KEMAS 12 (1) (2016) 131-138

Jurnal Kesehatan Masyarakat

http://journal.unnes.ac.id/nju/index.php/kemas

TRANSOVARIAL TRANSMISSION AND DENGUE VIRUS SEROTYPES IN *AEDES AEGYPTI* IN KUPANG

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Article Info

Article History:
Submited Frebruary 2016
Accepted June 2016
Published July 2016

Keywords:
Virus; Transovarial;
Ae. Aegypti; Dengue

DOI
http://dx.doi.org/10.15294/kemas.v12i1.4993

Abstract

Dengue is an endemic disease in almost all provinces in Indonesia, including NTT province and Kupang City. DHF prevention and control efforts need to be accompanied by understanding of the epidemiological and entomological aspects. This study proves the presence of transovarial transmission and serotypes of Dengue virus in *Ae. aegypti* in areas with different endemics in Kupang City. The study was conducted in endemic, sporadic and Dengue-free villages. Samples from each location were 30 head squash *Ae. aegypti* females for examination of Dengue virus by IHC methods and 10 *Ae. aegypti* females for serotypes check of Dengue virus with RTPCR. Data presented in tables and images then analyzed descriptively. This study finds that transovarial infection rates 9.2% with serotype Dengue virus-1. As many 97.5% of respondents have heard about Dengue by most cadres resources (24.5%) and health workers (24%). Transovarial infection is found in endemic, sporadic and free areas so that the Dengue vector control needs to be done in the three regions with different endemicity.

Introduction

Dengue Hemorrhagic Fever (DHF) is found in tropical and subtropical regions. Data from around the world shows that Asia ranks first in the number of Dengue fever patients annually. Meanwhile, starting from 1968 until 2009, WHO notes that Indonesia as the country with the highest Dengue cases in Southeast Asia. Dengue case was first reported in Indonesia in 1968 in Surabaya and Jakarta with 58 cases and Case Fatality Rate (CFR) reaching 41%. Since then, cases of Dengue fever was spread throughout Indonesia, from 2 provinces and 2 cities in 1968 to 32 provinces (97%) and 382 cities / counties (77%) in 2009. The cases of Dengue also increased from 58 cases in in 1968 to 158 912 in 2009 (Pusat Data Surveilans Epidemiologi, 2010). The incidence of Dengue fever continues to rise and is now an endemic disease in almost all provinces.

Dengue case in East Nusa Tenggara province always exists and occupies the lowest position in 2009, but in 2011, it became 19th out of 33 provinces, with most cases happened in Kupang. Dengue cases in Kupang have increased every year, from 273 cases in 1998 to 525 cases in 2011. In 2012, an extraordinary events/outbreak occurred in 51 villages in Kupang with the number of 867 cases with 3 deaths cases (Dinkes Kota Kupang, 2013). Figure 1 shows that Kupang has higher IR than the national average since 2007-2012.

DHF is caused by Dengue virus and there are four serotypes which are known, namely Den-1, Den-2, Den-3 and Den-4 (Guerdan, 2010). DHF is transmitted through the bite of female *Aedes sp*, where *Aedes aegypti* (*Ae. Aegypti*) is the main vector and *Ae. albopictus* as the secondary vector in Indonesia, but for other countries such as Costa Rica *Ae. albopictus* is the main vector (Calderon, 2010). Climatic factors and changes in the ecological and socio-demographic factors play an important role in the increased incidence and expansion of

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P-ISSN 1858-1196
E-ISSN 2355-3596

