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Standardization of immunocytochemical method for the diagnosis of dengue viral infection in *Aedes aegypti* Linn mosquitoes (Diptera: Culicidae)

Sitti Rahmah Umniyati
Department of Parasitology
Faculty of Medicine, Gadjah Mada University
Yogyakarta

ABSTRACT

Sitti Rahmah Umniyati - ***Standardization of immunocytochemical method for the diagnosis of viral infection in Aedes aegypti Linn mosquitoes (Diptera: Culicidae)***

Background: *Aedes aegypti* is the important vector of dengue haemorrhagic fever (DHF), and there are several methods for virus detection in the mosquito, such as the direct fluorescent-antibody test on head squashes. However, it has the disadvantages of being labor-intensive and requiring fluorescent microscope as well as cryofreezer. Newer methods involving enzyme conjugates such as peroxidase in conjunction with either polyclonal or monoclonal antibodies are greatly improved. With new methods of immunocytochemistry, it is now possible to detect dengue viral antigen in a variety of tissues.
Objective: This study was aimed to standardize an immunocytochemical streptavidin-biotin-peroxidase-complex assay for diagnosis of dengue infection in *Aedes aegypti* using monoclonal antibody DSSC7.
Methods: The infected mosquitoes were held in small cylindrical cages covered with mosquito netting, and incubated at $27 \pm 1^{o}C$ and at relative humidity of $88 \pm 6\%$. The specificity of the immunocytochemical procedure was validated by negative and positive controls showing that the antibody was bound to an appropriate structure. The sensitivity and specificity were also evaluated based on Herrmann's Formula. The presence of dengue antigen on head squash preparation was detected based on ISBPC assay using monoclonal antibody against dengue. The validity and reliability of the measurement were evaluated based on kappa values, according to Landis and Koch.
Result: Positive result was detected as discrete brownish granular deposits throughout most visual fields of brain tissue. Dengue viral antigen was immunolocalized to the cytoplasm of brain cells. The immunocytochemical test under light microscope at magnification of 400x was 86.67% sensitive, 96.00% specific, and the kappa value is 0.64. Meanwhile the kappa value between two observers was 0.92, with sensitivity and specificity of 96% and 97% respectively at magnification of 1000x.
Conclusion: The monoclonal antibody DSSC7 was sensitive, specific, valid, and reliable as primary antibody to detect dengue viral infection in *Ae. aegypti* head squash preparation based on immunocytochemical streptavidin-biotin-peroxidase-complex assay under light microscope.

Key words: antigen - denguevirus - *Aedes aegypti* – immunocytochemistry - monoclonal antibody DSSC7

ABSTRAK

Sitti Rahmah Umniyati ***Standarisasi metode imunositokimia untuk diagnosis infeksi viral Aedes aegypti Linn (Diptera: Culicidae).***

Latar belakang: *Aedes aegypti* merupakan vector utama demam berdarah dengue (DBD). Dan ada beberapa metode untuk deteksi virus pada nyamuk, seperti *direct fluorescent-antibody test* (DFAT) pada pencetan kepala (head squashes) nyamuk. Namun demikian, metode tersebut memerlukan waktu yang lama dan peralatan mikroskop fluoresens serta *cryofreezer*. Metode lebih baru yang menggunakan konjugat enzim

Sitti Rahmah Umniyati, Department of Parasitology,Faculty of Medicine,
Gadjah Mada University Yogyakarta

seperti peroxidase bersama dengan antibodi poliklonal maupun antibodi monoklonal poliklonal telah banyak digunakan untuk melokalisasi dan mendeteksi antigen di bawah mikroskop cahaya. Dengan metode baru imunositokimia tersebut sekarang ini dimungkinkan untuk mendeteksi antigen viral dengue di berbagai jaringan.

Tujuan: Penelitian ini bertujuan untuk melakukan standarisasi teknik imunositokimia *streptavidin-biotin-peroxidase-complex* (SBPC) untuk diagnosis infeksi virus dengue pada *Aedes aegypti* menggunakan antibody monoclonal DSSC7.

