Laboratory Testing of Transovarial Transmission in Aedes Aegypti Mosquito

Guntur Benedict Hutadjulu[1*], Sitti Rahmah Umniyati[2], Jarir At Thobari[3]
[1]Undergraduate Student. [2]Department of Parasitology. [3]Department of Pharmacology,
Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia

*Corresponding author: gunturbenedict@hotmail.com

ABSTRACT

Introduction: Ae. aegypti is one of the vectors causing Dengue Hemorrhagic Fever (DHF) that feeds on human blood suffering from DHF and transmit it to another healthy human. The ability of Dengue Virus (DENV) to survive in nature is due to 2 mechanisms i.e. horizontal transmission among viremic vertebratae infected by Aedes mosquitos and vertical (transovarial) transmission from infected female mosquito to another generation.

Objectives: To know the differences between next generation's viral carriage, percentage of mosquito descendants carrying DENV, and the distribution of DENV in its infected mosquitos.

Methods: Study design was a quasi experimental posttest-only design with control group. The study was conducted on 53 second-generation female Ae. aegypti mosquitos which are grouped into treatment and control groups. Dengue Viral antigen in both groups were detected by immunohistochemistry method using Streptavidin Biotin Peroxidase Complex (SBPC) in paraffin embedding preparation of mosquitos aged 1-7 days. Fisher exact test was used to compare proportion of virus detected in moaquitos between 2 groups.

Results: The virus detected in second generation of DEN-infected and uninfected mosquitos were 100% and 85.7%, respectively. The virus was distributed from day 1 to 7 in all organs such as brains, thorax, salivary gland, gastric epithelial cells, and ovarium of the infected mosquitos. Similar distribution was not detected in control mosquitoes.

Conclusion: There was no difference in next mosquito generation's transovarial infection among DENV-infected and uninfected Ae. aegypti mosquitos.

Keywords: Ae. aegypti mosquito; Dengue virus (DENV); transovarial transmission; immunohistochemistry method, Streptavidin Biotin Peroxidase Complex (SBPC).

INTISARI

Pendahuluan: Ae. aegypti adalah salah satu jenis nyamuk vektor penyebar penyakit Demam Berdarah Dengue (DBD), yaitu penyakit yang ditularkan melalui gigitan nyamuk Aedes yang menggigit penderita DBD kemudian ditularkan kepada orang sehat. Kemampuan virus Dengue (DEN) untuk mempertahankan keberadaannya di alam dilakukan melalui dua mekanisme, yaitu transmisi horizontal antara vertebrata viremia yang ditularkan oleh nyamuk Aedes dan dengan transmisi vertikal (transovarial) yaitu dari nyamuk betina infektif ke generasi berikutnya.

Tujuan: Mengetahui ada tidaknya perbedaan antara nyamuk Ae. aegypti yang diinfeksi virus DEN dengan yang tidak dalam hal kandungan virus pada keturunan selanjutnya, persentase keturunan nyamuk yang mengandung virus DEN, serta persebaran virus DEN pada tubuh nyamuk.

Metode: Penelitian ini menggunakan metode quasi experimental dengan desain penelitian post only design with control group terhadap 53 ekor nyamuk Ae. aegypti betina generasi kedua. Sampel kemudian dibagi menjadi kelompok intervensi dan kontrol. Pada kedua kelompok dilihat kandungan virusnya dengan metode imunohistokimia Streptavidin Biotin Peroxidase Complex (SBPC) pada sediaan embedding paraffin pada nyamuk berumur 1-7 hari, kemudian proporsi virus yang telah didapat dari 2 kelompok dianalisis menggunakan formula Fisher exact test.
Hasil: Hasil penelitian menunjukkan kandungan virus DEN pada generasi ke II kelompok intervensi mencapai 100 % dan 85,7 % pada kelompok kontrol. Persebaran virus pada kelompok intervensi terjadi secara merata pada hari 1-7 di setiap organ seperti otak, thorax, kelenjar saliva, sel epitel, lambung, dan ovarium set, sedangkan pada kelompok kontrol persebarannya tidak merata.
Simpulan: Tidak ada perbedaan antara nyamuk Ae. aegypti yang diinfeksi virus DEN dengan yang tidak diinfeksi virus (kelompok kontrol) dalam hal adanya infeksi transovarial pada nyamuk keturunan selanjutnya.

