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J Med Sci
Volume 44, No. 1, March 2012: 35 - 40

Effect of temperature and storage duration of *Aedes aegypti* mosquito specimens artificially infected with dengue-3 virus on the results of immunohistochemical examination

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ABSTRACT

To confirm the presence of any dengue viral in a mosquito, mosquito's head can be squashed on a slide and stained with immunohistochemical staining. The remaining samples then can be stored in the cryo freezer at -80°C to avoid specimen damage. However, for laboratories with limited facilities, with only a refrigerator with a temperature range of -20°C to 4°C is available, examination to evaluate whether the dengue antigen can still be detected in specimens stored at these temperature is necessary. It was a quasi-experimental study. Three to five-day-old adult female *Aedes aegypti* mosquitoes were injected intrathoracically with dengue-3 (DENV-3) strain H-87 virus and then maintained for about 7 days. The dengue viral antigen on mosquitoes was identified using immunohistochemical method after stored at a temperature of 4°C, -20°C, -80°C for 2, 4 and 8 weeks. Mosquito specimens that were not stored were used as a positive control. Kappa value was counted to analyze level of agreements between two observers. Two-way Anova was used to analyze mean positive rates. Kappa value showed poor agreement (0.00-0.16) between two observers when the specimens were stored at 4°C for 2-8 weeks, and showed good agreement (Kappa value of 0.77), when stored at -20°C for 4 weeks. The kappa value showed very good agreement (0.90-0.92) when the specimens were stored at -20°C for 2 weeks, and at -80°C for 2-8 weeks. Mean positive rates of the specimens stored at 4°C were significantly lower ($p<0.005$) than stored at -20°C and -80°C, but there were no significant differences between specimens stored at -20°C and -80°C ($p>0.05$). In conclusion, availability of the dengue viral antigen on mosquito specimens was influenced by temperature and storage duration of the specimens.

ABSTRAK

Untuk memastikan adanya virus dengue dalam nyamuk, dapat dibuat preparat *head squash* nyamuk dan pewarnaan immunohistokimia. Sisa sampel selanjutnya dapat disimpan pada *cryo freezer* suhu -80°C untuk menghindari kerusakan. Untuk laboratorium dengan fasilitas terbatas hanya tersedia lemari es dengan suhu -20°C dan 4°C, pemeriksaan untuk memastikan apakah antigen virus dengue masih bisa dideteksi pada sampel yang disimpan pada suhu tersebut diperlukan. Penelitian ini merupakan penelitian eksperimental semu. Nyamuk *Aedes aegypti* dewasa betina umur 3 hari diinfeksi dengan virus dengue-3 (DENV-3) strain H-87 dan kemudian dipelihara selama sekitar 7 hari. Antigen virus dengue nyamuk diidentifikasi menggunakan metode imunohistokimia setelah disimpan pada temperatur 4°C, -20°C, -80°C selama 2, 4 dan 8 minggu. Spesimen nyamuk yang tidak disimpan digunakan sebagai kontrol positif. Nilai Kappa dihitung untuk mengetahui derajat kesepakatan antara 2 pemeriksa. Anova 2 jalan digunakan untuk

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menganalisa rerata *positive rates.* Nilai Kappa menunjukkan kesepakatan yang rendah (0,00-0,16) antara 2 pemeriksa jika spesimen disimpan pada suhu 4°C selama 2 sampai 8 minggu, tetapi menunjukkan kesepakatan yang baik (nilai Kappa 0,77) jika disimpan pada suhu -20°C selama 4 minggu. Nilai Kappa menunjukkan kesepakatan sangat baik (0.90-0.92) jika spesimen disimpan pada suhu -20°C selama 2 minggu dan pada suhu -80°C selama 2 sampai 8 minggu. Rerata *positive rate* spesimen yang disimpan pada suhu 4°C lebih rendah secara bermakna ($p < 0,05$) dibandingkan disimpan pada suhu -20°C dan -80° C, tetapi tidak ada perbedaan bermakna pada penyimpanan suhu -20°C dan -80°C ($p > 0,05$). Dapat disimpulkan adanya antigen virus dengue pada spesimen nyamuk dipengaruhi oleh suhu dan lama penyimpanan.