Metode: Nyamuk yang telah diinfeksi dengan virus dengue dipelihara dalam sangkar silindris yang mempunyai penutup dari kain kasa kedap nyamuk pada temperature $27 \pm 1^{o}C$ dan kelembapan relatif $88 \pm 6\%$. Spesifisitas prosedur imunositokimia divalidasi dengan kontrol negatif dan kontrol positif yang menunjukkan bahwa antibodi diikat oleh struktur antigen yang sesuai. Sensitivitas dan spesifisitas juga divalidasi berdasarkan Herrmann's Formula. Keberadaan antigen dengue pada preparat *head squash* dideteksi berdasarkan uji imunositokimia SBPC menggunakan antibodi monoklonal DSSC7 terhadap antigen dengue. Validitas dan reliabilitas diukur dengan menghitung nilai kappa menurut Landis dan Koch.

Hasil: Hasil positif terdeteksi sebagai deposit granular kecoklatan pada bidang pandang yang terdapat sel-sel otak. Antigen viral dengue terlokalisasi secara imunologis pada sitoplasma sel-sel otak. Sensitivitas dan spesifisitas uji imunositokimia di bawah mikroskop cahaya pada perbesaran 400x berturut-turut sebesar 86,67% dan 96,00%, sedangkan nilai kappanya 0,64. Sementara itu nilai kappa antara dua pemeriksa sebesar 0,92, berturut-turut dengan sensitivitas dan spesifisitas sebesar 96 dan 97% pada perbesaran 1000x.

Simpulan: Antibodi monoklonal DSSC7 memberikan reaksi sensitif, spesifik, sahih, dan terandalkan sebagai antibodi primer untuk deteksi infeksi viral dengue pada *head squash Ae.aegypti* berdasarkan metode imunositokimia streptavidin-biotin-peroxidase-complex di bawah mikroskop cahaya.

INTRODUCTION

Dengue hemorrhagic fever (DHF) is an important health problem in subtropical and tropical countries, including Indonesia. This disease is caused by dengue virus (DENV), a member of Flaviviridae family that is transmitted by *Aedes aegypti* Linn mosquito as the main vector. A lot of evidence showed dengue virus transmission in *Aedes sp* may occur horizontally, vertically, and transovarially.[1,2]

Various methods have been developed to identify DENV infection in vector mosquito, such as viral isolation using Toxorrhynchites mosquito and cell culture C6/36, followed by antigen detection with immunofluorescence method in mosquito head squash preparation.[3,4] This method is adequately sensitive, but it needs several days to give result, and needs a relatively expensive fluorescent microscope. Recently, a molecular biology method - reverse transcriptase-polymerase chain reaction (RT-PCR), has been developed to identify specific DENV in vectors. This method is adequately sensitive and specific, and gives a quick result for diagnosis of DEN viral infection in vector mosquito; however, a specific skill is needed, because the risk of false positive and false negative is high when the method is performed by unprofessional technicians. Besides, the material and tools for RT-PCR are expensive and not available in general laboratories. Therefore, a new breakthrough in diagnosis of DEN viral infection in vector mosquito, which only needs light microscope available in many laboratories, such as immunohistochemistry method using streptavidin-biotin-peroxidase-complex (SBPC) should be considered. This method utilizes secondary antibody labeled with biotin, that recognizes primary antibody (monoclonal or polyclonal antibodies), and uses streptavidin conjugate labeled with horseradish peroxidase enzyme and chromogen substrate to detect antigen in cells or tissues with very high sensitivity, so that even low level of antigen may be detected. The main basis of SBPC immunocytochemistry reaction is a very strong bond between streptavidin and biotin.

Dengue Team in Gadjah Mada University (GMU) has successfully produced hybrid cells (clones) which released monoclonal antibodies against dengue virus.[5] The growth of hybrid cells DSSC7 was good, even though they were maintained in liquid nitrogen container for several years. The 50-ml dengue-specific monoclonal antibody, which did not cross-react with Chikungunya virus, was successfully produced from hybrid cells DSSC7 in Balb/c mice ascites preparation. This antibody is in class IgG1 and able to detect DEN viral antigen in *Ae. aegypti* mosquito frozen paraffin tissue sliced at 1 day incubation period, and in Den-3-virus-infected *Ae. aegypti* head squash preparation at 11 days incubation period in the concentration of 1:50.[6] This antibody has been stored in freezer for 4 years, so that to detect DENV in mosquito, like the other general laboratory diagnostic method, a detection/diagnostic method is needed to fulfill the criteria: specificity, sensitivity, validity, and reliability.

Based on the background, a standardization of SBPC immunohistochemistry method was conducted using DSSC7 monoclonal antibody as primary antibody to detect DEN viral antigen in *Ae. aegypti* head squash preparation, which is specific, sensitive, valid, and reliable.

