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Characterization and Application of WDSSB5 Monoclonal Antibody for the Detection of Dengue Virus In C6/36 Cell Line Using Immunocytochemical Method

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ABSTRACT

Introducition: Dengue Fever (DF) and Dengue Hemorrhagic Fever (DHF) are caused by Dengue virus that consists of 4 serotypes of Dengue Virus (DENV) 1, 2, 3 and 4. Isolation of Dengue virus using C6/36 cell is considered as a gold standard for the diagnosis of Dengue virus infection. Dengue Team of Gadjah Mada University successfully produced monoclonal antibodies of DENV 3 originating from hybrid cells of DSSC7, DSSE10 and WDSSB5. The detection of Dengue virus's antigens of *Ae. aegypti* in human blood smear with Streptavidine Biotin Peroxide Complex (SBPC) immunocytochemistry method using DSSC7 primary antibody is highly sensitive and specific, whereas using WDSSB5 monoclonal antibody yet to be characterized.
Objective: The study aimed to identify characterization and application of WDSSB5 monoclonal antibody as primary antibody for detection of Dengue virus originating from serum of patients with Dengue infection which was inoculated in C6/36 cell line using SBPC immunocytochemistry method.
Methods: The study was experimentally designed. Propagation of WDSSB5 hybridoma cell was performed *in vitro* and *in vivo*. The characterization consisted of classification of WDSSB5 monoclonal antibody, examination of WDSSB5 ascites protein level, sensitivity and specificity of immunocytochemical SBPC method using WDSSB5 primary antibody and specificity of monoclonal antibody against Dengue antigen with Dot Blot method. Dengue virus obtained from patients was inoculated in C6/36 cell. Detection of Dengue virus antigen was performed by SBPC immunocytochemistry method with WDSSB5 monoclonal antibody as primary antibody. Positive control was made using C6/36 cell infected with DENV 1, 2, 3, 4 and inoculated in C6/36 cell, whereas negative control uses cell C6/36 not infected with Dengue virus.
Results: There was WDSSB5 monoclonal antibody detected in this research which was belonged to IgG class and IgG1 subclass. The least content of WDSSB5 monoclonal antibody that can detect Dengue antigen in C6/36 cell was 2.2 µg/µL. The WDSSB5 monoclonal antibody was sensitive to detect DENV 1, 2, 3, 4 antigens in C6/36 cell using SBPC immunocytochemistry method.
Conclusion: There was WDSSB5 monoclonal antibody specific againts Dengue virus identified in this study. WDSSB5 monoclonal antibody belonged to class IgG and subclass IgG1. WDSSB5 Monoclonal antibody can be applied to detect Dengue virus originating from serum of patients positively carrying Dengue virus inoculated in C6/36 cell using SPBC immunocytochemistry method.

Keywords: Dengue Virus, immunocytochemical, monoclonal antibody, C6/36 cell

INTISARI

Pendahuluan: Demam Dengue (DD) dan Demam Berdarah Dengue (DBD) disebabkan oleh virus Dengue yang terdiri dari 4 serotype virus Dengue (DENV) 1, 2, 3 dan 4. Isolasi virus Dengue menggunakan kultur sel C6/36 merupakan baku emas untuk menegakkan diagnosis infeksi virus Dengue. Team Dengue UGM telah berhasil memproduksi antibodi monoklonal terhadap DENV 3 antara lain yang berasal dari sel hibrid DSSC7, DSSE10 dan WDSSB5. Deteksi antigen virus Dengue pada organ nyamuk *Ae.aegypti* dan pada sediaan apus darah manusia dengan metode imunositokimia *Streptavidine Biotin Peroxide Complex* (SBPC) menggunakan antibodi primer DSSC7 mempunyai sensitivitas dan spesifisitas yang tinggi, sedangkan sel hibrid WDSSB5 belum dikarakterisasi.

Tujuan: Tujuan penelitian ini adalah untuk melakukan karakterisasi dan mengaplikasi antibodi monoklonal WDSSB5 sebagai antibodi primer untuk mendeteksi virus Dengue dari serum pasien yang positif mengandung virus Dengue yang diisolasi pada sel C6/36 dengan metode imunositokimia SBPC.

Metode: Desain penelitian ini eksperimental. Pada penelitian ini propagasi sel hibridoma WDSSB5 dilakukan secara *in vitro* dan *in vivo*. Karakterisasi yang dilakukan meliputi uji klasifikasi antibodi monoklonal WDSSB5, pemeriksaan kadar protein asites WDSSB5, sensitivitas dan spesifisitas metode imunositokimia SBPC menggunakan antibodi primer WDSSB5 serta spesifitas antibodi monoklonal terhadap antigen Dengue dengan *Dot Blot*. Virus Dengue yang berasal dari serum pasien yang positif mengandung virus Dengue diinokulasi pada sel C6/36. Deteksi antigen virus Dengue dilakukan dengan menggunakan metode imunositokimia SBPC dengan antibodi monoklonal WDSSB5 sebagai antibodi primer. Kontrol positif digunakan sel C6/36 yang diinfeksi virus DENV 1, 2, 3, 4 dan diinokulasi pada sel C6/36, sedangkan kontrol negatif adalah sel C6/36 yang tidak diinfeksi virus Dengue.

Hasil: Hasil penelitian didapatkan antibodi monoklonal WDSSB5 yang termasuk kelas IgG dan sub subkelas IgG1. Kadar antibodi monoklonal WDSSB5 terkecil yang dapat mendeteksi antigen Dengue pada sel C6/36 adalah 2,2 µg/µL. Antibodi monoklonal WDSSB5 sensitif dan spesifik mampu mendeteksi antigen DENV 1, 2, 3, 4 pada sel C6/36 dengan metode imunositokimia SBPC.

Simpulan: Didapatkan antibodi monoklonal WDSSB5 spesifik terhadap virus Dengue. Antibodi monoklonal WDSSB5 termasuk kelas IgG dan subkelas IgG1. Antibodi monoklonal WDSSB5 dapat diaplikasikan untuk mendeteksi virus Dengue yang berasal dari serum pasien yang positif mengandung virus Dengue yang diisolasi pada sel C6/36 dengan metode imunositokimia SPBC.