Kata kunci: Nyamuk Ae. aegypti, virus Dengue (DEN), transmisi transovarial, metode imunohistokimia, Streptavidin Biotin Peroxidase Complex (SBPC).

INTRODUCTION

Ae. aegypti is a common type of mosquito to Indonesian societies because this type of mosquito is one of the vectors transmitting Dengue Hemorrhagic Fever (DHF). Dengue Hemorrhagic Fever (DHF) is a Dengue viral infectious disease (DEN) transmitted by the bite of *Aedes* mosquitoes (Stegomyia), primarily *Aedes* (Stegomyia) *aegypti* (Linaeus)[1,2,3]. This type of Dengue attacks more than 100 countries in the tropics and subtropics with more than 2.5 billion people suffering this disease. It means that most of the world's population is at risk of developing this disease.

The role of *Ae. aegypti* mosquitoes as a disseminator of Dengue Virus (DENV) has been revealed since the announcement by Cleland, Brandley, and Mc. Donald in 1916 supported in 1923 by Chandler and Rice[4]. Rosenau explained that *Ae. aegypti* and *Ae. albopictus* (Skuse) in an optimum state are capable of transmitting the virus.

Transovarial transmission of DENV in Indonesia was first conducted in 2004 by Umniyati[5] in Klitren Village of Yogyakarta using immunohistochemical methods of Streptavidin Biotin Peroxidase Complex (SBPC) on head squash of mosquitoes. The result showed that the Transovarial Infection Rate (TIR) of for *Ae. aegypti* mosquitoes was 27.27%, resulted from the colonies between pupae and larvae obtained from wells. The method which was then standardized by Umniyati raises a strong presumption that *Ae. aegypti* mosquitoes play an important role in maintaining the DENV innature, particularly when climatic conditions are not favorable for mosquitoes[6]. This method is highly sensitive, specific, reliable, and valid for the diagnostic purposes of DENV infection in *Ae. aegypti* mosquitoes[7].

Since there has been no causal therapy and immunization with vaccines that are effective to prevent dengue infection, understanding in depth knowledge of the epidemiological aspects of DHF mainly the ones associated with disease agents (DENV), their host (humans and mosquito vectors), and environmental factors become significant. Particularly, vector control strategy remains the most possible method to overcome this problem. Control of mosquito vectors especially for *Ae. aegypti* has a strategic position, i.e. in the short term, to stop transmission of DENVes taken by vectors and in the long term, to prevent the expansion of endemic areas[2].

This study aimed to determine whether there is a difference between the *Ae. aegypti* mosquitos infected with DENVes compared with

the ones that are not in terms of virus content on the next offspring.

MATERIALS AND METHODS

This study was a quasi-experimental study, the design of this study was *post only design with control group*, in which the researcher measured the effect of the intervention by assessing the control group.

The subjects of this research were *Ae. aegypti* mosquitoes which were then divided into 2 groups. The treatment group consisted of *Ae. aegypti* mosquitoes infected orally by DENV, whereas the control group consisted of *Ae. aegypti* mosquitoes that were not infected by DENV. The research population were all *Ae. aegypti* mosquitoes which were the second offspring of the parent mosquitoes (parent mosquitoes were not examined). The sample was some female *Ae. aegypti* mosquitoes which constituted the second offspring of the parent mosquitoes. Then, the spread of the virus in the sample was analyzed from the first day until the seventh day. The samples used for each intervention were 35 female *Ae. aegypti* mosquitoes of the second offspring. In so doing, the total number of samples required was as many as 70 female *Ae. aegypti* mosquitoes of the second offspring. Having been matched with the inclusion and exclusion criteria, the size of the sample was reduced to 53 female *Ae. aegypti* mosquitoes of the second offspring, consisting of 32 second-offspring mosquitoes derived from parent mosquitoes infected by viruses and 21 mosquitoes derived from parent mosquitoes that were not infected by viruses.

Initially, 30 female *Ae. aegypti* mosquitoes of the first offspring and 30 cages equipped with an egg trap (ovitrap tubes) were prepared. Then, each treatment sample was given a code. The mosquitoes were divided into 2 groups (each group consist of 15 mosquitoes). Group I was infected with the DENV-3 (i.e., the mosquitoes were given the opportunity to suck blood missed with the suspension of the virus and sucrose 10% at a ratio of 1:1:1 through membrane feeding until they felt full), while group II was not infected (as a control).