Keywords: dengue virus - head squash - immunohistochemistry - kappa agreement - temperature

INTRODUCTION

Dengue virus infections are significant causes of morbidity and mortality in many areas of the world, including South-East Asia, Western Pacific and Americas Regions. The infection is transmitted by *Aedes aegypti* and *Ae. albopictus*. In nature, the transmission can occur through two mechanisms i.e. horizontal transmission through infected vertebrates to mosquitoes and vertical transmission through infected mosquitoes to another mosquitoes.[1] Several methods to detect dengue infection on *Aedes* mosquito have been developed. Direct fluorescent antibody test (DFAT) is used for detection of dengue viral antigen on head squash preparation under a fluorescent microscope, whereas reverse transcription polymerase chain reaction (RT-PCR) is used for specific detection of dengue virus genome in a sample.

Umniyati[2] has developed a method for detection dengue viral antigen on head squash preparation of *Ae. aegypti*. This method was developed based on immunocytochemical streptavidin biotin peroxidase complex (ISBPC) assay using monoclonal antibody DSSE10 (mAb DSSE 10) against dengue virus. Positive antigen was detected under light microscope as granular, bright, brownish coloration dots that are scattered throughout most fields of brain tissue and negative result demonstrated as blue coloration. The method has been evaluated by Widiastuti[3] in comparison with RT-PCR as a gold standard. The result showed that the method provided a sensitivity and specificity of about 100% and 91% respectively with Kappa value of 0.64.[3.]

The method as described by Umniyati[2] was then widely used for detection the presence of transovarial transmission of dengue virus in *Ae. aegypti* in the field by some researchers.[4-10] For this purpose, the trapped eggs, larvae and pupae from the field were reared to adult in different cages and newly adults were provided with 10% sugar without blood feeding. After they were 7 days old, the *Ae. aegypti* mosquitoes were killed by being put into the freezer for 5 minutes. The mosquito specimens sometimes were stored in a cryo freezer for several months before stained with immunocytochemical staining method in order to avoid specimes damage due to high temperature effect. It is well known that temperature influences the stability of mosquito specimen in storage.[11] However, for laboratories with limited facilities, with only a refrigerator with a temperature range of -20ºC to 4ºC is available, examination to evaluate whether the dengue antigen can still be detected in the specimens stored at these temperatures is necessary. This study was conducted to evaluate the effect of temperature and storage duration on dengue antigen in mosquito specimen.

MATERIALS AND METHODS

Infection of Mosquitoes

Three to five-day-old *Ae. aegypti* mosquitoes were intrathoracically injected with

dengue-3 (DENV-3) strain H-87 virus. The injected mosquitoes were then divided into 3 groups. Each group was divided into 3 sub-groups, and each sub-group contained ten mosquitoes. They were reared in a cylindrical cage covered with mosquito netting and incubated for seven days in a styrofoam box in the special insectary at the Department of Parasitology, Faculty of Medicine, Gadjah Mada University, Yogyakarta.

Detection of Dengue Antigen

After 7 days, the mosquitoes were killed by being put into the freezer for 5 minutes. The mosquito specimens of the 1st, 2nd, and 3rd group were stored in a refrigerator, freezer, and cryo freezer, respectively. The 1st, 2nd and 3rd sub-groups were stored for 2, 4, and 6 weeks, respectively. Un-stored mosquito specimens were used as positive control, and un-infected mosquitoes specimens were used as negative control.

The presence of dengue viral antigen on head squashes preparation was detected using mAb DSSE10 (1:50) obtained from Dr. Sitti Rahmah Umniyati from Department of Parasitology, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta. The mAb DSSE10 showed high immunoreactivity toward DENV-1, DENV-2, DENV-3, and DENV-4 and no cross reactivity toward Chikungunya antigen based on indirect ELISA.[2] Therefore, the mAb DSSE10 is appropriately used as primary antibody for detection of dengue antigen in *Aedes* mosquitoes.