Kata kunci: virus Dengue , imunositokimia, antibodi monoklonal, sel C6/36.

INTRODUCTION

Dengue Disease (DD) and Dengue Hemorrhagic Fever (DHF) are caused by Dengue virus, an Arbovirus group B which belongs to the family of Flaviviridae, genus of Flavivirus and is composed of four serotypes (DENV 1 to 4). Dengue virus infections in humans result in a spectrum of clinical manifestations ranging from the mild undifferentiated febrile illness, dengue fever, Dengue Hemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS).

Dengue virus is spread all over the world increasingly mixed each other of Dengue 1,2,3,4 following human mobility. All serotypes are still endemic in Indonesia. The number of dengue cases in Indonesia, according to WHO data during 2006 to 2008, were 106,425 cases of dengue in 2006 with the mortality rate was 1,132

(Case Fatality Rate (CFR) was 1.06%). In the year 2007 there were 188,115 cases (CFR was 1.01%), whereas in 2008 there were 101,656 cases (CFR was 0.73%).

Course of the DHF is very rapid so if not immediately treated, it is often fatal. Until now, the diagnosis of Dengue fever in Indonesia is still primarily based on clinical diagnosis without followed by virological diagnosis confirmation. Virological confirmation actually can be done early since viremia occurred 2 days before the fever and during fever. However, diagnostic tool for this purpose has not been affordable for the most patients.

Laboratory tests that may be performed for confirmation of dengue virus infection are virus isolation, detection of viral genome using Polymerase Chain Reaction (PCR), viral antigen detection and serological tests. Virus isolation using cell culture is the gold standard for definitive diagnosis of dengue virus infection. Cell cultures which widely used are *Ae. pseudo scutellaris* (AP/61) cells, *Ae. albopictus* (C6/36) and *Toxorrhynchites amboinensis* (TRA-284). Culture results are usually identified using Direct Immunofluorescent Assay (DFA) or Indirect Immunofluorescent Assay (IFA) methods. Although it is the best way to ensure Dengue virus infection, this method requires sophisticated laboratory equipment and requires a long time. The advantage of dengue virus isolation is it produced local dengue virus isolates that can be used for further research.

An immunocytochemistry method commercially available now is Streptavidin Biotin Peroxidase Complex (SBPC). This technique is using biotin-labeled with secondary antibody that can recognize the primary antibodies (monoclonal antibodies or polyclonal antibodies) and using enzyme-labeled streptavidin conjugated to horseradish peroxidase. A substrate of chromogenic mixture is used to detect antigens on the cell or tissue with a very high sensitivity so that the low levels of antigen can be detected. The main basis of SBPC immunocytochemistry reaction is a strong bond between streptavidin and biotin. Immunocytochemistry method can be used to detect Dengue virus in various organs of the mosquito, paraffin tissue or on buffycoat, whereas those in C6/36 cell have not been reported. Examination using immunocytochemistry preparations can be done with a light microscope that is widely available in the laboratory, whereas immunofluorescent preparations should be examined under an expensive fluorescent microscope and rarely available in the laboratory. This method has advantages because it does not require special equipment since it can be done with a light microscope that is widely available in laboratories and does not require any particular skills. It is required specific monoclonal antibodies against dengue virus as a diagnostic reagent. Dengue Team of Universitas Gadjah Mada has successfully producing a monoclonal antibody against the DENV 3 secreted by hybrid cells (clone) of WDSSB5.

The purpose of this study was to characterize and apply WDSSB5 monoclonal antibody as the primary antibody for detection of dengue virus from serum of patients positive for dengue virus that was isolated in cell C6/36 with SBPC immunocytochemistry method.

MATERIALS AND METHODS

This study was an experimental study. In this study, the propagation of hybridoma WDSSB5 was performed in vitro and in vivo. In in vitro propagation, hybridoma clones cultured in RPMI PBS-enriched culture medium, penicillin-streptomycin and antifungal amphotericin B on

24 wells microplate and incubated at 37 °C with 5% of CO_2. Cell growth was observed every day under an inverted microscope until reaching logarithmic phase. Then, recloning was done to produce hybridoma cell line. Classification of monoclonal antibody was determined using an antigen-mediated ELISA method. Specificity of WDSSB5 monoclonal antibodies was analyzed using Dot Blot method.

Isolation of viruses from patient's serum performed on mosquitoes and cell C6/36. Serum was diluted with PBSBA (1: 5). Samples were intrathoracically inoculated into each of *Toxorrhyinchites sp* aged 2-4 days. After inoculation, mosquitoes were incubated at 32°C with 75-85% humidity for 9-10 days and fed with 10% sugar solution. The mosquitoe's heads were cut and other body parts are crushed and made a suspension with buffer containing protein stabilizers and antibiotics so it becomes a 10% suspension, then centrifuged and filtered.

The confluent's C6/36 cell taken from the medium and spared little and the suspension was inoculated on C6/36 cell, then adsorbed for 60 minutes in laminary flow hood and shaken every 10 minutes. Five mL of maintenance medium were added then the cells were incubated at 29-30°C for 4-5 days without CO_2. Every day the infected cells were observed under an inverted microscope and compared with control. After the cells on each bottle showed symptoms of infection include the presence of giant cell or cell lysis has begun, cells were scraped and cell then fit with the medium into 15 mL centrifuge tube and centrifuged at 450 g at temperature of 4°C for 10 minutes. Supernatant was stored at temperature of -84°C. The pellets were made into preparation for infectivity examination with immunocytochemistry techniques. Uninfected cells or control is also made into preparation on poly-L-lysine glass objects. Once dried, the preparation were fixed with cold methanol and dried up at room temperature. The WDSSB5 monoclonal antibodies of 2.2 ug/mL was used as primary antibodies and then the results were observed under a light microscope. Positive controls were prepared using C6/36 cells infected with DENV 1,2,3,4 and the negative controls were C6/C36 cells that were kept uninfected. After inoculation, the cells were incubated for 1-4 days. The C6/36 cell preparations were infected by dengue virus is not given the primary antibody the primary antibody at the time of SBPC immunocytochemistry test. The results are called positive for dengue antigen if the cell cytoplasm is brown, while the results are called negative if the cell cytoplasm is blue.