Each mosquito was maintained individually in cages within which there was an ovitrap for 7 days until they laid their eggs. Every day, the mosquitoes were fed with sucrose 10% absorbed in cotton inserted on the cover of the cage made of gauze. Once the mosquitoes lay eggs, the eggs were separated into 2 containers. The first container contained eggs from parent mosquitoes infected with viruses while the second one contained eggs from parent mosquitoes that were not infected with viruses.

The eggs that had been classified earlier were incubated in plastic trays containing water, and as soon as they hatch the larvae were fed with chicken livers that had been frozen until dried in the refrigerator. Larvae that had turned into a pupa were taken and moved into a small plastic cup which was later put in a cage. Pupae that hatch into mosquitos were moved to another cage and their age was recorded. As many as 5 mosquitoes aged 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, and 7 days were taken both from the treatment group and the control group, then they were taken to the Anatomic Pathology Laboratory for the process of mosquitoes' tissue slices (*paraffin embedding*).

After paraffin embedding, the distribution and the content of the virus was determine with immunohistochemistry methods of SBPC using monoclonal antibodies DSSE10 as primary antibodies in mosquito organs such as brain cells, thorax, salivary glands, gastric epithelial cells, and ovarium. The detection of the content and distribution of DENV-3 was performed under a light microscope at a magnification of 1000x. Positive results were indicated with brown color and negative results were indicated with blue color.

The data obtained was calculated and the results were analyzed with *Fisher exact test*.

RESULTS AND DISCUSSIONS

The total number of the samples of female *Ae. aegypti* mosquitoes of the second offspring

Table 1. A comparison of the number of samples between each group

Day	Treatment Group			Control Group		
	(+)	(-)	(X)	(+)	(-)	(X)
1	4	0	1	3	1	1
2	4	0	1	3	0	2
3	5	0	0	1	1	3
4	5	0	0	3	0	2
5	4	0	1	4	0	1
6	5	0	0	0	1	4
7	5	0	0	4	0	1
Total	32	0	3	18	3	14

Definition:

Treatment group : *Ae. aegypti* mosquitoes obtained from parent mosquitoes infected with viruses.

Control group : *Ae. aegypti* mosquitoes obtained from parent mosquitoes that were not infected with viruses.

(+) : *Ae. aegypti* mosquitoes that were proved to contain a virus after an investigation had been made.

(-) : *Ae. aegypti* mosquitoes that were proved to contain no viruses after an investigation had been made.

(X) : *Ae. aegypti* mosquitoes that did not comply with the inclusion criteria (excluded) after an investigation had been made.

Table 1 showed that there were 4 groups as follow: infected treatment group (32 mosquitoes), non-infected treatment group (0 mosquitoe), infected control group (18 mosquitoes) and non-infected control group (3 mosquitoes). In addition, 17 mosquitoes were excluded, 3 from the treatment group and 14 from the control group.

The *Fisher exact test* analysis showed that the treatment and control groups, both of which originally were derived either from the *Ae. aegypti* mosquitoes infected with viruses or the ones that were not infected with viruses was not significantly different ($p = 0.057$). In other words, the proportion of *Ae. aegypti* mosquitoes containing viruses in the treatment and control groups did not differ significantly, which at the same time prove the occurrence of transovarial virus infection.

Every day for seven days, 5 *Ae. aegypti* mosquitoes each from treatment and control groups were examined. Figure 1 showed a comparison of the percentage of the *Ae. aegypti* mosquitoes from the treatment group and the control group that were infected with viruses since Day 1 to Day 7.