METHODS

Materials

This study was done in the Parasitology Laboratory Faculty of Medicine Gadjah Mada University, Yogyakarta, (study year 2004-2007). Research subjects were *Ae. aegypti* mosquito from the colony in Parasitology Laboratory, Faculty of Medicine, GMU, which infected with DEN-3 virus or has sucked blood from DHF patients, and *Aedes sp* mosquito from ovitrap in DHF non-endemic area in Timika, caught by the entomology team from Namru-2 Jakarta.

Other materials for this study were: blood from DHF patients who were IgM positive and DEN viral antigen positive; DENV-3 strain H-87 acquired from Virology Laboratory in Namru-2 Jakarta; DSSC7 monoclonal antibody from Balb/c mice ascites preparation maintained in freezer for 4 years; commercial DENV-specific monoclonal antibody (Chemicon International a Serological Company); Novostain Universal Detection Kit NCL-RTU-D consisted of 3 ready-to-use reagents: (a) Prediluted normal horse serum; (b) Prediluted biotinilated secondary antibody, which recognizes rabbit serum (IgG) and mice serum (IgG and IgM); (c) Prediluted streptavidin peroxidase conjugate, and DAB (Diaminobenzidine tetrachloride).

METHODS

Sample and sample size

Sample size was calculated based on the formula with expected sensitivity and specificity of 90%, 10% deviation, and confidence interval of 95%, or significance level of 5%, so that Za for 2-way test = 1.96. Based on that, sample size was calculated based on the formula by Pusponegoro *et al.*[7] was as follows. N1 = N2 = $zá^2$ (0.90 x 0.10)/0.10^2 = 1.96^2 (0.90 x 0.10)/0.01 = 34.57, or around 35. Therefore, for sensitivity and specificity study, 35 + 35 = 70 samples were needed.

Dengue-viral-infected mosquito sample preparation

Cylindrical mosquito cages were prepared, with a diameter of 7 cm and 10 cm high, and a cubical cage (20x20x20 cm^3) in special insectariums for infectious mosquito in Parasitology Laboratory, Faculty of Medicine, GMU, was also prepared. Female *Ae. aegypti* mosquito who were fasting overnight were put into 5 cylindrical cages, each cage was filled with 30 female mosquitoes.

A suspension of DENV serotype 3 strain H-87 warmed on water bath (37°C) was mixed with erythrocytes from healthy volunteer and sucrose 10% in the ratio of 1:1:1. Suspension for control negative consisted of PBSBA, erythrocytes from healthy volunteers, and sucrose 10% in the ratio of 1:1:1. The viral suspension was put into centrifuge tubes and sealed with fresh rat skin. The tube was put in cylindrical cage with the position of the seal (fresh rat skin) downwards, adhered to the upper netting of cylindrical cage, so that the mosquito might penetrate the rat skin and sucked the viral suspension. This treatment was given to the mosquitoes in cage I-IV; meanwhile mosquitoes in cage V were given erythrocyte suspension and sucrose without virus as the negative control. After

the mosquitoes were satisfied (after around 3 hours), the tubes were taken and burned. In the next day, the cages were filled with cottons containing 10% sugar solution, and the cotton was replaced everyday. After 2, 5, 8, and 11 days of incubation, mosquitoes in cages I, II, III, and IV were killed, respectively, by putting them in the freezer. The dead mosquitoes were transferred into 1.5 ml tubes and kept in liquid nitrogen container or cryofreezer in Natural Sciences Laboratory, GMU, until the head squash preparation and DEN antigen detection with SBPC immunocytochemistry were ready to perform. Infectious mosquitoes were also acquired from *Ae. aegypti* mosquitoes from the colony in Parasitology Laboratory, Faculty of Medicine, GMU, that has been given DHF patients blood which is IgM positive and DEN viral antigen positive.

Head squash preparation

Mosquito head was separated from its cervix with mosquito surgery needle on objective glass. On the other object glass, caput was pressed under cover glass with the eraser part of a pencil. The cover glass was removed, and was put into a bottle filled with alcohol 70%. The crude tissue on object glass was taken and put into a bottle filled with alcohol 70%.

The slide was dried in room temperature for around 30 minutes. Afterwards, it was fixed with cold acetone (-20°C) in freezer for 3-5 minutes, and then dried in laminary flow. If the slide was not ready to identify, wrapped in aluminium foil and kept in freezer for a maximum of 1 week. The ready slides were processed as follows.