RESULTS AND DISCUSSION

Recloning of WDSSB5 hybridoma cell clones

The propagation of WDSSB5 hybridoma clones results in obtaining monoclonal antibodies in WDSSB ascites fluid in volume of 19.5 mL.

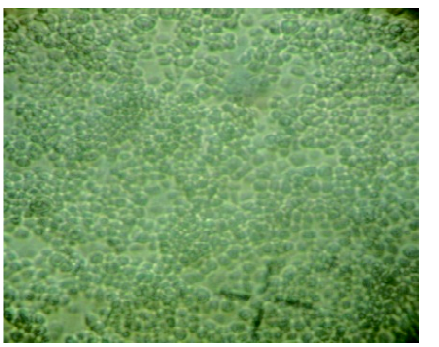

Figure 1. Producer of hybridoma cell line under an inverted microscope

Characterization of WDSSB5 monoclonal antibodies

Classification Test Classification test of monoclonal antibody was conducted using an antigen-mediated ELISA method. The result shows that the WDSSB5 monoclonal antibody belong to the IgG class and IgG1 subclasses.

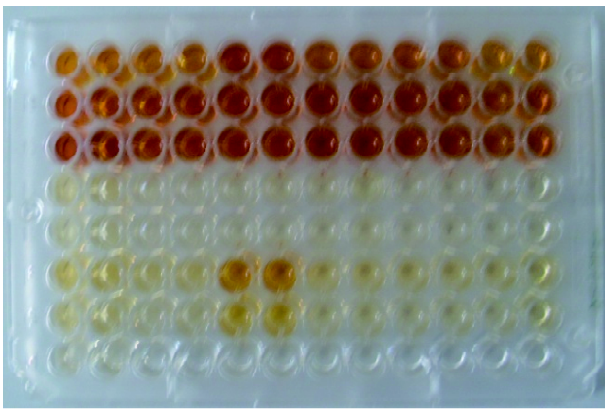

Figure 2. Result of classification test of WDSSB5 monoclonal antibody (B5, well number 5 and 6 arise brown color)

Examination of WDSSB5 ascites protein levels

Examination results obtained WDSSB5 ascites protein levels was 11 mg/mL. Optimization results obtained WDSSB5 ascites protein levels was 2.2 mg/mL. This level is optimal for detecting dengue virus in C6/36 cell preparations based on the method of immunocytochemistry SBPC with a positive rate of 100% (Table 1). The results of microscopic examination of SBPC immunocytochemistry using WDSSB5 as a primary antibody successfully detect dengue antigens in of C6/36 cells preparations and showed a positive reaction indicated by the brown color of the cytoplasm of the cell.

Negative reaction is indicated by the appearance of blue color in the negative control and in C6/36 cell were infected by dengue virus but not given the primary antibody when SBPC immunocytochemistry test was performed. The results of microscopic examination of the SBPC immunocytochemistry of optimization WDSSB5 ascites protein levels as the primary antibody on C6 / 36 cell preparations are presented in Table 1.

Table 1. SBPC immunocytochemistry examination for optimization of WDSSB5 primary antibody levels in C6/36 cell preparations which were infected by DENV 3 and incubated for 4 days

Protein levels of WDSSB5 ascites (µg/µL)	Number of cell						Positive Rate (%)
	I		II		III		
	(+)	(-)	(+)	(-)	(+)	(-)	Mean
2.2	465	0	478	0	459	0	100
1.1	389	61	405	53	411	49	88.0
0.22	347	84	367	71	353	61	83.2
0.11	309	132	317	108	299	118	72.1

Photomicrographs of SBPC immunocytochemistry of C6/36 that were infected by DENV 3 and incubated for 4 days for optimization WDSSB5 monoclonal antibody levels are presented in Figure 3.

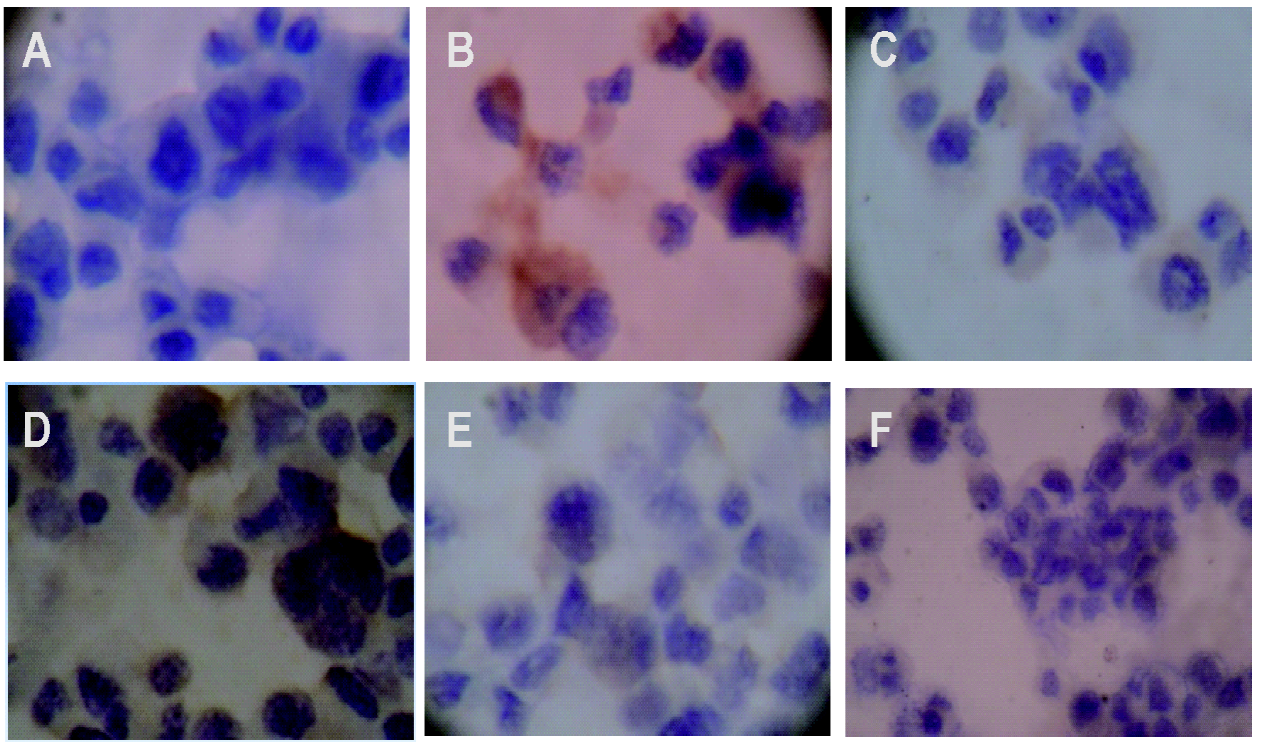

Figure 3. Photomicrographs showing brown color (positive) for dengue virus antigen in the cytoplasm of C6/36 cells (magnification of 100 x 10 times). B (2.2 µg/µL), C (1.1 µg/µL), D (0.22 µg/µL), E (0.11 µg/µL). A and F showing C6/36 cell cytoplasm with negative reaction (blue).