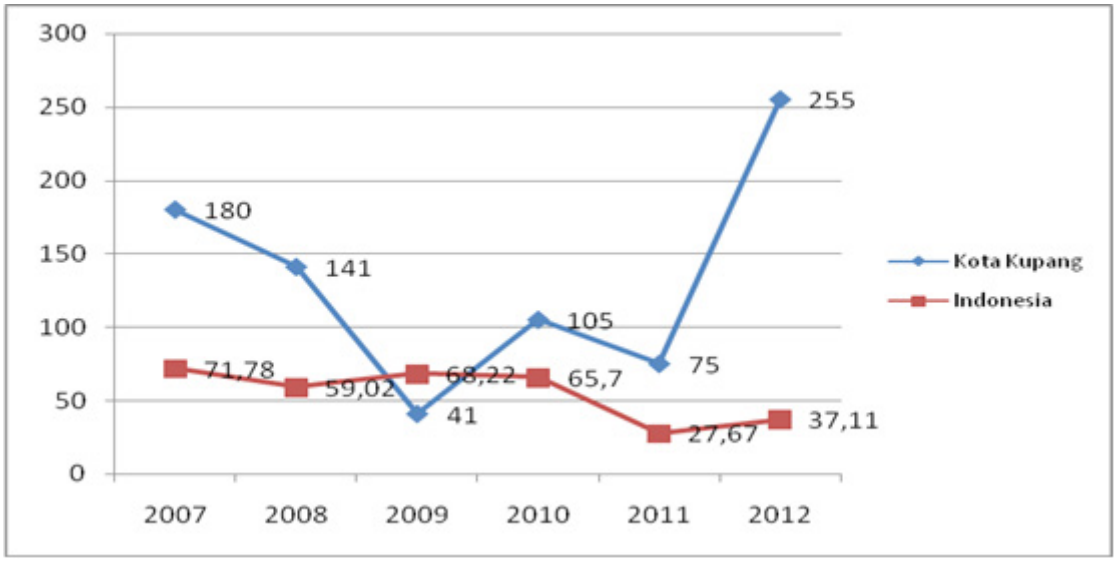


Figure 1. Incidence of DHF Rate per 1,000 Population 2009-2012 in Kupang and Indonesia Source: Dinkes Kota Kupang (2013 and MoH RI (2013)

endemic areas of Dengue disease. Therefore, epidemiological aspects including mechanisms of transmission of Dengue virus (Dengue virus) is important aspect to learn. Transmission of Dengue virus can occur horizontally i.e. from transmission of Dengue virus from sick patients to mosquitoes and vice versa, as well as vertically (transovarial) i.e. the transmission of Dengue virus from female mosquitoes into the ovum in the uterus or transmission from male mosquitoes infected with Dengue virus to female mosquitoes which occurs during the venereal activities (Mardihusodo, 2007). This vertical transmission of Dengue virus causes the transmission of the Dengue virus will never stop because once mosquito is infected with Dengue virus, then the virus remains in the animal for the rest of its life and it is also found throughout the season (Angel, 2008; Rohani, 2008).

Until now there is no cure or right vaccine for Dengue disease. Therefore, in the prevention and control efforts incidence of DHF, it is necessary to understand the epidemiological aspects including climatic conditions, the vector density and the presence of transovarial transmission and serotypes of Dengue virus in *Ae. Aegypti*. This study aims to prove the presence of transovarial transmission and serotypes of Dengue virus in *Ae. aegypti* in endemic, sporadic and free of Dengue areas in the city of Kupang.

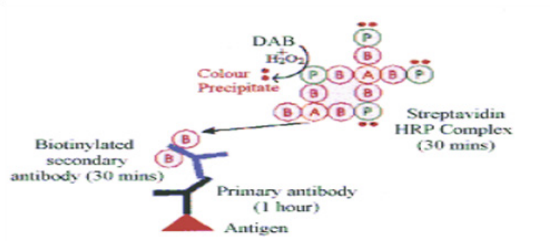
Method

This study was an observational study using a descriptive study through cross-sectional design where the study variables were studied in the same period. The study was conducted in endemic areas (Oebobo village Oebobo District and Alak Village, Alak District), sporadic area (South Oesapa South Village, Kelapa Lima District) and free area (Fatukoa village, Maulafa District). Therefore, four villages in four districts in Kupang were involved in this study. Every survey was conducted in 30 houses per village.

