed a diagnostic band of 511 bp for DENV screening and 290 bp for DENV 3 serotyping, the same as the female mosquitoes that had undergone mating process. This proved that infectious male mosquitoes with DENV 3, within 5 to 14 days of incubation period were able to mate with non-infectious and DENV 3 infected female mosquitoes. The venereal transmission was also proven by the existence of fertile eggs (eggs which had been fertilized) from female mosquitoes, in which the eggs can be hatched and continue to develop into the larvae stage. Female mosquitoes require blood as provision for their eggs. However, there was no fertility in such eggs that they were not able to hatch, or if they did hatch it would not be able to reach immature stadium. The results of One-Step RT-PCR in mosquito pools of female *Ae. aegypti* mosquitoes that lay fertile eggs by pooling is presented in Table 1.

The following table is the numbers of female DENV 3 positive *Ae. aegypti* mosquitoes and the numbers of fertile eggs according to the incubation period of male *Ae. aegypti* mosquitoes, treatment and control.

The next step is to see whether the infectious *Ae. aegypti* mosquitoes with 5 and 14 days incubation period have the same polygamy potency as the non-infectious male *Ae. aegypti* mosquitoes (control). The variable analyzed was

Table 1. The Result of *One-Step RT-PCR* in Individual Male *Ae. aegypti* Mosquitoes and in Total Female *Ae. aegypti* Mosquitoes which had Laid Fertile Eggs by Pooling.

Male Mosquitoes Incubation Period	The Numbers of Male Mosquitoes	One Step RT-PCR (Individual) Result	The Numbers of Female Mosquitoes that Produce Fertile Eggs	One Step RT-PCR (Pooling) Result
5	1	positive	5	positive
14	1	positive	2	positive

Table 2. The Numbers of Fertile Eggs Each Female *Ae. aegypti* Mosquitoes with Positive DENV 3 Laid According to the Incubation Period of Male *Ae. aegypti* Mosquitoes, Treatment and Control.

	TREATMENT		CONTROL	
	Incubation Period		Incubation Period	
	5 days	14 days	5 days	14 days
The numbers of Fertile eggs laid per DENV 3 Positive Female *Ae. aegypti* mosquitoes	35	25	21	31
	29	22	56	47
	34		33	6
	6		31	
	17		45	

the numbers of hatched eggs or the numbers of fertile eggs from DENV 3 positive female mosquitoes. The ANOVA test was deployed as the distribution of the data was normal. The number of female mosquitoes which laid fertile eggs and the average fertile eggs produced by female mosquitoes, on experimental and control groups are presented in table 3.

The analysis of the table by ANOVA test gave a p value of 0.365, thus can be concluded that there was no significant difference between the average amount of the fertile eggs laid by DENV 3 positive female mosquitoes within the four experimental and control groups.

The result of the study proved that male *Ae. aegypti* mosquitoes have an important role in transmitting DENV 3 through their polygamy behavior potency and ability in transmitting DENV 3 sexually. The result of the study also indicated that the polygamy behavior in DENV 3 infected male *Ae. aegypti* mosquitoes have the same potency as the non-infected male *Ae. aegypti* mosquitoes. This fact lead to the conclusion that one DENV 3 infected male

Table 3. The number of female mosquitoes which lay fertile eggs and the average fertile eggs produced by female mosquitoes, on experimental and control groups.

	Male mosquitoes incubation period (in days)	The numbers of Female mosquitoes that produce fertile eggs	The average of fertile eggs/mosquitos (Mean ± SD)	P
Experimental	5	5	24.20 ± 5.56	0.365
	14	2	23.50 ± 1.50	
Control	5	5	39.80 ± 6.24	
	14	3	28.00 ± 11.93	

mosquito, through their polygamy behavior, is able to mate with non-infectious female mosquitoes in certain numbers and eventually the infectious female mosquitoes can lay fertile eggs.

Inside the infected female mosquitoes, when the DENV 3 reaches their salivary gland, it will transmit DENV 3 potentially (horizontally) to vertebrate host, and if the female mosquitoes were infected through venereal transmission, their fertile eggs will be infected via trans-ovarian pathway and the transmission of dengue case will get distributed into wider areas.

The existence of DENV in nature is maintained through horizontal and vertical transmission. The best documented mechanism is the transmission of dengue virus via horizontal transmission, which is maintained by the human–*aede*s mosquito–human cycle. However, in vertical transmission, the infected female mosquito is able to transmit the virus directly to their progeny, which has been reported experimentally. Rohani (2008), proved that DENV 2 is detected positive until the next five generations of *Ae. aegypti* mosquitoes, with MIR 30 – 45/1000.