Sensitivity and Specifity Tests of WDSSB5 monoclonal antibody by SBPC immunocytochemistry method

The results of examination SBPC immunocytochemistry showed that 2.2 ug/mL of WDSSB5 primary antibody was able to detect infection of DENV 1, 2, 3, 4 in C6/36 cell from 1-day of incubation period with a positive rate of 100%. The results are depicted in Table 2.

Table 2. The results of SBPC immunocytochemistry on C6/36 cell which were infected by DENV 1, 2, 3, and 4 with the incubation period of 3-4 days

Dengue Virus Serotype	Incubation period (Days)	Number of cell Rate (%) I (+)	I (-)	II (+)	II (-)	III (+)	III (-)	Positive rate (%) Mean
DENV 1	1	338	0	389	0	356	0	100
	2	353	0	333	0	367	0	100
	3	272	0	267	0	289	0	100
	4	109	0	121	0	133	0	100
DENV 2	1	348	0	327	0	356	0	100
	2	346	0	337	0	359	0	100
	3	301	0	279	0	287	0	100
	4	255	0	223	0	248	0	100
DENV 3	1	428	0	456	0	433	0	100
	2	366	0	349	0	357	0	100
	3	325	0	317	0	329	0	100
	4	208	0	198	0	187	0	100
DENV 4	1	413	0	422	0	435	0	100
	2	375	0	368	0	397	0	100
	3	305	0	314	0	321	0	100
	4	188	0	176	0	197	0	100

Table 2 shown that 2.2 ug/mL of WDSSB5 primary antibody was able to detect infection of DENV 1, 2, 3, 4 in C6/36 cell from 1-day of incubation period with a positive rate of 100%. This suggests that the WDSSB5 primary antibody is sensitive and able to detect infection of DENV 1, 2, 3, 4 from the onset of infection.

Photomicrographs of SBPC immunocytochemistry of C6/36 cell that were infected by DENV 1,2,34 and incubated for 1 to 4 days for are presented in Figure 4.

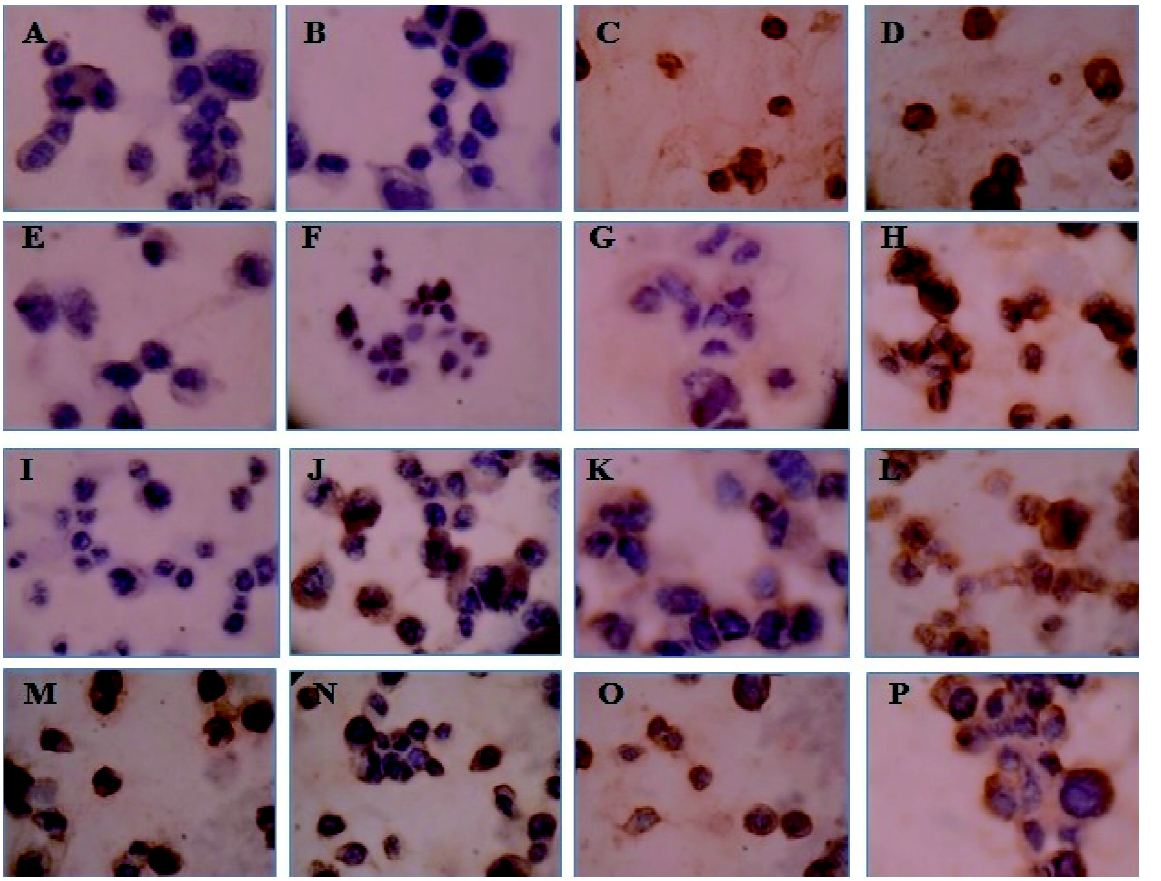

Figure 4. Photomicrographs of SBPC immunocytochemistry preparations of C6/36 cells that were infected by DENV 1, 2, 3, 4 and incubated with 2.2 µg/µL of WDSSB5 primary antibody (incubation days 1, 2 3, and 4) showing brown color (positive) for dengue virus antigen in the cytoplasm of C6/36 cells (magnification of 100 x 10 times). B (2.2 µg/µL), C (1.1 µg/µL), D (0.22 µg/µL), E (0.11 µg/µL). A, B, C, D = incubation days 1,2,3,4 of dengue virus type 1; E, F G H = incubation days 1,2,3,4 of DENV 2; I, J K, L = incubation days 1,2,3,4 of DENV 2; M N, O, P = incubation days 1,2,3,4 of DENV 4.

