Sensitivity and specificity of immunocytochemical assay for detection of Dengue virus 3 infection in mosquito

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Abstrak

Latar belakang: *Survei virologi pada nyamuk vektor dapat digunakan sebagai Sistem Kewaspadaan Dini untuk mencegah penularan Demam dengue di suatu daerah. Pemeriksaan laboratoris untuk deteksi virus Dengue pada nyamuk seperti isolasi virus, Polymerase Chain Reaction (PCR) dan Direct Fluorescent-Antibody (DFA) memerlukan keahlian yang tinggi, peralatan yang mahal dan waktu yang lama. Suatu metode berdasarkan imunositokimia menggunakan antibody monoclonal DSSE10 memiliki beberapa kelebihan. Tujuan penelitian ini untuk mengevaluasi sensitifitas dan spesifitas pemeriksaan imunositokimia dibandingkan metode Reverse Transcription-Polymerase Chain Reaction (RT-PCR) untuk mendeteksi infeksi Virus Dengue 3.*

Metode: *Penelitian eksperimental dilakukan di laboratorium Parasitologi Fakultas Kedokteran Universitas Gajah Mada (UGM) pada bulan Mei 2009-Oktober 2010. Sebanyak 22 Ae. aegypti yang diinfeksi virus Dengue 3 digunakan sebagai kelompok infeksius dan 35 nyamuk yang tidak diinfeksi sebagai kelompok non infeksius. Pemeriksaan imunositokimia Streptavidin Biotin Peroxidase Complex (SBPC) menggunakan antibodi monoklonal DSSE10 dilakukan pada sediaan head squash Ae .aegypti untuk mendeteksi antigen virus Dengue 3. Pemeriksaan RT-PCR sebagai baku emas diaplikasikan pada toraks nyamuk.*

Hasil: *Nilai Kappa menunjukkan kesepakatan yang baik antara dua orang pemeriksa (0,63). Imunositokimia mendeteksi antigen virus Dengue-3 dengan sensitivitas yang sama dengan RT-PCR (sensitivitas 100%). Namun spesifisitas IC lebih rendah dibanding RT-PCR (spesifisitas 91%) karena beberapa hasil positif palsu muncul pada pemeriksaan ini.*

Kesimpulan: *Metode IC memiliki nilai sensitivitas dan spesifisitas yang tinggi dibandingkan dengan metode RT-PCR. Metode IC ini dapat digunakan untuk surveilans virus Dengue pada nyamuk vektor. **(Health Science Indones 2011;2:87-91)***

Kata kunci: *imunositokimia, DSSE10, head squash, dengue*

Abstract

Background: Virological surveillance provides an early warning sign for the risk of transmission in an area. Laboratory tests for dengue virus infection on mosquitoes include isolation of the virus, Polymerase Chain Reaction (PCR) and Direct Fluorescent-Antibody (DFA) requires a high level of technical skill, expensive equipment, and time-consuming. A method based on immunocytochemical (IC) using monoclonal antibody DSSE10 has several advantages. This study aimed to evaluate sensitivity and specificity IC assay compared with Reverse Transcription-Polymerase Chain Reaction (RT-PCR) as gold standard to detect Dengue Virus (DENV)-3 infections in mosquito *Aedes aegypti*.

Methods: An experimental study was conducted in laboratory of Medical Parasitology, Faculty of Medicine, Universitas Gadjah Mada (UGM) in May 2009 until October 2010. A total of 22 artificially-infected adult *Ae. aegypti* mosquitoes of DENV 3 were used as infectious samples and 35 non-infected adult *Ae. aegypti* mosquitoes were used as normal ones. The IC Streptavidin Biotin Peroxidase Complex (SBPC) assay using monoclonal antibody DSSE10 was applied in mosquito head squash to detect Dengue virus antigen. RT-PCR as a gold standard was applied in mosquito thorax.

Results: The kappa value showed a good agreement between two observers (kappa value 0.63). IC could detect dengue virus antigen as sensitive as RT-PCR (sensitivity 100%). But IC was less specific than RT-PCR (specificity 91%) because some false positive results were found in this method.

Conclusion: The IC method has a high sensitivity and high specificity compared with RT-PCR. This IC method may be useful for virological surveillance of dengue infected Aedes mosquitoes. ***(Health Science Indones 2011;2:87-91)***

Keywords: immunocytochemical, DSSE10, head squash, dengue

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Dengue is a prominent disease in tropic and subtropic areas. The major disease burden is found in Southeast Asia and the Western Pacific.[1] There are four antigenically related but distinct serotypes of dengue virus, designated DEN-1, DEN-2, DEN-3, and DEN-4.[2] DEN-3 has been recognized as the predominant serotype in many recent epidemic occurrences of DHF in Indonesia.