Fieldwork was conducted to survey the knowledge and attitudes about Dengue and its prevention as well as for the collection of eggs of *Aedes sp*. The egg collection was done by mounting ovitrap which was contained 200ml glass painted black on the outside, filled with water 2/3, then mounted filter paper (ovitsrip) with a size of 5cm x 20cm full circle in the glass limited to surface water so ovistrip became moist. Coarse filter paper was mounted with hard section facing inward as mosquito breeding ground. Ovitrap was set inside or outside the house in a dark place that was expected to be potential spawning grounds for *Aedes sp*. Each house was fitted two pieces of ovitrap inside and outside the house. After three

days, the water of ovitrap was checked and was taken after 1 week. Each place was done for 3 weeks or 3 repetitions.

The dried ovistrip with eggs was then separated according to location and was sent to a parasitology laboratory of Medical Faculty, Gadjah mada University to do rearing until the first offspring (F1) and the examination of the Dengue virus in *Ae. Aegypti*. The eggs became adult mosquitoes colonized up to expectations when there was a virus in eggs then they were replicated during the development of the egg to adult mosquitoes, making it easier to detect the presence of Dengue virus. Positive eggs Ovistrip*s* were laid in the tray. After they were hatched into larvae, they were fed with meat of dried liver. Pupae emerged later were transferred to mosquito breeding nest and waited 1-2 days to become adult mosquitoes. Adult mosquitoes that had appeared were separated by location in a mosquito cage sized 20x20x20cm and then were maintained until the age of 7 days to be only given sugar water 10%. The samples for examination of transovarial transmission with IHC method were 30 *Ae.Aegypti* female mosquitoes. Therefore, 120 *Ae. aegypti* females were involved in four locations. While samples for examination serotype of Dengue virus in mosquitoes *Ae.Aegypti* method RTPCR were 10 mosquitoes *Ae. aegypti* females coming from 4 locations.



Source: Umniyati (2004)

Figure 2. ISBC Method Scheme to Detect Antigen Virus in body tissue/cell

The detection of Dengue virus in mosquitoes used Immunohistochemistry Streptovidin Biotin Peroxidase Complex (ISBPC) method by setting preparation of head squash which had been developed and standardized by Umniyati (2004). The steps were: preparations of head squash were made by pressing the head of *Ae. aegypti* aged > 7 days that had been killed and separated from the body on an object glass coated with Poly L Lysine and made per location; preparations were fixed with cold methanol -20°C for 3-5 minutes, then stored at -20°C if not immediately checked; washing the preparations under distilled water and then with Phosfat Buffer Saline (PBS) until it was stagnant; activities of *Peroxidase Blocking Solution* in a room temperature for 10 minutes or under tap water for 5 minutes; preparations were incubated in *prediluted Horse Serum* for 10 minutes at a temperature of 25°C or the tray of damp then wrapped; Primary antibody commercial *monoclonal antibody* 1:10 that had been prepared was added as much as 100µl per preparation (until stagnant) and were then incubated in a moist tray at room temperature (25°C) for 60 minutes or overnight in the refrigerator; further, the preparations were put in fresh PBS for 5 minutes; Biotinylated Universal Secondary Antibody (BUSA) as much as 100µl per slide was added, and then incubated at a temperature of 25°C for 10 minutes or in a tray of moist, then the preparations were washed for 5 minutes with fresh PBS; the preparations were incubated in ready-to-use streptovidin/peroxidase complex reagent as much as 100µl for 10 minutes in a tray of moist, after the preparations were washed with PBS for 5 minutes; preparations incubated in 100µl DAB/preparations for 2-10 minutes (the thicker the longer the incubation preparation) at room temperature (25°C); preparations were washed using tap water, then Mayer hematoxylin (counterstain) of 100µl was added and incubated for 5 minutes, then washed under tap water; The slide was dipped into 95% alcohol, then dipped into xylol and was cleaned for contrasting colors; The slide was dropped using mounting media and was then covered with a cover slip, then a dry slide was ready to be examined under a microscope at 400x and 1.000x distribution; brown preparation meant that it was positive with Dengue virus antigen, whereas pale blue preparation meant that it did not contain the Dengue virus antigen.