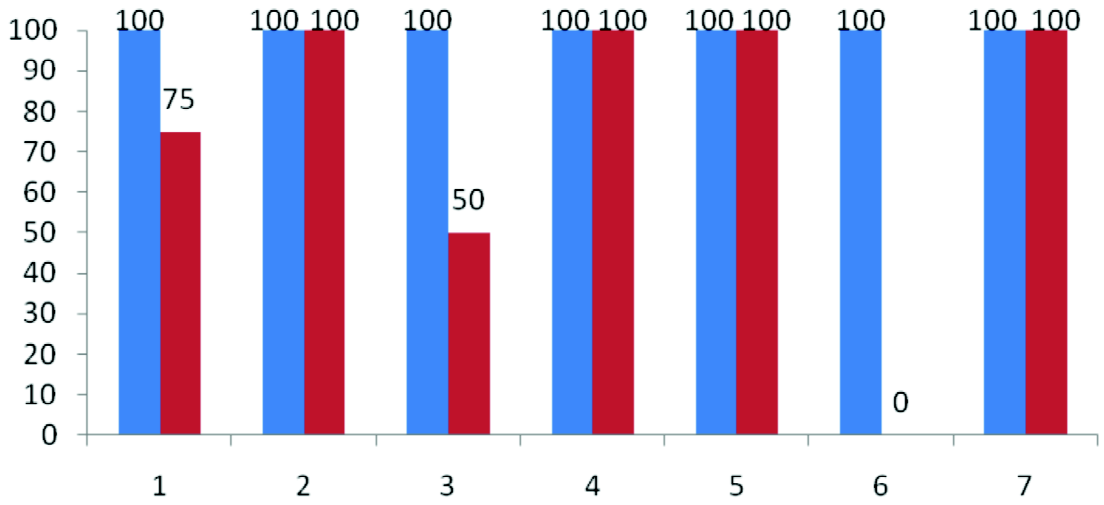

Figure 1. A comparison between the percentage of *Ae. aegypti* mosquitoes from the treatment group (blue) and the control group (red) that were infected with viruses since Day 1 to Day 7.

From the treatment group, the number of samples starting from Day 1-7 (after being filtered with the inclusion and exclusion criteria) was each 4, 4, 5, 5, 4, 5 and 5, respectively, and in short the total number of the samples for the treatment group was 32. Of the 32 samples of the treatment group, it was revealed that all of them were virus-infected (+).

From the control group, the number of samples starting from Day 1-7 (after being filtered with the inclusion and exclusion criteria) was each 4, 3, 2, 3, 4, 1 and 4, respectively, and in short the total number of the samples for the control group was 21. Of the 21 samples of the treatment group, it was revealed that 18 of them were virus-infected (+) and 3 of them were not (-). Note that Day 1 and Day 2 indicated the results by 75% and 50 % suggesting that on those day not all mosquitoes were infected with viruses (+). Meanwhile, Day 6 indicated the results that were totally negative (-). This was because from 5 mosquito samples, 4 of them were excluded and there was only 1 sample that was truly negative. The high number of samples excluded was assumed to be caused by less perfect mosquito tissue slices (*paraffin embedding*) making many mosquito organs lose and finally excluded.

Using data from the diagram above, it can be calculated that the percentage of the infected treatment group compared to the number of the group's samples is (32 / 32 x 100 %) = 100 %, while the percentage of the infected control group and the number of the group's samples is: (18/21 x 100 %) = 85.7 %. There result shows that both the treatment and control groups indicate a proportion that was not much different. In other words, the proportion of *Ae. aegypti* mosquitoes containing viruses in the treatment and control groups did not differ significantly.

In this study each *Ae. aegypti* mosquitoes was examined in terms of the virus spread since Day 1 to Day 7 in 5 different organs (the brain, thorax, salivary glands, gastric epithelial cells, and ovaries).