Dengue viruses are transmitted to humans by the bite of infective female mosquitoes of the genus A*edes*. *Aedes (Ae.) aegypti* is considered the main vector because this species is closely associated with human habitation, but in some regions other *Aedes* species, such as *Ae. albopictus*[3] and *Ae. polynesiensis*[4], are also involved. Virological surveillance provides an early warning sign for the risk of Dengue virus transmission in an area.[5]

Laboratory tests for detection mosquitoes infected with dengue viruses include isolation of the virus and demonstration of a specific viral antigen or RNA. Isolation of the virus is the most definitive approach, but the techniques involved require a relatively high level of technical skill, equipment, and are time-consuming.[6]

Detection of nucleic acid is an alternative method to detect infected mosquitoes. Polymerase Chain Reaction (PCR) is one technique available for the laboratory diagnosis of dengue infection. This molecular technique is rapid, highly sensitive and specific.[6] However PCR requires a relatively expensive equipment, such as a thermalcycler.

Direct detection of dengue antigen, such as the Direct Fluorescent-Antibody (DFA) test is labor-intensive and requires fluorescent microscope and cryo-freezer.[6] Therefore, a method based on immunocytochemistry (IC) involving enzyme conjugates such as peroxidase and phosphatase in conjunction with either polyclonal or monoclonal antibodies has been developed to detect dengue antigen. This IC method is a common laboratory technique that uses antibodies that target specific peptides or protein antigens in the cell via specific epitopes. This method has 94.3% sensitivity, 90% specificity for whole blood samples.[7] Therefore, IC is useful in detecting dengue virus infection.

Monoclonal antibody against DENV-3 was produced by the Dengue Team of Universitas Gadjah Mada (UGM).[8] In this study, the newly developed MAbs DSSE10, which recognize NS1 of dengue virus serotype 3 (DEN-3), respectively, were used in staining mosquito head squash.

This study aimed to evaluate sensitivity and specificity IC assay compared with RT-PCR to detect Dengue Virus-3 infection in mosquito *Aedes aegypti.*

METHODS

An experimental study was conducted in laboratory of Medical Parasitology, Faculty of Medicine, UGM from May 2009 until October 2010. A total of 22 artificially-infected adult *Ae. aegypti* mosquitoes of DENV 3 were used as infectious samples and 35 non-infected adult *Ae. aegypti* mosquitoes were used as normal ones.

Dengue virus type 3 (H-87) in C6/36 cell lines was obtained from Naval Medical Research Unit 2 (NAMRU-2), Jakarta. Eggs of laboratory colony *Ae. aegypti* on dry filter paper, were reared to adults in the laboratory of Medical Parasitology, Faculty of Medicine, UGM. Three days old adults female *Ae. aegypti* were collected by manual aspirators for use in the experiments.

Three-day old adult females *Ae. aegypti* were experimentally infected with DENV-3 using a sterile parenteral inoculation technique.[9] Mosquitoes were immobilized over wet ice for 5-10 minutes before being injected with virus suspension in the membrane area of the intrathoracic. Inoculation procedures took place under a dissecting microscope using a calibrated capillary needle and syringe plunger. Infected mosquitoes were held in small cylindrical cages covered with mosquito netting, and they were incubated at 27±1°C and a relative humidity of 88±6% and maintained on 10% sucrose for 7 days. They were collected at 5, 6, and 7 days after inoculation and separated into caput and thorax. RT-PCR was applied in mosquito thorax, while caputs were kept at -70ºC for IC test.

Negative controls comprised uninfected *Ae. aegypti* from non-endemic area of DHF and *Anopheles* mosquitoes from Salatiga district, Central Java province. The Anopheles mosquitoes were used as negative control tissue because they are not the vector of dengue virus.

RNA extraction was done in accordance with the protocol of High Pure Viral Isolation Kit (Roche, Germany). Dengue viral RNA in mosquitoes were detected by reverse transcriptase polymerase chain reaction (RT-PCR) using DENV-3 -specific primers.[10] The RT-PCR product was analyzed by agarose gel-electrophoresis on a 1.5% agarose gel (invitrogen) containing ethidium bromide (0.5 µg/ml). For the DNA size marker, 100 bp DNA ladder (0.1 mg/ml) was used. Electrophoresis was set at 100 volts/cm2 and was run for 30-45 minutes. The expected size of 538 bp was identified as being of DENV-3 respectively.