Staining procedure and dengue viral antigen identification

Slide was fixed with cold methanol (-20°C) for 3-5 minutes, and washed with PBS. To eliminate the endogenous peroxidase activity, the slide was soaked in peroxidase blocking solution (1 part of hydrogen peroxide 30% + 9 part of absolute methanol) at room temperature for 10 minutes. Slide was incubated in prediluted blocking solution for 10 minutes in room temperature (25°C), then 100 µl primary antibody (DSSC71:10 monoclonal Antibody) was added to the slide (adjusted until all part was soaked) and incubated on damp tray at room temperature (25°C) for 60 minutes or overnight in the refrigerator. Then it was washed with (fresh) PBS for 5 minutes; 100µl biotinylated universal secondary antibody was added, and the slide was incubated at room temperature (25°C) for 10 minutes, and then washed with fresh PBS for 5 minutes. The slide was incubated with ready-to-use streptavidin-peroxidase-complex reagent for 10 minutes, and then washed with PBS for 5 minutes; incubated in 100µl peroxidase substrate solution (DAB) for 2-10 minutes (the thicker the sample, the longer the incubation time), and then washed with tap water; then 100µl Mayer hematoxylin (counter stain) was added, incubated for 1-3 minutes, and then washed with tap water. The slide was then soaked in alcohol, washed, and then soaked in xylol. Drops of mounting media were added on the slide, and covered with cover glass. After dried, the slide was ready to be evaluated under light microscope with magnification of 40x, 100x, 400x, and 1000x.

If the slide showed a brown colour, it meant that the preparation contained DEN viral antigen, meanwhile, if the preparation showed blue or pale colour (as in the negative control), it meant that the preparation did not contain DEN viral antigen.

It must be noted, that each staining positive and negative controls have to be available. Positive control was DENV-infected mosquito slide reacted with commercial DENV-specific primary antibody. Negative control was non-infectious slide reacted to primary antibody dilution or primary antibody.

Analysis

Sensitivity and specificity were measured based on Hermann formula.[8] **Validity and reliability was determined based on kappa value by Landis and Koch.**[9]

RESULTS AND DISCUSSION

Immunocytochemistry is a potential method for protein or antigen identification in cells and tissues,

but this method depends on antibody specificity in binding the protein epitope used as immunogen. Its specificity depends on antibody and method used. Antibody specificity is best determined by immunoblotting or immunoprecipitation. Method specificity is best determined with negative control by replacing primary antibody with non-immune serum, and positive control antibody with cells containing antigen they recognized.[10]

Microscopic examination of dengue antigen localization in the head squash preparation of *Ae. aegypti* infected with dengue-3 virus with 11 day incubation using SBPC immunocytochemistry method with commercial dengue-specific monoclonal antibody as control positive is shown in Figure 2. Dengue antigen localization in the head squash preparation of *Ae.aegypti* who sucked the blood of DHF patients and infected with oral DENV-3 for 8 day incubation period, using SBPC immunocytochemistry method with dengue-specific monoclonal antibody secreted by hybrid cell DSSC7 is shown in FIGURE 2.

The description proved that monoclonal antibody DSSC7 produced by GMU had a potential to detect DEN viral antigen in head squash preparation of *Ae.aegypti* mosquito infected with DENV under light microscope, using SBPC immunocytochemistry method. In this study, severe infection (++++) was shown as discrete brownish granules between the whole brain tissues, and more than 100 cells infected per visual field showed brown colour in the cytoplasm at the magnification of 400x, more distinct with the magnification of 1000x. Negative samples, as well as negative control, showed blue brain tissue.

The FIGURE 2 indicated that DEN viral infection for 8 day incubation period might be detected in head squash preparation of *Ae.aegypti* who sucked blood of DHF patients who were IgM positive, using SBPC immunocytochemistry method with DENV-specific monoclonal antibody DSSC7 produced by GMU, examined with light microscope, although the monoclonal antibody has been kept in freezer for more than 4 years.