Dengue virus examination was done separately for each location and conducted only for female mosquitoes. Examination of Dengue virus with RTPCR method began with the isolation of viral RNA from the head of females

Ae. aegypti using the High Pure Viral nucleid Acid Kit from Roche. Viral RNA obtained was stored in a temperature (-20°C) prior to RTPCR.

Primary data included data on transovarial transmission rates of Dengue virus in *Ae. aegypti* females which were obtained by observation/direct examination in the laboratory with ISBPC method. Mosquitoes implied transovarial transmission when the virus antigen was detected by the formation of brown color on inspection method of ISBPC. No transovarial transmission in mosquito was shown when brown color was not formed; instead, the preparations slide became blue on the examination by the ISBPC method. Dengue virus serotype data was obtained by observation / inspection using RTPCR. The primary data was about people's behavior in elimination of DHF mosquito breeding sites was directly obtained through interviews. The collected data was processed and presented in the form of tables and pictures, and then analyzed descriptively.

Results and Discussion

Based on education, the majority of

Table 1. The Knowledge about DHF an DHF Prevention in the Endemic, Sporadic and Free Dengue Regions in Kupang

Variables	Endemic 1	Endemic 2	Sporadic	Free	Kupang
Education					
No School	3,3	6,7	0,0	6,7	4,2
SD	6,7	30,0	6,7	46,7	22,5
SLTP	10,0	16,7	6,7	23,3	14,2
SLTA	46,7	33,3	36,7	16,7	33,3
College	33,3	13,3	50,0	6,7	25,8
Already Heard about DHF					
Never	0,0	3,3	0,0	6,7	2,5
Already	100,0	96,7	100,0	93,3	97,5
Information Source					
Health Officers	21,5	30,4	16,7	33,3	24,0
Village Officials	2,8	2,9	1,7	2,7	2,4
Cadres	18,7	31,9	19,2	34,7	24,5
Family	24,3	23,2	13,3	5,3	16,7
Electronic Media	14,0	4,3	25,0	17,3	16,4
Printed Media	18,7	7,2	24,2	6,7	15,9
Mosquitoes transmitting DHF					
Anopheles	0,0	0,0	0,0	3,6	0,8
Aedes	56,7	34,5	90,0	53,6	59,0
Culex	0,0	0,0	0,0	0,0	0,0
DHF Mosquitos	43,3	62,1	10,0	35,7	37,6
Do not know	0,0	3,4	0,0	7,1	2,6
Breeding sites of *Aedes*					
Shower Tub	100,0	100,0	100,0	100,0	100,0
Closet Tub	96,7	89,7	93,3	75,0	88,9
Crock	20,0	0,0	56,7	32,1	27,4
Used tires	10,0	0,0	3,3	7,1	5,1
Vase	20,0	3,4	10,0	3,3	11,1
Bird drinking pot	13,3	0,0	3,3	10,0	6,0
Used can/bottle	46,7	6,9	23,3	0,0	29,9
Bamboo/Branch	3,3	6,9	0,0	27,4	3,4
Preventive Action for DHF					
Draining TPA	100,0	89,7	100,0	92,9	95,7
Closing TPA	96,7	86,2	96,7	82,1	90,6
Burying used materials	80,0	82,8	80,0	50,0	54,7
Pouring abate	96,7	89,7	96,7	71,4	88,9
Using mosquito repellent substances and mosquito net	33,3	6,7	33,3	21,4	26,5

respondents have high school education (33.3%) and only 4.2% are not in school, as shown in Table 1.

Table 1 shows that 97.5% of respondents have heard about Dengue and only 2.5% have never heard of Dengue. Their sources of information about Dengue are from cadres (24.5%), health officers (24%) and village officials only 2%. This study finds that only 59% of respondents who know the mosquito-borne Dengue fever is *Aedes*.

This research probes at 120 mosquito *Aedes aegypti*. The transovarial infection rates (TIR) in Kupang is 9.2%. The highest TIR which is found in endemic areas is 13.3% and in the free areas of Dengue fever, transovarial transmission is still found although their TIR is lower than in most endemic and sporadic areas as shown in Table 2.