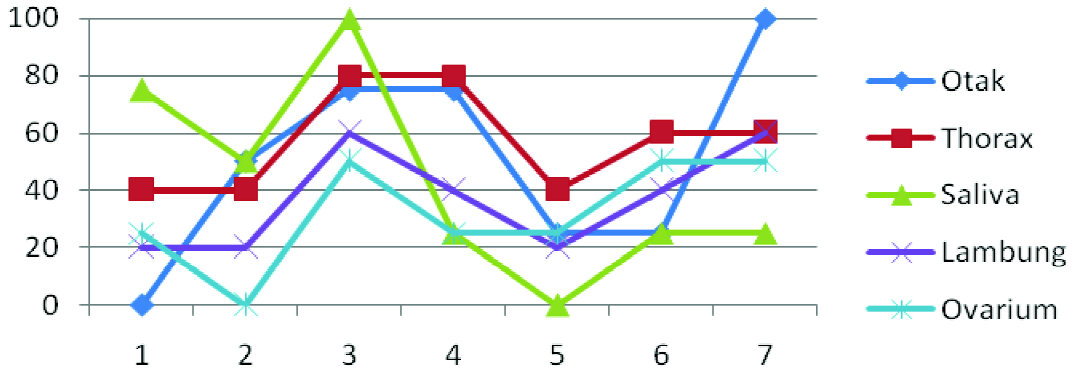

Figure 2 A. Treatment Group

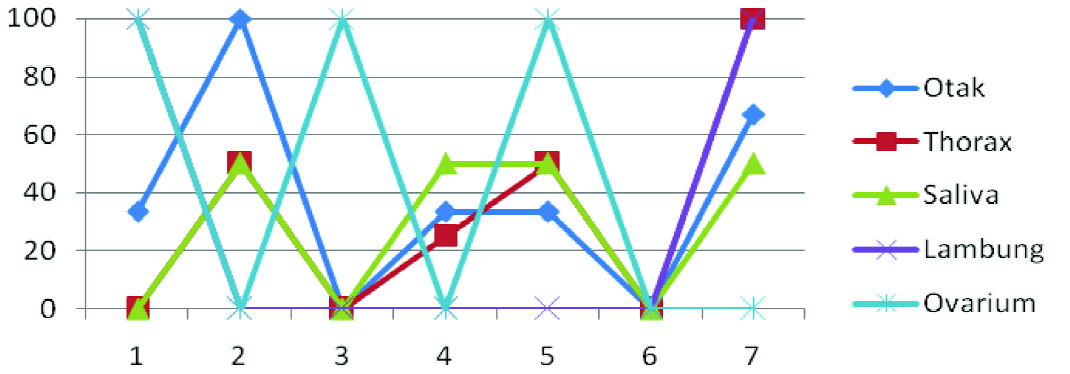

Figure 2 B. Control Group

Figure 2. Charts showing the percentage comparison of viral spread in each organ of *Ae. aegypti* mosquitos from Day 1 to Day 7 among treatment (A) and control (B) groups

Figure 2 showed that the spread of the virus in the treatment group occurred evenly in each organ every day since Day 1 to Day 7, while in the control group it was not. It may be because the treatment group consisted of the second-offspring *Ae. aegypti* mosquitoes whose parent had been infected with the virus previously, having high level of virus, lead the spread of the virus in the second-offspring *Ae. aegypti* mosquitoes more rapid and even, while the control group, consisted of the second-offspring *Ae. aegypti* mosquitoes whose parent had not been infected with the virus previously, having low level of virus, lead the spread of the virus in the second-offspring *Ae. aegypti* mosquitoes that less rapid and even. However, it did not matter because the virus remained spread in the second-offspring mosquitoes, which also proves the existence of the virus infection in the *Ae. aegypti* mosquitoes in transovarial ways. The DENV-3 is persistently transmitted transovarially and its frequency increases until the 7th offspring while its infection remains occurring in the later offspring[8].