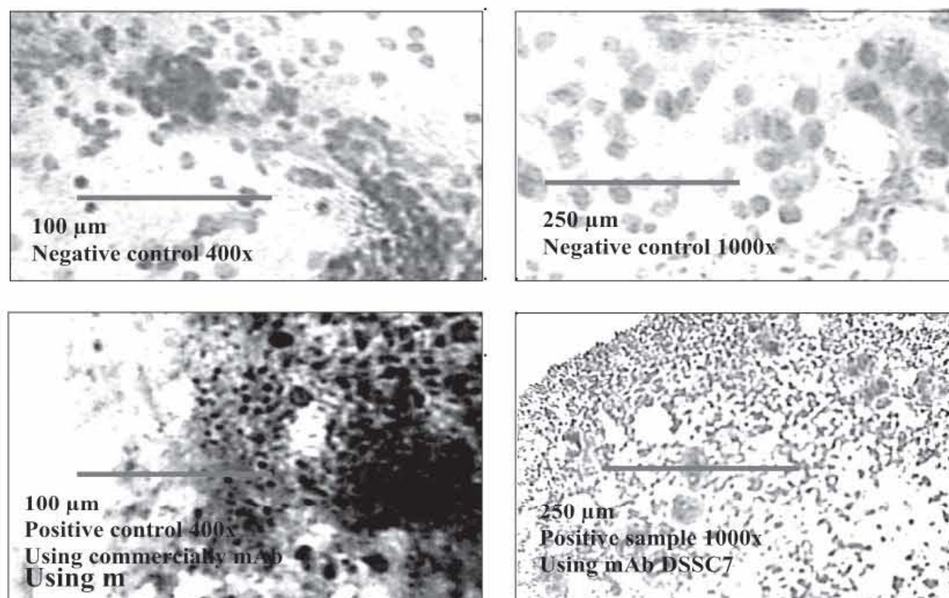

FIGURE 1. Head squashes immunocytochemical preparation of orally-infected *Ae. aegypti* with DENV-3 strain H87 at incubation period of 11days showing positive reaction as brownish coloration in the cytoplasm of infected cells and discrete brownish granular deposits with mAb DSSC7 as primary antibody and negative reaction was shown on head squashes of uninfected *Ae. aegypti* preparation as blue coloration