Caputs of *Ae. aegypti* from infectious and non-infectious group were put on the object glasses then pressed under cover glass with the eraser part of a pencil. Each object glass can be filled with 10-15 caputs. The cover glass was removed, and the object glass was put into a bottle filled with alcohol 70%. The preparation was dried in room temperature for around 30 minutes. Afterwards, it was fixed with cold acetone (-20^{0}C) in freezer for 3-5 minutes, then dried in laminary flow.[11] Preparation was fixed with cold methanol (-20^{0}C) for 3-5 minutes and washed with PBS. To eliminate the endogenous peroxidase activity, the preparation was soaked in peroxidase blocking solution (1 part of hydrogen peroxide 30% + 9 part of absolute methanol) at room temperature for 10 minutes. Preparation was incubated in prediluted blocking solution for 10 minutes in room temperature (25^{0}C).[11]

One hundred mL primary antibody (DSSE10 1:10 monoclonal antibody) was added to the preparation (adjusted until all part was soaked) and incubated on damp tray at room temperature (25^{0}C) for 60 minutes or overnight in the refrigerator. Then it was washed twice with (fresh) PBS for 2 minutes; 100mL biotinylated universal secondary antibody was added, and the preparation was incubated at room temperature (25^{0}C) for 10 minutes, and then washed twice with fresh PBS for 2 minutes.[11]

The preparation was incubated with ready to use streptavidin-peroxidase-complex reagent for 10 minutes and then washed twice with PBS for 2 minutes; incubated in 100 mL peroxidase substrate solution (DAB) for 2-10 minutes (the thicker the preparation, the longer the incubation time), and then washed with tap water; then 100 mL Mayer hematoxyllin (counter stain) was added, incubated for 1-3 minutes, and then washed with tap water. The preparation was then soaked in alcohol, washed, and then soaked in xylol. Drops of mounting media were added on the preparation and covered with cover glass. When it was already dry, the preparation was ready to be evaluated under light microscope with magnification of 40x, 100x, 400x, and 1000x.

The microscopic examinations involved two observers. The first observer was an experienced technician of Medical Parasitology laboratory, Faculty of Medicine UGM. The second observer was the researcher. If the preparation showed a brown color, it meant that the preparation contained DEN viral antigen. Meanwhile if the preparation showed blue or pale color (as in the negative control) the preparation did not contain DEN viral antigen.

Laboratory mosquitoes infected with DENV-3 were dissected into 2 parts (head and thorax) on day 5, 6, and 7 days post-inoculation. Each mosquito thorax was amplified by RT- PCR to detect dengue virus. All of RT-PCR product of uninfected mosquito showed negative result, meaning there were no DENV inside these mosquitoes. These groups were used as non-infectious group.

Sensitivity and specificity were measured based on Hermann formula. Validity and reliability was determined based on kappa value by Landis and Koch.[12]

RESULTS

All head squash preparation of infectious groups showed positive result based on IC (Figure 1). DENV-3 infection in *Ae. aegypti* was shown as discrete brownish granules between the whole brain tissues. Most of head squash preparation of non-infectious group showed blue and pale brain tissues, meaning there is no DENV-3 antigen inside these mosquitoes.

Figure 1 showed IC staining positive result on A (5 days), B (6 days), and C (7 days) post-inoculation and negative result on non-infected mosquito (D).

For reliability, an agreement has achieved for kappa between two observers. Inter observer agreement result shown in Table 1. The first and the second observers were in agreement for detecting Dengue viral antigen on head squash preparation by immunocytochemistry using mABs DSSE10 (Kappa value was 0.63).