Figure 4 shows that those WDSSB5 monoclonal antibodies as primary antibodies are capable of recognizing all serotypes of dengue virus. The C6/36 were infected by DENV 1, 2, 3, 4 and incubated for 1-4 days showed a positive reaction (brown cytoplasm). The negative control and C6/36 cells were infected by dengue virus but not given the primary antibody showed blue cytoplasm. At the microscopic observation brown color intensity in C6/36 cell which were incubated 1 to 2 days showed brown cytoplasmic and more cell number, whereas cells which were incubated 3-4 days showed positive dark brown cytoplasmic. In C6/36 cell which were incubated 4 days showed less cell number due to lysis of cells and there are many brown spots around the cells and even the nuclei also appear brown. This is due to a longer incubation time so that the titer of virus was getting higher.

Specificity test of monoclonal antibody using Dot Blot method

The results showed that the WDSSB5 monoclonal antibody showed a positive reaction with the formation of black color against dengue type 1 2, 3, 4 antigens. This test used dengue type 1 2, 3, 4 antigens as a positive control, dengue type 1, 3, 4 are from local isolates, whereas the negative control using PBS. Result of specificity test of the monoclonal antibody test with Dot blot method were presented in Figure 5.

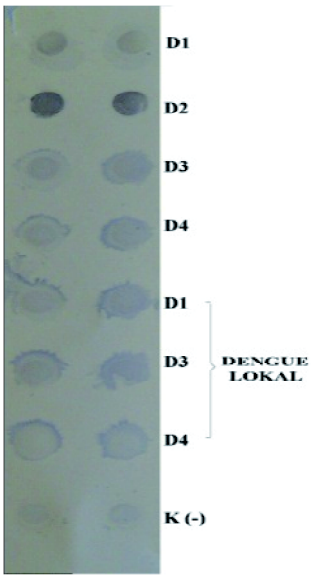

Figure 5. Positive reaction to DENV type 1 and 2 antigens showing black dot color.

Isolation of Dengue Virus from *Toxorrhynchites sp* mosquitoes and C6/36 Cells

Photomicrographs of SBPC immunocytochemistry of mosquito's head squash showing a positive reaction. The results showed that granule brown colored like sand on the cytoplasm of mosquitoes's brain cells which were injected with Dengue virus (Figure 6).

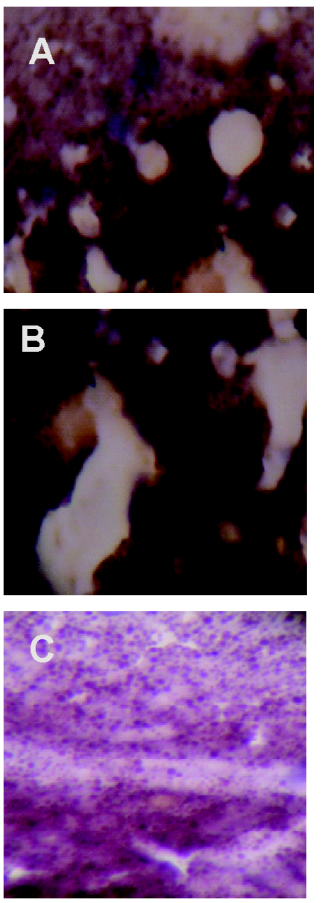

Figure 6. Photomicrographs of SBPC immunocytochemistry of head squash of *Toxorrhynchites sp* mosquitoes which were intrathoracally injected with the serum of patients positive for Dengue virus. A & B showing positive results (brown color cytoplasm). Negative control (C) shows a blue cytoplasm.

Results of virus inoculation in C6/36 cell showed a positive reaction to an infection, the presence of giant cells and cells began to lyse. Photomicrographs of C6/36 cells were presented in Figure 7.