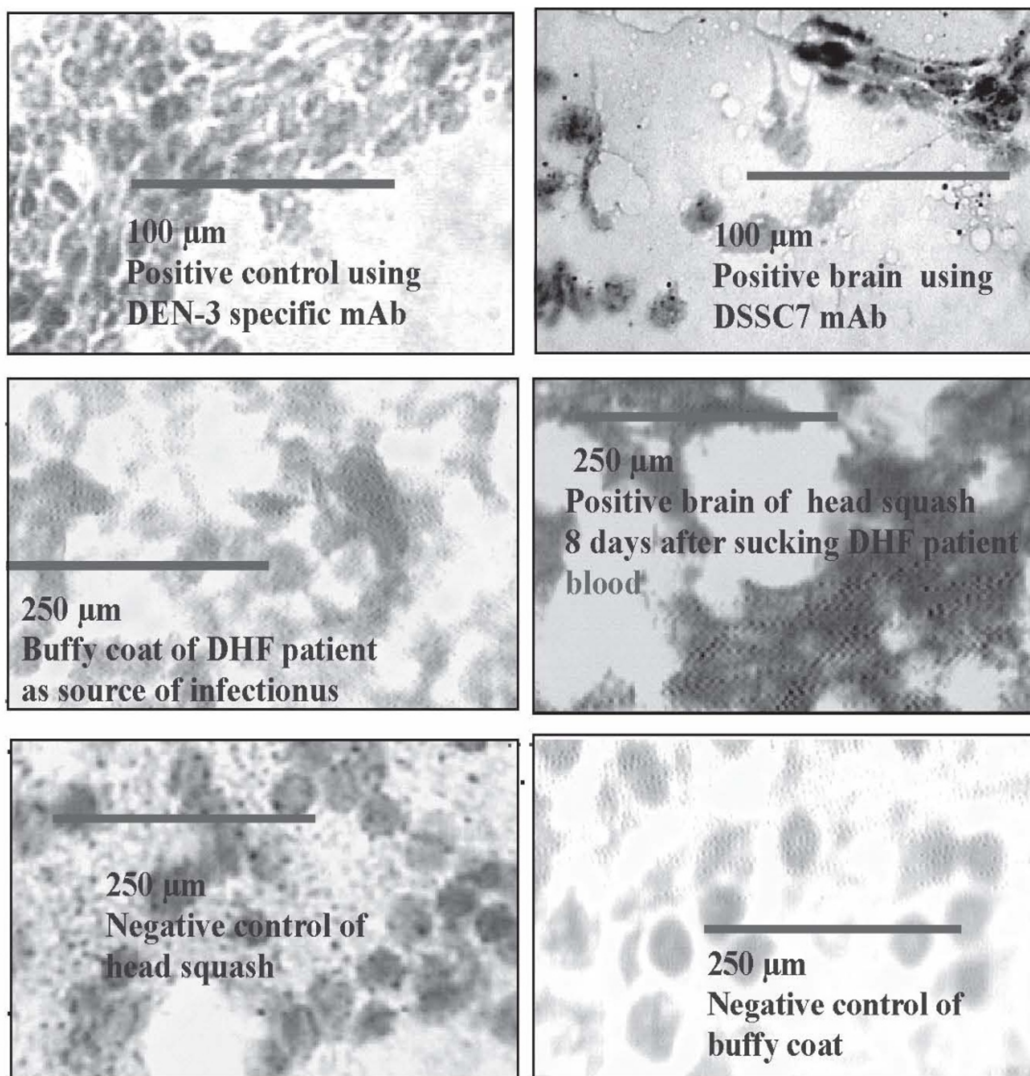

FIGURE 2. Positive head squash of orally-infected *Ae. aegypti* with DENV-3 and dengue patient who were IgM postive at incubation period of 8 days showing dengue viral antigen in the brain based on ISBPC assay using monoclonal antibody DSSC7 (1:50)

The description showed brown colour in brain cell cytoplasm and it was included in infectious level of (+++), because at the magnification of 400x more than 10 cells showed positive sign per visual field. At 5 day and 2 day incubation periods, most cell showed infection level of (++), because at the magnification of 400x, there were 1-10 positive cells per visual field, and showed infection level of (+), because there were only discrete brownish granule without any positive cells.

DEN viral antigen detection in head squash preparation was initially developed by Rosen and Gubler[3] based on direct fluorescence antibody technique (DFAT) with infection criteria of (+, ++, +++, and ++++). Infection level of (+++) was shown as discrete fluorescent granules and more than 10 cells per visual field showing greenish fluorescent in brain cell cytoplasm. Infection level of (++) showed as fluorescent granules with 1-10 positive cells per visual field at the magnification of 400x.

The lowest infection level was shown as discrete fluorescent granules without any fluorescence in the cytoplasm, or if at all, there was only one cell per visual field. In this study, the infection level of (+++) and (++) were observed after 8 day and 5 day incubation periods, while infection level of (+) was observed after 8 day, 5 day, and 2 day incubation periods.

Ideal diagnostic test is a test that gives positive result for ill subjects and gives negative result for healthy subjects. Almost in all diagnostic tests, there is a possibility to observe positive results in healthy subjects (false positive), and negative results in ill subjects (false negative). Therefore, a new diagnostic test is needed to be tested for its sensitivity and specificity, compared with gold standard. Gold standard is a standard verification of the availability or unavailability of diseases in patients, and is the best available diagnostic tool. On the other side, if an adequate gold standard is not available, an agreement is made to choose a gold standard.[7]

In this study, sensitivity and specificity tests were conducted on monoclonal antibody DSSC7 from Balb/c mice ascites preparation kept in freezer for around 4 years, because monoclonal antibody utilization for field samples was only conducted 4 years post-production of the monoclonal antibody. Microscopic examination showed that the monoclonal antibody was not sensitive if it was used in 1:50 dilution. Therefore, monoclonal antibody was diluted 10 times. The microscopic examination of preparation of *Ae.aegypti* mosquito infected with DEN-3 virus after 2, 5, 8, and 11 day incubation periods with monoclonal antibody produced by GMU from Balb/c mice ascites preparation DSSC7 as primary antibody are shown in TABLE 1.

TABLE 1. Result of microscopic examination of dengue antigen on head squash preparation of *Ae. aegypti* orally infected with DENV-3 at incubation period of 1,2,3 days based on ISBPC assay using mAb DSSC7 1:10 from ascitic fluid of Balb/c mice

Incubation period (day)	Result of immunocytochemistry		Infection
	Positive	Negative	rate (%)
2	6	4	60
5	8	2	80
8	7	3	70
11	9	3	75*
Total	30	12	71,43

TABLE 1 showed that infection rate of *Ae.aegypti* mosquito infected with DEN-3 virus was between 60-80% with average of 71.43%. This result was not different with infection rate of *Ae. aegypti* from Torres Strait Australia colony against DEN-2 viral infection, which was 76% after 2 week incubation period.[11]