This study has been approved by the Medical and Health Research Ethics Committee, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta.

Statistical Analysis

Kappa value was counted to analyze the level of agreements between two observers, according to Landis and Koch.[12] Two-way Anova was used to analyze mean positive rates among experimental and control groups.

RESULTS

Head squash smear of *Ae. aegypti* mosquito infected with DENV-3 strain H-87 was made. Dengue antigen was detected under light microscope based on ISBPC assay using mAb DSSE10 (1:50) as primary antibody. Positive antigen was detected as brownish color in the cytoplasm of infected cells in most of mosquito brain tissue. Negative result was detected as blue color in most of brain tissue, no brownish color other than the chitinous mosquito tissues and non-specific background was distinctly different from specific positive result. The results of immunohistochemical assays for detection of dengue viral antigen on head squashes preparation were influenced by temperature and storage duration of specimen as shown in TABLE 1 and TABLE 2.

Strength of agreement

Strength of agreement between two observers was evaluated based on Kappa value according to Landis and Koch.[12] The result is presented in TABLE 1.

TABLE 1. Kappa value between two observers according to temperature and duration of storage of artificially infected *Ae. aegpyti* with DENV-3 strain H-87

Storage condition		Kappa value	Strength of agreement
Temperature (°C)	Duration (week)		
4	2	0.16	very poor
4	4	0.00	very poor
4	8	0.16	very poor
-20	2	0.90	very good
-20	4	0.77	good
-20	8	0.80	very good
-80	2	0.90	very good
-80	4	0.92	very good
-80	8	1.00	very good

Kappa value showed poor agreement between two observers (Kappa value from 0.00 to 0.16), when the specimens were stored in a refrigerator at 4°C for 2-8 weeks. However, it showed good agreement (Kappa value of 0.77), when the specimens were stored in a freezer at -20°C for 4 weeks. The Kappa value showed very good agreement (Kappa value from 0.90 to 0.92) when specimens were stored in a freezer at -20°C for 2 weeks, and cryo freezer at -80 °C for 2-8 weeks.

Mean positive rates

Mean positive rates were 5.56%, when specimens were stored in the refrigerator at 4°C for 2 weeks, and 0.00% when they were stored for 4-8 weeks. The MPRs were 27.7% and 38.89%, when the specimens were stored in the freezer at -20°C and in the cryo freezer -80°C for 2 weeks, respectively. The MPRs were 60.00% and 53.17% when the specimens were stored for 4 weeks in the freezer and the cryo freezer, respectively. The MPRs were 16.67% and 21.75%, when the specimens were stored for 8 weeks in the freezer and the cryo freezer, respectively (TABLE 2).

TABLE 2. Mean positive rates of artificially infected *Ae. aegyti* with DENV-3 strain H-87 specimen according to temperature and duration of storage

Temperature (°C)	Duration (week)	MPR (%)	vMPR+0.5
4	2	5.56	2.36
4	4	0.00	0.71
4	8	0.00	0.71
-20	2	27.78	5.27
-20	4	60.00	7.75
-20	8	16.67	4.14
-80	2	38.89	6.24
-80	4	53.17	7.29
-80	8	21.75	4.66

TABLE 2 showed that the MPRs were 0%, when the specimens were stored in a refrigerator at 4°C) for 4 and 8 weeks. Therefore for the statistical analysis purposes each data of MPR was transformed into "MPR+0.5 for analyzing the data using two-way Anova. In this study the MPRs of specimens stored in the refrigerator at 4°C were significantly ($p<0.05$) lower than the ones stored in the freezer at -20°C and cryo freezer at -80°C, but there were no significant differences ($p>0.05$) of the MPRs between specimens stored in freezer at -20°C and the cryo freezer at -80°C.