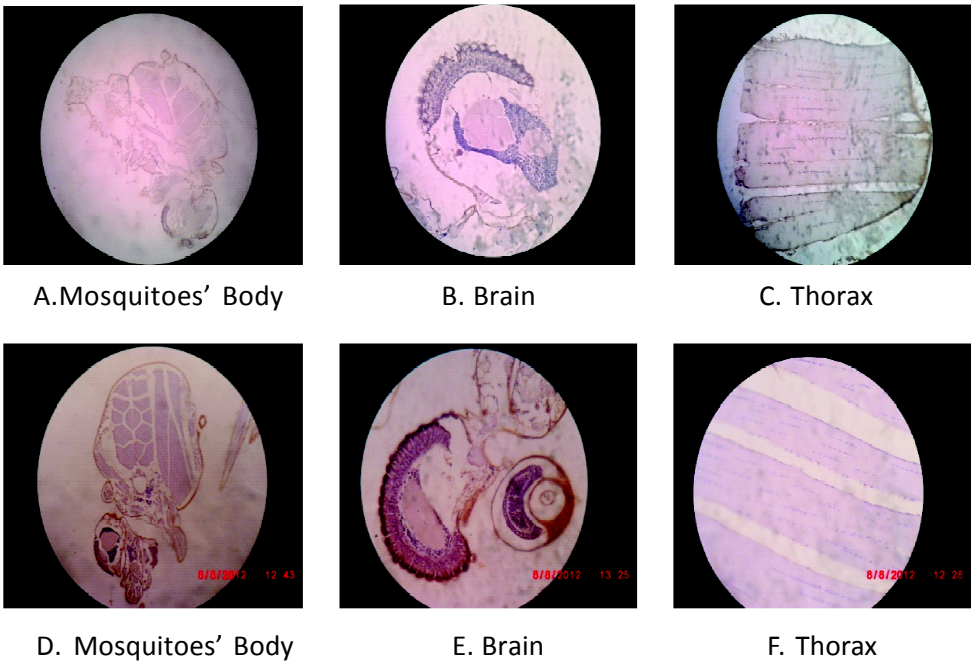

Figure 3. Microscopic pictures of the organs of *Ae. aegypti* mosquitoes. Mosquitoes' organs that contain the DENV as positive, brown-colored (A, B and C). Mosquitoes' organs that do not contain the DENV as negative, blue-colored (D, E, F)

Our results suggest that understanding the epidemiological aspects of DHF such as disease agents (DENV), their host and environmental factors becomes highly significant because so far there has been no causal therapy and immunization with vaccines that are effective; thereby vector control remains the only way to overcome it.

CONCLUSION

There is no difference between *Ae. aegypti* mosquitoes infected with the DENV and the ones that were not infected with the virus (control group) in terms of the occurrence of transovarial infection happening to mosquitoes of the latter offspring. The percentage of *Ae. aegypti* mosquito offspring containing DENV from virus-infected parents was 100 %, whereas for the control group was 85.7 %. The spread of the virus in the *Ae. aegypti* mosquito offspring containing DENV from virus-infected parent occurred evenly in the brain, thorax, salivary glands, gastric epithelial cells, and ovaries every day from Day 1 to Day 7, whereas for the control group the virus did not spread evenly.

It is suggested that intensive efforts of DHF vector control are made considering the high content of DENV distributed to the eggs of *Ae. aegypti* mosquitoes in transovarial ways. Further research is needed on the variation of the organs under study in order to determine the spread of the virus in *Ae. aegypti* mosquitoes.

ACKNOWLEDGEMENTS

The author would like to thank the Head of Parasitology Department and the Head of Anatomical Pathology Department for the permission and supports during the study. Our gratitude is also for Mr. Purwono, Mr. Yunadir, and Ms. Atin for their helpful assistance.

REFERENCES

1. WHO SEARO. *Pencegahan dan Penanggulangan Penyakit Demam Dengue dan Demam Berdarah Dengue*. Depkes RI. Jakarta, 2003.
2. Mardihusodo SJ. *Manajemen Pengendalian Vektor Demam Dengue.* Dalam Simposium Dengue Control Update. Pusat Kedokteran Tropis, Universitas Gadjah Mada, Yogyakarta, 2005.
3. Depkes RI. *Pencegahan dan Pemberantasan Demam Berdarah Dengue di Indonesia*. Jakarta, 2005.
4. Mardihusodo SJ, Noerhayati S, Moesfiroh S, Baidlowi CA, Moetrarsi SK. Survei *Aedes aegypti L. (Diptera; Culicidae*) di Yogyakarta. I. Pengukuran Index Breteau. *Berita Ilmu Kedokteran*, 1974;VI(3):95-101.
5. Umniyati SR. *Prelimenary Investigation on the Transovarial Transmission of Dengue Virus in The Population of Ae. aegypti in The Well*. Dalam Seminar Hari Nyamuk IV, 21 Agustus 2004 di Surabaya, 2004.
6. Soegijanto S. *Demam Berdarah Dengue*. Edisi 2. Airlangga University Press, Surabaya, 2006;248-9:253-4
7. Mardihusodo SJ, Satoto TBT, Mulyaningsih B, Umniyati SR, & Ernaningsih. *Bukti Adanya Virus Penularan Dengue secara Transovarial pada Nyamuk Ae. aegypti di Kota Yogyakarta*. Simposium Nasional Aspek Biologi Molekuler, Patogenesis, Manajemen dan Pencegahan KLB, 16 Mei 2007. Pusat Studi Bioteknologi Universitas Gadjah Mada, Yogyakarta, 2007.
8. Joshi V, Mourya DT, Sharma RC. Persistence of Dengue-3 Virus Through Transovarial Transmission Passage In Successive Generations of *Ae. aegypti* Mosquitoes. *Am. J. Trop. Med. Hyg*, 2002;67:158-61.