Statistical analysis with Fisher's exact test showed that there was no significant difference between the examination results of mosquitoes infected with DEN-3 virus after 2 and 5 day incubation periods ($p = 0.2438$), between 5 and 8 days of incubation periods ($p = 0.348297$), and between 2 and 8 days incubation periods ($p = 0.325077$), using monoclonal antibody DSSC7 1:10 as primary antibody based on SBPC immunocytochemistry method. Similar result was shown between examination result of mosquitoes infected with DEN-3 after 2 and 11 day incubation periods ($p = 0.270898$), between 5 and 11 day incubation periods ($p = 0.37594$), and between 8 and 11 day incubation periods ($p = 0.353826$) using monoclonal antibody DSSC7 as primary antibody. A very significant difference was shown in the result of negative control and mosquitoes infected after 2 day incubation period ($X^2 = 17.45224$; db = 1; $p < 0.001$), 5 day ($X^2 = 27.5152439$; db = 1; $p < 0.001$), 8 day ($X^2 = 22.33073$; db = 1; $p < 0.001$), and 11

day ($X^2 = 26.74389$; db = 1; $p < 0.001$). This result indicated that DEN-specific monoclonal antibody produced by GMU kept in freezer (-20°C) was still sensitive to detect DEN viral antigen in head squash preparation if the concentration was 1:10, and the result was not different with monoclonal antibody DSSC7 which kept only for 1 year.

Sensitivity and specificity of DEN viral infection diagnostic test for head squash at the magnification of 400x compared with gold standard at the magnification of 1000x is shown in TABLE 2.

TABLE 2. Result of performance analysis of immunocytochemical SBPC assay for detecting DEN viral antigen on head squash preparation using mAb DSSC7 from ascitic fluid of Balb/c mice under light microscope at magnification of 400x

Magnification 400x	Gold standard		Total
	Positive	Negative	
Positive	39	1	40
Negative	6	24	30
Total	45	25	70

Sensitivity = *86.67%*
Spesificity = *96.00%*
Positive predictive value = *97.50%*
Negative predictive value = *80.00%*

The table showed that there were true positive in 39 samples, false positive in 1 sample, false negative in 6 samples, and true negative in 24 samples. The sensitivity was 86.67%, indicated that the proportion of infectious subjects showing positive result was 86.67%. Specificity was 96%, indicated that the proportion of non-infectious subjects showing negative result was 96%. Analysis showed that positive predictive value was 97.5%, which meant that the probability that the samples infected with DEN virus was 97.5% if diagnostic test showed positive result at the magnification of 400x. Negative predictive value was 80%, which meant that the probability of the samples not infected with DEN-3 virus was 80% if diagnostic test result showed negative result at the magnification of 400x.

Validity and reliability test was conducted to *Ae.aegypti* mosquito samples infected with DEN-3 virus after 5 and 8 day incubation periods, and caught by backpack aspirator. Examination result of head squash preparation on DEN antigen with SBPC immunocytochemistry using primary antibody DSSC7 1:10 with magnification of 400x and 1000x is shown in TABLE 3.

TABLE 3. Chi square analysis of immunocytochemical SBPC assay under light microscope at 400x and 1000x magnification for detecting DEN viral antigen on head squash preparation using mAb DSSC7

Magnification 400x	Magnification 1000x		Total
	Positive	Negative	
Positive	23	1	24
Negative	6	10	16
Total	29	11	40

Chi Square = 0,00004 db=1 P>0,05

TABLE 3 showed that there was no significant difference between examination results with magnification of 400x and 1000x (p >0.05). For reliability, an agreement has achieved for kappa between examination results with magnification of 400x and 1000x. Kappa agreement result is shown in TABLE 4.

TABLE 4. Strength of agreement of examination results of immunocytochemical SBPC assay under light microcope between magnification of 400x and 1000x for detecting DEN viral antigen using mAb DSSC7

Magnification 400x	Magnification 1000x		Total
	Positive	Negative	
Positive	23	1	24
Negative	6	10	16
Total	29	11	40

Kappa value= 0,62 (good).

TABLE 4 shows that kappa agreement level between examination results with magnification of 400x and 1000x was 0.62 and according to Landis and Koch[9], the agreement was considered good if the value is more than 0.60. Kappa agreement result is also shown in TABLE 5.