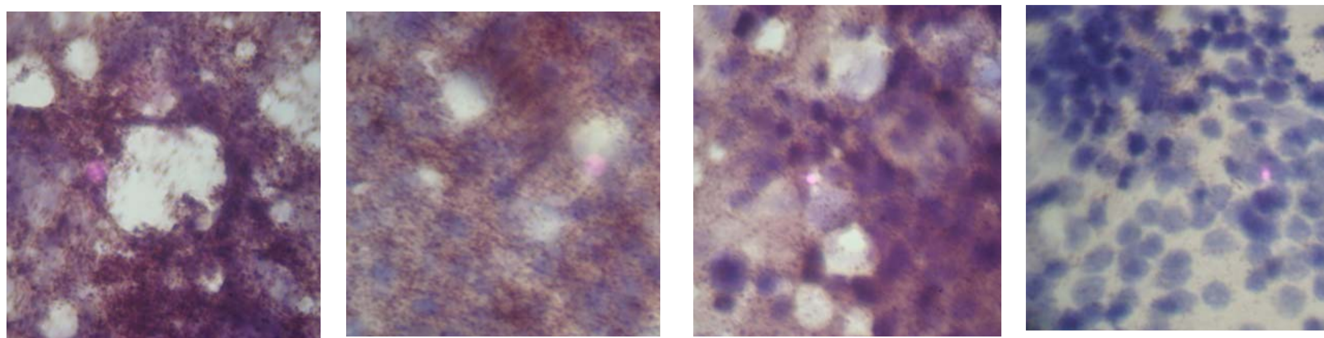

Figure 1. All head squash preparations of infectious groups

Table 1. Inter-observer agreement of immunocytochemical assay

		Observer 2 (+)	Observer 2 (-)	Total
Observer 1	(+)	15	10	25
	(-)	0	32	32
Total		16	41	57

Kappa value = 0.63

Table 2 showed that IC has good sensitivity (100%) and good specificity (91%). Furthermore, analysis showed probability that the samples infected with DEN virus was 88% if diagnostic test showed positive result at IC assay. In addition, the probability of samples non-infected with DENV-3 was 100% if diagnostic test result showed negative at IC assay.

Table 2. Sensitivity and specificity of immunocytochemical assay

		RT-PCR (+)	RT-PCR (-)	Total
Immunocytochemistry	(+)	22	3	25
	(-)	0	32	32
Total		22	35	57

Sensitivity = 100% Specificity = 91%
Positive predictive value = 88% Negative predictive value = 100%

DISCUSSION

Immunocytochemistry is a powerful method for the identification of proteins or antigen in cells and tissues. The monoclonal antibody used in this study was secreted by DSSE10 clone belonging to IgG class and IgG1 subclass. The monoclonal antibody was secreted by a single hybrid (DSSE10) which was generated from the third fusion recognized as DENV complex specific epitope and showed no cross-reactivity to Chikungunya and Japanese Enchephalitis antigens based on Western blotting analyses. The mAb DSSE10 reacts to non-structural protein (NS1).[11]

The detection of DENV-3 antigen in mosquitoes by IC assay using monoclonal antibody DSSE10 has high sensitivity (100%), which was more sensitive than the detection in human thick blood smears. Detection of Dengue virus in human thick blood smears by IC using monoclonal antibody DSSE10 gives 94.3% sensitivity and 90% specificity.[7] This difference in sensitivity value may be caused by the different preparation between head squash and blood smears. Head squash preparation was dried by wet-fixed smear, meanwhile blood smear preparation was dried by air drying. Air dried preparation often exhibits relatively weak immunoassaying. This is probably because the dried cells exhibit an overall lower antigen density.[13]

The IC stains are indispensable for problem solving in detection of infectious agent. In order to evaluate them appropriately, it is critical to be aware of a true positive stain and a false positive stain. A "true positive" stain shows chromogen deposition in cells or structures that truly contain the antigen of interest. In contrast, a "false positive" stain is one where the chromogen is localized to cells or structures that in reality lack the antigen of interest.[14]

The main cause of non-specific background staining is endogenous peroxidase activity which is found in many tissues and can be detected by reacting fixed tissue sections with DAB substrate[13]. To eliminate endogenous peroxidase activity in this study was by the pretreatment of the tissue section with hydrogen peroxide prior to incubation of primary antibody. However Immunocytochemical stains in this study showed false positive in 3 samples of non-infected mosquitoes.

The IC assay has several advantages over the PCR method. First, IC process is easier than PCR. Besides, IC assay does not require specific equipment such as thermalcycler, so this assay could be done in every laboratory and more cost-effective. Moreover, head squash preparation of IC assay could be stored for along times. So, with these advantages, IC has a chance to replace other method of dengue virus detection.

Detection of Dengue virus in mosquitoes would give valuable information as early warning system to prevent Dengue Fever transmission. Chow noted that by detecting of Dengue virus infection in the mosquito vectors before its introduction into the human population it was possible to predict an outbreak six weeks in advance of the occurrence of the first human case in Singapore.[15] When the number of dengue infection in mosquitoes increases the Local Health Office should make an effective vector control program to prevent the virus introduction to the human populations. Therefore, it needs further study to evaluate the application of immunocytochemical assay for detection Dengue virus infection in mosquito head squash in the field level to develop an effective early warning system for Dengue Fever prevention.

In conclusion, immunocytochemical assay could be used in detection DENV-3 infection on mosquito head squash. This method has a high sensitivity, high specificity and good inter observer agreement.

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