Male *Ae. aegypti* mosquito has been considered to have minor role in transmitting DENV, because male mosquito is unable to draw blood from human host. Nevertheless, they have an opportunity to be infected with the virus by vertical transmission from infected parents and naturally infected males may be the main force initiating venereal transmission. However, several studies proved that DENV is originally reared through vertical to progeny (F1) of male *Ae. aegypti* mosquitoes in nature. Arunachalam (2008) in Tamil Nadu India, stated that DENV 2 and DENV 3 were detected in male *Ae. aegypti* caught in the wild, with the highest MIR of 28/1000. DENV 3 was detected in male *Ae. aegypti* collected by using ovitrap in Minas Gerais, Brazil with MIR of 10/1000 (Vilela, 2010). (Mulyatno, 2012) also proved that the transovarial transmission of DENV 1 on *Ae. aegypti* has an MIR of 16 – 26/1000.

This study proved that the principal species of dengue is the *Ae. aegypti* mosquito. Male *Ae. aegypti* mosquito is able to mate with a maximum number of 14 female mosquitoes in the rearing cage with the ratio of male : female = 1 : 30. With the strong polygamous mating behavior of *Ae. aegypti* males, venereal transmission of the DENV could be one effective mechanism to maintain the existence of DENV in nature during the absence of viremic vertebrate host or when the climate condition is not favorable for a dense mosquito population.

Unfortunately, little information is available on mating of mosquito especially in nature. New mosquitoes leaving the pupa has not reached maturation, because the external genitalia has not developed. The external genitalia matures within 24 hours after leaving the pupa. In most species, male accessory gland matures within days after adulthood (emerging from pupa), so the sperm will succeed to get transferred. Almost all species of mosquitoes need 24 – 48 hours after the males emerged until mating. The mating does not need the egg to have matured and developed, but eggs of most of mosquito species are able to be kept whenever the fertilization occurs (Howell, 2009; Oliva, 2011).

Mating is initiated when the male mosquito grabs the female mosquito and orients himself so he is in "venter to venter" position with the female. *Ae. aegypti* copulation is completed in an average of 10 seconds (Ponlawat, 2009). During the mating process of *Ae.aegypti* mosquitoes, the accessory gland will secrete fluid in the semen (seminal fluid), which will be kept in the female bursa inseminalis. Seminal fluid from the male accessory gland functions as the media in which the sperms swim to female spermatecha organ (Arthur, 2014). Seminal fluid of the male *Ae. aegypti* contains a large number of protein that get transferred into the female during mating and may have important influences on female biology and behavior (Sirot, 2009).

Used immunofluorescent techniques to stain the organs of orally and parenterally DENV 1 infected *Ae. albopictus* and *Ae. aegypti*. DENV infections were found in the female ovarioles, oviducts, and accessory glands, suggesting that venereal transmission DENV may occur at fertilization. While the fertilization is happening, DENV is spreading to the eggs through microfil and transmit

through transovarial pathway to the progeny of *Ae.aegypti*, both male and female. Progeny male mosquitoes have the potency to transmit DENV through venereal transmission and polygamy behavior whereas the female will transmit to vertebrate host.

Ae. aegypti mosquito is the principal vector of DENV, and since it has been proven that it can transmit the virus through venereal transmission with its polygamy behavior, it can be concluded that male *Ae. aegypti* mosquito has an important role as the transmitter of dengue case. However, it is still unknown about the venereal transmission of the virus whether it can potentially transmit dengue to vertebrate host, most importantly human. The virus transmission as a result of venereal transmission is already proven by (Mavale, 2010). Female *Ae. aegypti* is potentially transmitting its CHIKV to the babies mice. Thus, it will be the focus for the next study in molecular entomology and dengue epidemiology.

Several studies also proved that vertical study can predict dengue outbreak cases by monitoring the immature stage of Aedes sp. (H.L. Lee, 2005) reported that dengue cases and transovarial transmission of DENV on *Ae. albopictus* were analyzed at the same time, reported that the transovarian transmission occurred before the dengue reported as positive in human and the intervals between transovarial dengue virus detection and first human case ranged from 7 – 41 days. The statement supported by (Thongrungkiat, 2011) stated that the survey of dengue virus on immature stage from wild environment and the information from dengue-GIS (Martins, 2012), potentially indicated the transmission of earlier dengue epidemic and is useful to complete an early warning system on dengue outbreaks during inter-epidemic periods**.** However, the explanation of the study above has not proven statistically the association between the increasing DENV infection in immature stage and the occurrence of dengue outbreaks, therefore it needs further study.

Conclusions

The conclusion of this study is that the male *Ae. aegypti* mosquitoes are proven to have important role in transmitting DENV 3 through their polygamy behavior and their ability to transmit DENV 3 through venereal transmission. It needs further study on venereal transmission in *Ae. aegypti* mosquitoes whether they have any potential in transmitting dengue to human. It needs further statistical comprehension of the association between the increasing of DENV infections in immature stage and the occurrence of dengue outbreaks. This study will give more important information in developing strategy control toward DBD in vector aspect and it can be used to complete an early warning system on dengue outbreaks.

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