TABEL 5. Strength of agreement between first observer and second observer for detecting DEN viral antigen on head squash preparation using mAb DSSC7 based in immunocytochemical SBPC assay

Observer-2	Observer-1		Total
	Positive	Negative	
Positive	43	1	44
Negative	2	29	31
Total	45	30	75

Kappa value = 0.92 (very good).
Sensitivity = 43/45x100% = 95.66% = 96%
Spesificity = 29/30x100% = 96.66% =97%

Srength of agreement between the first and the second observers for detecting DEN viral antigen on head squash preparation using mAb DSSC7 based in immunocytochemical SBPC assay is very strong (0.92). According to Landis and Koch[9], the strength of agreement is very good, if kappa value is 0.81-1.00. Validity and reliability test results indicated that microscopic examination result with magnification of 400x and 1000x on DEN viral antigen with SBPC immunocytochemistry using monoclonal antibody DSSC7 as primary antibody were valid and reliable.

These findings is needed to be followed through by applying monoclonal antibody DSSC7 to complete the DHF vector surveillance in Indonesia that previously aimed to pra-imago stadium density in the form of larva free rate without knowing the prevalence of infectious mosquito. Therefore, a new breakthrough of DEN viral antigen detection in Aedes mosquito caught at pra-imago stadium (DEN viral transovarial transmission) which is simple and only needs light microscopic examination with immunocytochemistry method is needed to be considered.

CONCLUSION

SBPC immunocytochemistry method using monoclonal antibody DSSC7 as primary antibody successfully detected dengue viral antigen in

Ae.aegypti mosquito head squash with light microscope. The diagnostic test was specific, sensitive, valid, and reliable.

SUGGESTION

Monoclonal antibody secreted by hybrid cell DSSC7 is needed to be applied for verifying dengue viral transovarial infection in *Ae.aegypti* mosquito descendants from colony in the laboratory which already sucked the blood of DHF patients, using SBPC immunocytochemistry method.

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REFERENCES

1. Mourya DT, Joshi V. Horizontal and Vertical Transmission of Dengue Virus in Aedes aegypti Mosquitoes and its Persistence in Successive Generations, 6th International Symposium on Vectors and Vector Borne Diseases. Bhubaneswar: National Academy of Vector Borne Diseases & Regional Medical Research Centre, 2002.
2. Malavige GN, Fernando S, Fernando DJ and Seneviratne SL. Dengue viral infections. Postgraduate Medical J, 2004; 80:588-601
3. Rosen L and DJ Gubler. The use of mosquitoes to detect and propagate dengue viruses. Am J Trop Med Hyg 1974;21:1153-60.
4. Gubler DJ, Nalim S, Tan R, Saipan H, Sulianti Saroso J. Variation in susceptibility to oral infection with dengue viruses among geographic strains of Aedes aegypti. Am J Trop Med Hyg 1979; 28(6):1045-52
5. Sutaryo, Umniyati SR, Wahyono D. Produksi antibodi monoklonal terhadap virus DEN-3 untuk Deteksi penderita Demam Berdarah Dengue dan vektornya. Laporan Penelitian RUT-3 Tahun I, FK UGM, Yogyakarta, 1996.
6. Umniyati SR, Soeyoko, Mulyaningsih B. Pengembangan antibodi monoklonal anti dengue-3 produksi lokal Universitas Gadjah Mada untuk deteksi infeksi virus Dengue pada nyamuk Aedes spp. Laporan Penelitian Hibah Bersaing X/3, Universitas Gadjah Mada, Yogyakarta, 2004.
7. Pusponegoro HD, Wirya IGNW, Pudjiadi AH, Bisanto J, Zulkarnain SZ. Uji Diagnostik dalam S Satroasmoro dan S Ismael.(eds) Dasar-dasar metodologi penelitian klinis. Jakarta: Bagian Ilmu Kes. Anak FK UI. 1995; pp 126-62.
8. Herrmann JE. Immunoassays for the diagnosis of infectious diseases. In: PR Murray (ed): Manual of Clinical Microbiology, 6th ed. Washington DC: ASM Press, 1995; pp. 110-22.
9. Landis JR, Koch GG. The measurement of obser-veragreement for catagorical data. Biometrics 1977; 197(33):159-74.
10. Burry RW. Specificity Controls for Immunocyto-chemical Methods. J Histochem Cytochem 2000;48:163-66
11. Knox TB, Kay BH, Hall RA, Ryan PA. Enhanced vector competence of Aedes aegypti (Diptera: Culicidae) from the Torres Strait compared with mainland Australia for dengue 2 and 4 viruses. J Med Entomol 2003;40(6):950-56.