DISCUSSION

The results of immunohistochemical assays for detection of dengue viral antigen on head squashes preparation in this study were influenced by temperature and storage duration of specimen. This results supported the previous study that reported that the existance of a virus was influenced by temperature and the virus would be unsustainable in high temperature.[11] In this study, head squashes preparations of mosquito specimens stored in the refrigerator at 4°C for 2-8 weeks did not strongly attach on the object glass. Therefore, the immunohistochemical staining yielded a slight brown color on observed brain tissue led difficulties to distinguish positive results of specimes from the negative ones.

In contrast, the head squashes preparations of mosquito specimens stored in the freezer at -20°C or cryo freezer -80°C strongly attached on the object glass. Therefore, the immunohistochemical staining yielded a positive result clearly as showed by discrete brownish granular deposits. Morover, it was easy to differentiate between positive and negative results of the specimens.

Since it was difficult to distinguish positive results of specimens after storage in the refrigrator at 4°C, the strength of agreement between two observers was very poor. In

contrast, after storage in the freezer at -20°C or cryo freezer -80°C the strength of agreement between two observers was very good. Morover, the MPRs of the specimens in this storage conditions were significantly lower than in the storage conditions of the freezer and cryo freezer. However, no significant difference of the MPRs between specimens stored in freezer at -20°C and the cryo freezer at -80°C was observed.

Immunohistochemical staining method is a powerful method for the identification of an antigen or a protein in cells and tissues. The success of this identification method depends on the antibody binding to the epitope of the protein used as an immunogen. The specificity of the results obtained depends on the specificity of the antibody and method used. The specificity of antibody specificity is determined by immunoblot and/or immunoprecipitation. The specificity of the method used is determined by both a negative control, replacing the primary antibody with non-immune serum, and a positive control, using the antibody with cell, known to contain the protein or antigen.[13]

The method used in the study is based on the principle of immune streptavidin biotin peroxidase complex techniques to identify antigen in cells or tissue. The endogenous peroxidase activity of head squashes preparations of mosquitoes specimen is destroyed by treating the specimen with hydrogen peroxide blocking solution. The non-specific background is eliminated by incubating the specimen with non-immune serum. The primary antibody to specific antigen is incubated to target antigens. This process is followed by addition of biotinilated second antibody which serves as the linker between the primary antibody and peroxidase streptavidin conjugate. Streptavidin-peroxidase is then added to bind to the biotin residues on the linking antibody. The presence of enzyme can be revealed by addition of a mixture of substrate chromogen solution. The enzyme peroxidase will catalyse the substrate, hydrogen peroxide, and convert the chromogen to a brown colored deposit demonstrating the location of the antigen.[14]

Surveillance of mosquitoes infected by dengue viruses provides an early warning sign for risk of transmission in an area and the specific predominant circulating serotype in the vector population.[15] Based on the finding of this study, the mosquito specimens should not be stored in the refrigerator at 4°C before immunohistochemical staining method is performed. It is recommended to store head squashes preparations of mosquito specimen in the freezer or cryo freezer.

This immunohistochemical staining method was widely used to detect the presence of transovarial transmission of dengue virus on *Ae. aegypti* in the field. This method has been used in different areas in Indonesia as reported by some authors.[3-10] The presence of dengue virus in their progeny is lower than their parent mosquitoes that are horizontally transmitted through infected vertebrates. Therefore, further study is suggested to be performed to evaluate the result of immunocytochemical assays for detecting the presence of dengue viral antigen in the brain on head squashes of *Ae. aegypti* reared from larvae and pupae collected from the field after storing them in different temperatures with different duration.

CONCLUSION

The results of immunohistochemical assays for detection of dengue viral antigen on head squash preparation of mosquito specimens were influenced by temperature and storage duration of the specimen. A refrigerator is not recommended for storing specimens, whereas a freezer at -20°C is a good tool and a cryo freezer is the best tool for storing specimens of

Ae. aegypti mosquitoes before the immunohistochemical staining is performed.

ACKNOWLEDGEMENTS

The authors would like to thank the Head of Department of Environment Health, and the Director of Health Polytechnic of Pontianak, Ministry of Health for their support during this study. The authors would also like to thanks Mrs Suprihatin, Mr Purwono, Mr Heru, Mrs Kuswati for their valuable assistance in the laboratory work.

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