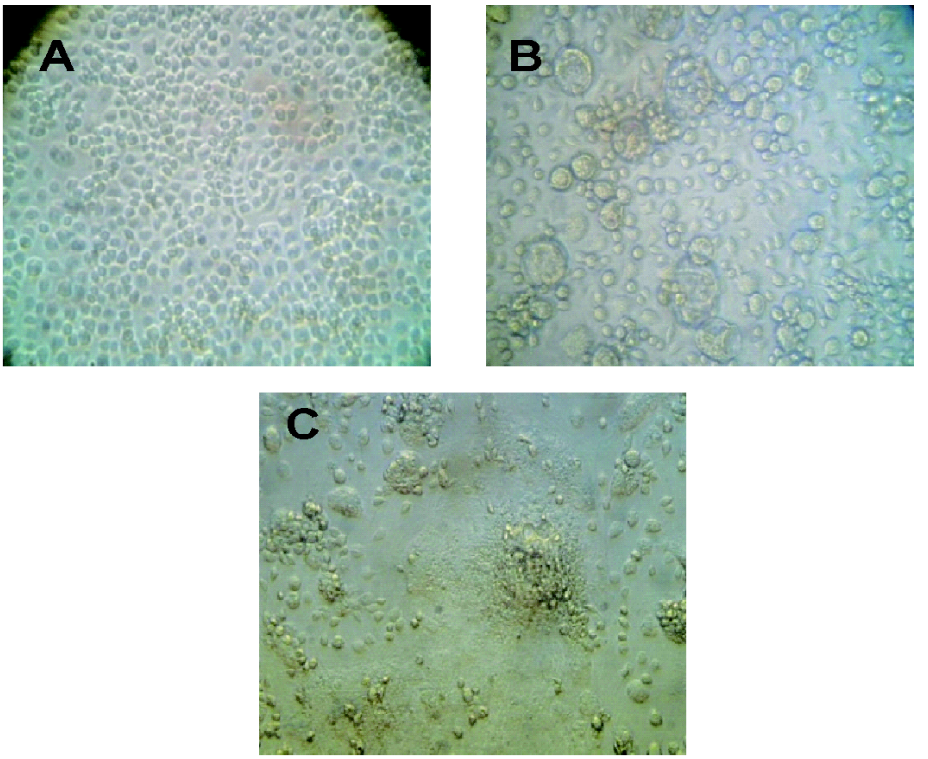

Figure 7. Photomicrographs under an inverted microscope (magnification of 100 x 10 times) show sign of infection. A: the confluent of C6/36 cell which were uninfected by dengue virus; B: the presence of *cytophatic epec* in C6/36 cell which were inoculated with the serum of patients positive for dengue virus; C: C6/36 cell which were inoculated with dengue virus type 4.

Application of WDSSB5 monoclonal antibodies to detect dengue virus from serum of patients positive for dengue virus which were inoculated in C6/36 cell using SBPC immunocytochemistry method

The results of microscopic examination of SBPC immunocytochemistry of C6/36 cells preparations which were derived from the serum of patients are presented in Table 3.

Table 3. Microscopic examination of SBPC immunocytochemistry of C6/36 cells preparations which were derived from the serum of patients

Sample No	C6/36 cell incubation (days)	Fever (days)	Dengue (RT PCR)	SBPC Immunocytochemistry	Positive Rate Mean (%)
1	9	5	1	Positive	100
2	9	4	1	Positive	100
3	11	4	3	Positive	100
4	9	5	1	Positive	100
5	7	5	1	Positive	100
6	10	5	1	Positive	100
7	10	3	1	Positive	100
8	10	1	1	Positive	68.52
9	9	4	3 & 4	Positive	91.62
10	7	4	1	Positive	100
11	10	5	1	Positive	100
12	10	5	1	Positive	91.38
13	9	5	1	Positive	52.01
14	10	4	1 & 3	Positive	91.68
15	9	3	1 & 3	Positive	55.95
16	10	1	1	Positive	90.19
17	10	4	1	Positive	64.38
18	11	4	1	Positive	100
19	9	3	1	Positive	93.07
20	10	5	1	Positive	83.78
21	5	4	3	Positive	61.82
22	5	5	1	Positive	35.29
23	5	7	1	Positive	55.15
24	10	4	1	Negative	0
25	7	5	1	Positive	100
26	5	4	1	Positive	53.36
27	7	4	3	Positive	100
28	7	4	1	Positive	100
29	5	4	4	Positive	84.22

Table 3 shows that with SBPC immunocytochemistry method, the primary antibody WDSSB5 successfully detect dengue antigens on the preparations C6/36 cell which were infected with Dengue virus from serum of patients positive for DENV 1, 3, and 4. Positive levels vary depending on the concentration of dengue virus and time of incubation of C6/36 cell. Of the 29 patients whom serum were inoculated on C6/36 cell, 96.5% showed positive results (28 positive and 1 negative results).

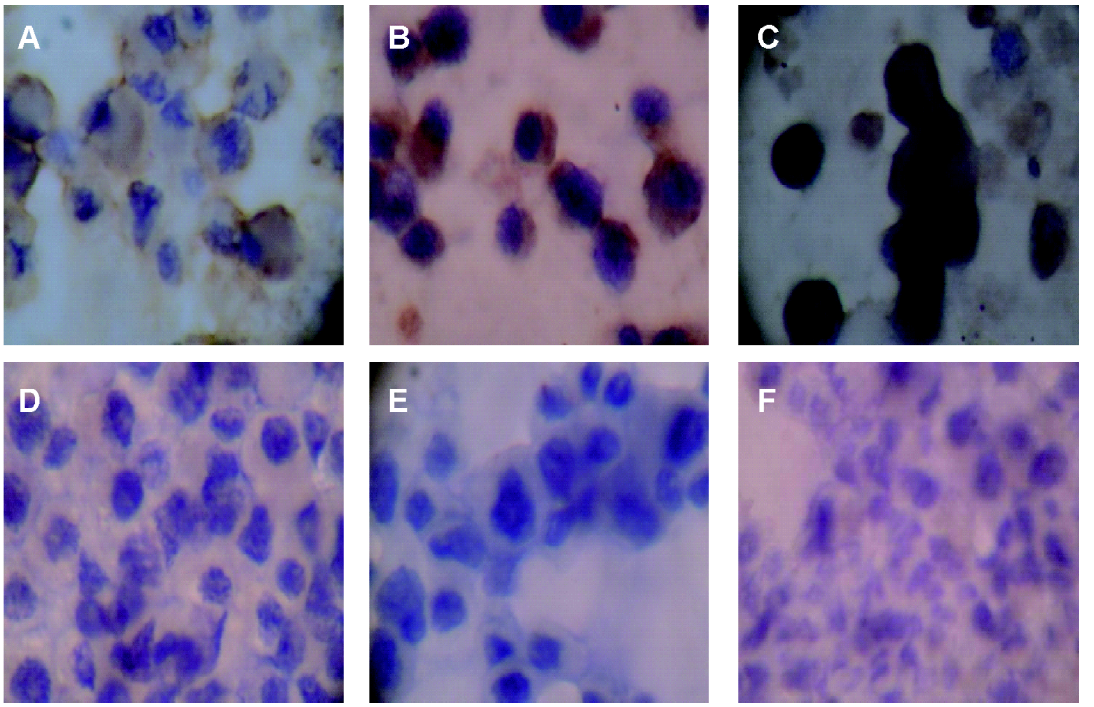

Figure 8. Photomicrographs of SBPC immunocytochemistry of C6/36 cell which were inoculated with serum of patients positive for dengue virus showing a positive reaction (brown cytoplasm). A (positive for DENV 1), B (positive for DENV 1 & 3), C (positive for DENV 3), D (C6/36 cell which were infected by dengue virus showing cell cytoplasm with negative result (blue). E (negative control) showing a blue cytoplasm, F (C6/36 cell which were infected by dengue virus without primary antibody) also showing a blue cytoplasm.