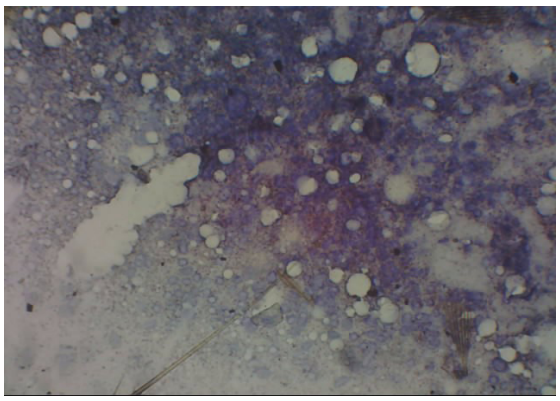
Table 2. The Number of Transovarial Transmission in the Endemic, Sporadic and Free Regions in Kupang 2013.

Endemicity	**VirDen (+)**	**Total** *Ae. Aegypti*	**TIR (%)**
Free	1	30	3,3
Sporadic	2	30	6,7
Endemic 1	4	30	13,3
Endemic 2	4	30	13,3
Total	11	120	9,2

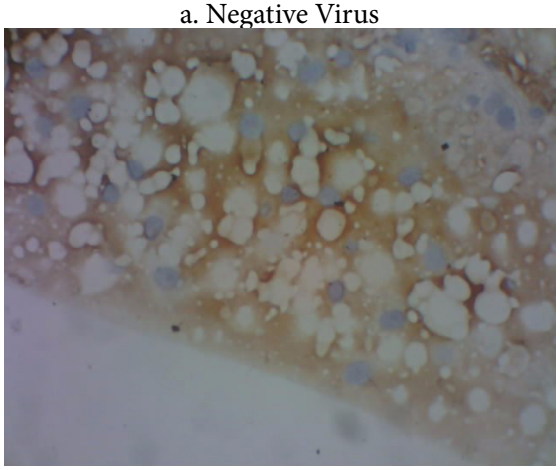
This study detects the presence of Dengue virus in the head squash of *Aedes aegypti* mosquitos using Immunohistochemistry method. It is said to be positive when the brown color on the head quash is mosquito *Aedes aegypti* formed. The presence of the Dengue virus is said to be negative when it does not form a brown color but instead blue color, as shown in Figure 1.

This study also examines the serotypes of the Dengue virus in *Ae. aegypti* which comes from Kupang and was only found Dengue virus serotype 1 and it is found in Oesapa (sporadic area) and Alak (endemic area). The other Dengue virus serotypes such as Den-2 virus, Den- 3 virus and Den-4 viruse are not found in Kupang.

This study finds the evidence of transovarial transmission of Dengue virus in *Ae. aegypti* in Kupang 13.3% and is found in all free, sporadic or endemic regions. Research on Dengue virus serotypes in Kupang has not been done so far and this study only finds the virus serotype Den-1 only in mosquitoes *Ae.Aegypti* originating from Kupang and is only found in Fatukoa Village (sporadic areas) and Alak village (endemic area). The other Dengue virus serotypes of virus 2, virus 3 and virus 4 are not found in of Kupang.



a. Negative Virus



b. Positive Virus

Figure 1. Negative Virus is Shown in Blue Head Squash (a) Positive Virus is Shown in Brown Head Squash (b) *Ae. aegypti* Females Mosquito with Immunohistochemistry

Research in Kupang is the same as the results of research in Surabaya in 2012 where virus 1 is the most dominant Dengue virus (60.56%) (Aryati, 2012). However, in previous studies in 2008 – 2009, the dominant serotype of Dengue virus in Surabaya is different, which is Den-2 virus. The change from the predominant serotypes of Dengue virus is likely to be an important role in transmission patterns