Photomicrographs of SBPC immunocytochemistry of C6/36 cell which were inoculated with serum of patients positive for dengue virus were presented in Figure 8. The results showed a positive reaction indicated by the brown color in the cytoplasm of positive control and C6/36 cell which were infected with dengue virus derived from patient serum. Negative reaction is indicated by the blue color of the negative control, as well as in the cytoplasm of C6/36 cells which were infected by dengue virus and not given the primary antibody when SBPC immunocytochemistry test was performed. In this study, immunocytochemistry examination found C6/36 cells that showed false positive results, namely the cells C6/36 preparation which were not infected by the dengue virus but showed positive results. Nonetheless, preparations which show false positive results can still be distinguished from positive preparations which containing antigens (Figure 9).

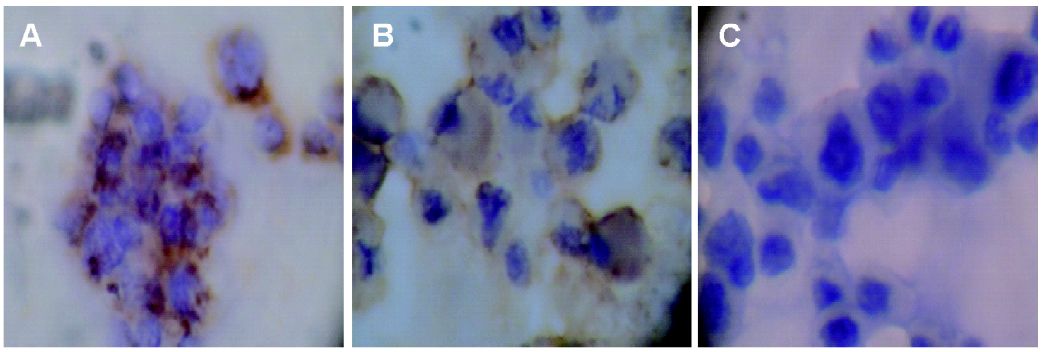

Figure 9. Photomicrographs of SBPC immunocytochemistry of C6/36 cell. A (false positive), B (C6/36 cell which were infected by dengue virus showing cell cytoplasm with positive result (brown), C (negative control of C6/36 cell which were not infected by dengue virus) showed a blue cytoplasm.

The propagation of WDSSB5 hybridoma clones

For in vitro propagation, WDSSB5 hybridoma clones were cultured in RPMI medium containing 20% of FBS. After the cells are in optimal growth, culture was continued using RPMI medium with 10% of FBS. After the growth, recloning process was conducted to yield the producers of hybridoma cells where there is only one cell in each well of a 96-microplate and from one cell derived hybridoma producer clones lines which producing monoclonal antibodies. After the growth of producer of hybridoma cells in a culture medium, subsequent propagation performed in vivo in Balb/c mice which have been sensitized with 0.5 mL of pristan intraperitoneally for 4 times at intervals of 2 weeks. The pristan is an immunosuppressive agent and accelerate the formation of ascites fluid. Then the mice were injected with suspension contains 10^6– 10^7 of producer clones cells per animal. Once abdomen was enlarged, ascites fluid was taken until the it becomes tumor. In this study, the volume of ascites fluid produced was 19.5 mL with twice-tap. This supports previous research conducted by Umniyati *et al.*[10], who have succeeded in producing monoclonal antibodies derived from DSSC7 hybrid cell *in vivo* in Balb/c mice.

According to Goding[17], productions of monoclonal antibodies in vitro and in vivo have both advantages and disadvantages. Concentration of monoclonal antibody *in vitro* culture were about 1-10 µg/mL, whereas the concentration of *in vivo* culture was 1000-fold (mg/mL) than that of *in vitro* culture. Levels of immunoglobulin produced in vitro is more advantageous in terms of purity, as only slightly contaminated with another protein derived from the medium, whereas *in vivo* may be contaminated with other proteins derived from mice. So for the further development of monoclonal antibodies such as monoclonal antibodies labeling, purification is necessary to obtain a pure one.

Characterization of WDSSB5 monoclonal antibodies

Specific character of monoclonal antibody is a major requirement for a variety of immunological reaction techniques. The main properties

of monoclonal antibodies is determined by the structure of the antibody molecule, which is determined by the amino acid sequence, including the presence of class and subclass of an antibody[18].

In this study, the results of classification tests showed that WDSSB5 monoclonal antibody is included in IgG class and IgGI subclass. This result supports research by Sutaryo *et al*[12,19,20], which has successfully produced monoclonal antibodies against DENV 3 derived from DSSC7 hybrid cells. The antibodies included in the IgG1 class and can recognize dengue antigens with the molecular weight of 48 kDa based on epitopes analysis by Western Blotting method.

In this study, we conducted a specificity test of monoclonal antibodies by Western Blotting method, but the results could not be concluded because the epitope was not detected. This is likely due to conformational difference of epitopes. In addition, another possibility is the absence of denaturation prior to SDS transfer, so there is no fragmentation of proteins and after immunoblotting, the antibodies do not recognize epitopes. In this study, to determine the specificity of monoclonal antibodies against dengue antigens, a Dot Blot method was carried out. The drawback is Dot blot method can only know the type of antigen but cannot determine the molecular weight of the protein. This method is good enough to use as a screening a large sample[16].

Results of the protein levels examination by the Biorad micro assay method showed that levels of WDSSB5 ascites protein was 11 mg/mL using Bovin Serum Albumin (BSA) as a standard solution. This proves that the *in vivo* production of monoclonal antibodies yields a high concentration which is 1000 times higher than that of *in vitro*[17]. The weakness in this study was that protein purification is not performed so that the levels of proteins obtained are still crude proteins which influenced by other proteins derived from mice.

In this study, the optimization of protein ascites dilution rate is conducted to establish the smallest levels (highest dilution) of a WDSSB5 monoclonal antibody that detects Dengue antigens optimally based on SBPC immunocytochemistry method. Optimization was performed using C6/36 cells infected by DENV 3 since WDSSB5 hybrid cells is the result of a fusion between splenocytes of immune mice against DENV 3. The results of microscopic examination of SBPC immunocytochemistry (Table 1) showed that 20 mL of monoclonal antibody as the primary antibody with concentration of 2.2 mg/mL successfully detect DENV 3 antigens which were inoculated in C6/36 cells for 4-days incubation with the positive rate of 100%.