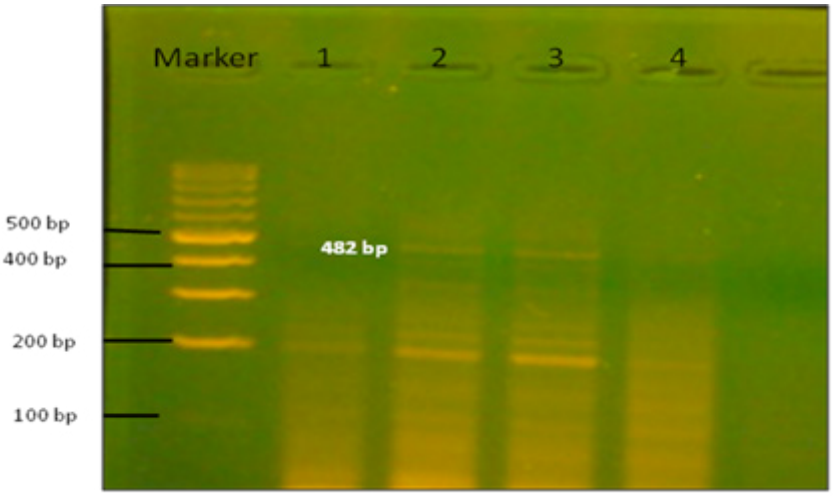


Figure 2. The result of RT-PCR using the primers of Lancioti shows *Ae. aegypti* generation 1 comes from District Oesapa Village (Lane 2) and Alak Village (Lane 3) and also the diagnostic band for Den-1 virus (482 bp), while those coming from Fatukoa and Oebobo villages are negative

of Dengue virus. Therefore, virus serotypes examination should be conducted to determine in the changes pattern of Dengue disease.

The frequency of each Dengue serotype according to Heyman (2008) is successively decreased starting from Dengue 2 virus, Dengue 3 virus, Dengue 4 virus and the last, Dengue 1 virus. In Indonesia, Dengue 3 virus is related to major cases of Dengue and is the most extensive serotype distribution followed by Dengue 2 virus, Dengue 1 virus and Dengue 4 virus (Ditjen PP & PL, 2013).

This study shows that the Dengue virus which causes the Dengue disease in Kupang is the virus Den-1, and this study proves also that the virus Den-1 can be transovarially transmitted, which is passed down from mosquitos parents to descendants (in this study, the first offspring (F1)). Previous research in Malaysia has also proven that the Dengue virus has been even descended to the *Ae. aegypti* F5, and it is proved in Den-2 virus (Spiritual et al., 2008). Minimum infection rate (MIR) in the study is approximately 30‰ - 45 ‰ in generation F1 - F5, and in F6 and F7 there is no Dengue virus (Rohani et al., 2008). Den-4 viruses can also be transovarially transmitted in *Ae. aegypti* females and males with a MIR of 10.5 ‰ (Da Cruz, 2014). Research in Thailand and Bantul even find that transovarial transmission in *Ae. aegypti* could happen to the virus Den-1, Den-2, Den-3 and Den-4 (Thongrungkiat 2011; Satoto, 2014).

In this study, the transovarial transmission of Dengue virus is only demonstrated in *Ae. aegypti* females, whereas in previous studies, it is also found in *Ae. aegypti* males, even minimum infection rate (MIR) which is higher in males than in females (Thongrungkiat et al., 2011). The finding shows that *Ae. aegypti* males can be infected with the virus from their females through venereal activities or male mosquitoes transmit Dengue virus to female mosquitoes during the reproduction (Thongrungkiat, 2011).

In this study, not all regions which are positive IHC virus are also positive at the time of inspection with RTPCR. This is because the mosquitoes used in the examination of IHC with RTPCR are different mosquitos. Therefore, the mosquitos used for RTPCR examination could be the ones without the Dengue virus. Other possibility is the sensitivity of IHC examination which is higher as compared to RTPCR examination, i.e. at a low titer of Dengue virus can be read by the IHC method but cannot be read by RTPCR. Dengue virus with RTPCR examination in this study is found in two regions (50%) which contain transovarial transmission. This is different from the research of Satoto (2014) who finds the 70.58% area contained transovarial transmission of Dengue virus in *Ae. Aegypti*.

Den-2 virus in the previous experimental

studies can also be descended to the offspring, where the number of transovarial transmission and hatchability of infected eggs of *Ae. aegypti* is lower than uninfected mosquitoes in both the F0 and F1 (Satoto et al, 2013). This study is different from experimental research. In this study, transovarial transmission rate is only 9.2% while in experimental research is 82.2% for *Ae. aegypti* first offspring (F1) (Satoto et al, 2013). This difference is due to a variety of possibilities, among others, not all of *Ae. aegypti* can be infected by the Dengue virus. This is because their immune system in the body which ends up being a good barrier in case of infection by viruses or other microbes in the body and is also a barrier in the middle of the stomach and salivary glands of mosquitoes (Luplertlop, 2014). The barrier causes not all Dengue virus can live and multiply in the mosquito's body. Besides, temperature and humidity also affect transovarial transmission. Besides the viral strain and strain mosquitoes geography also affect the amount of transovarial transmission (Rohani, 1997).

This study only proves the ability of *Ae. aegypti* in transmitting the Dengue virus transovarially (vertically), whereas previous studies have demonstrated that *Ae. albopictus* is capable of transmitting Dengue virus vertically namely Den-2 virus can be found in the larvae or *Ae. albopictus* that have not suck human blood (Cecilio, 2009). This proves that both *Ae. aegypti* and Ae albopictus are susceptible to the transovarial transmission of Dengue virus.

This study finds that transovarial transmission does not only occur in endemic areas, but also in sporadic and free areas of Dengue infection although the percentage number of transovarial transmission is higher in endemic areas. This shows that all regions in this study become the risk in the spread of Dengue fever because *Ae. aegypti* containing Dengue virus. And it is ready to transmit the Dengue virus to humans. Transovarial transmission of Dengue virus plays a role in the sustainability of the transmission of Dengue both at the time of epidemic as well as between epidemic because the mosquitoes that have been infected with Dengue virus can transmit the virus throughout their life, although they never suck infected human blood (Ditjen PP & PL, 2013; Espinosa, 2014) ,

The information on the ability of *Ae. aegypti* to transovarially transmit Dengue virus in Kupang and elsewhere is very useful in combating Dengue and Dengue vector control, especially also because the transovarial transmission of Dengue virus is not only found in endemic areas, but also free and sporadic areas. This means that the area which is free from Dengue remains a risk for the occurrence of Dengue fever since the existing vector and virus. Therefore, when the supporting environmental factors, immune factors people declined, and vector control are not being encouraged in the free areas, it is not impossible that there will be also an increase in Dengue cases in the area. So it is expected to add awareness to the community and the government to intensify vector control in all areas including Dengue-free areas.

In addition, area-based management needs to be done, namely the concept of prioritizing, working or focusing on controlling the source of the disease which is done early to prevent the occurrence of extraordinary events and carried out simultaneously with the search and eradication of breeding places based on the characteristics of the RT, RW, or village (Achmadi, 2010). DHF conditions between regions can be different, including the determinant factors of incidence of Dengue, for example on research in *Puskesmas* Sekaran in Semarang, it is found that the incidence of Dengue is associated with the presence of larvae in shower tub and clothes hanging habit (Sukowinarsih, 2011), but this does not necessarily apply equally in elsewhere, for the area-based management in the eradication of Dengue here, it is very necessary.

Conclusion

This study proves that the Dengue virus transovarial infection can be found in all endemic, sporadic and free areas with the highest rate is in endemic areas (13.3%) and the average of Kupang is 9.2%. Den-1 virus can be found only in sporadic and endemic areas. It is expected that Dengue vector control is not only performed in endemic areas but also in areas of sporadic and free of Dengue.

Acknowledgment

Our gratitude is addressed to the Director polytechnic Kemenkes Kupang, Chief of Health Offices Kupang, Head of Puskesmas Pasir Panjang, Head of Puskesmas Alak, Head of Puskesmas Oesapa, Head of Puskesmas Oebobo, Head of Parasitology Division, Medical Faculty Gadjah Mada University and staffs, as well as various parties for their supports and permission so the research can be carried out well.

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