Previous studies conducted by Umniyati *et al*[10] using a DSSC7 monoclonal antibodies with 1:10 concentration; 1:50 concentration; 1: 100 concentration as the primary antibody with SBPC immunocytochemical methods to detect dengue virus infection in the abdomen and head squash of *Ae. aegypti* after 5-day incubation yielded positive results of 33.35% and 75%. This study showed better results than those of previous study because of a shorter incubation time.

In this study, the sensitivity and specificity tests of WDSSB5 monoclonal antibodies were conducted by SBPC immunocytochemistry using C6/36 cells which were infected by dengue virus

type 1, 2, 3, 4 with an incubation time of 1-4 days. The results of microscopic examination of SBPC immunocytochemistry (Table 2) showed that the WDSSB5 monoclonal antibody as the primary antibody able to detect dengue type 1, 2, 3, 4 antigens in C6/36 cells and sensitive since 1 day of incubation. This suggests that the WDSSB5 monoclonal antibody as the primary antibody with a concentration of 2.2 ug/mL with SBPC immunocytochemistry method can detect dengue antigens early before the virus life cycle takes place perfectly. Thus, WDSSB5 monoclonal antibodies recognize common epitopes of Dengue antigen. The weakness of this test is that specificity test using C6/36 cells infected by Chikungunya virus was not performed, so the specificity of the Chikungunya virus is not known. However, the immunocytochemistry examination always includes a positive and negative controls.

Dengue virus isolation in the *Toxorrhynchites sp* mosquitos and C6/36 cell lines

Inoculation in the *Toxorrhynchites sp* mosquito aims to increase virus titers in serum, because the virus titers in serum are very low. In addition, to avoid contamination from other microorganisms due to the filtering process of scours mosquitoes, a 0.22 ì filter is used so that all other micro-organisms can be filtered unless the virus. It can prevent contamination at the time of inoculation of C6/36 cells.

In a previous study by Samuel and Tyagi (2006), inoculation performed on *Toxorrhynchites sp* and *Aedes sp* mosquitoes for dengue virus isolation, especially for increasing the viral titer. The density of dengue virus isolation in C6/36 cells was 36%, while the inoculation of mosquitoes were 80%.

In this study, result of inoculation of the virus in C6/36 cells was 96.5% positive. Of the 29 positive serum samples (by RT-PCR), after inoculation of C6/36 cells and microscopic examination of SBPC immunocytochemistry, the results showed that 28 samples were positive and 1 sample was negative. Inoculation of C6/36 cells needs a different incubation time ranged from 5-12 days, it depends on the onset of signs of infection such as the presence of giant cell or cell lysis. In samples that showed a positive reaction, cytophatic are evident, but not for the samples showed a negative reaction.

The C6/36 cells is a clone of cells line derived from *Aedes albopictus* mosquitoes that are most widely used for growing Arboviruses in cell culture media. C6/36 cells proved to have high sensitivity for dengue virus infection, easy to handle, has high stability and has the easy optimum temperature for growth than mammalian cells such as BHK-21, LLC-MK2 and Vero cells[21,22,23].

Previous research by Paula[24] and Osman[25] showed that Dengue virus isolation derived from patient serum in C6/36 cells and the detection of virus were compared using IFAT and RT-PCR methods. Virus detection by RT-PCR takes 1 day, whereas the IFAT requires 4 days after the virus was isolated.

Application of WDSSB5 monoclonal antibodies to detect dengue virus from the serum of patients positively carrying dengue virus which were inoculated in C6/36 cell lines using SBPC immunocytochemistry method.

Detection of DENV 1, 2, 3, 4 isolated from C6/36 cells was first performed by Igarashi[21] by using a fluorescent antibody staining technique. Positively Dengue antigen results as a green

fluorescent. In this study, the detection of DENV 1, 2, 3, 4 antigens was conducted using SBPC immunocytochemistry. Positive results were characterized by the presence of brown color in the cytoplasm of infected cells[15].

Dengue antigen detection by SBPC immunocytochemistry method using C6/36 cells which were infected by dengue virus type 1, 2, 3, 4. The basic principle is the presence of 40 and 45 kDa glycoprotein on the cell surface which is receptor for dengue virus[26]. Dengue virus replication process takes place in the cytoplasm of cells[2]. Dengue virus antigen localized to the cytoplasm of cells will bind to the WDSSB5 monoclonal antibody that will be recognized by biotin-labeled secondary antibody. Furthermore, with the addition of conjugate enzyme-labeled streptavidin radish peroxidase and chromogen substrate solution, the antigen can be detected by the presence of brownish color in the cytoplasm of infected cells[15].

In this study, a WDSSB5 monoclonal antibody as the primary antibody can detect dengue antigens on the C6/36 cells which were infected by dengue virus derived from serum positive for dengue virus in the acute phase of the disease include fever day 1 to day 7. Fever day or day of sampling related to the presence of dengue virus antigen that can be detected by SBPC immunocytochemistry test. According WHO[27], after the onset of the disease dengue virus can be detected in serum, plasma, blood cells and other tissues for 4-5 days. During this phase, virus isolation, nucleic acid detection and detection of dengue antigens can be used for diagnosis of dengue virus infection.

The results of microscopic examination of SBPC immunocytochemistry of C6/36 cells (Table 3) showed that there were 13 positive samples which had an average rate of 100%. Observations results showed a strong positive reaction with the brown color is very clear in the cytoplasm of cells, many giant cells, irregular cell shape, cell nuclei shrink and brown cell nucleus, cell lysis, and there are patches antigen around cell. The positive samples with average rate of 35.29% showed weakly positive reaction with the brown color not so clearly seen. This may be due to the less long incubation time so that less infection occured. At negative sample (T24) with incubation time of ten days, at the time of harvesting the cell culture, there are no obvious symptoms of infection. This is likely due to a less concentration of virus compared to number of *Toxorrhynchites sp* mosquito that successfully injected only one who lives despite repetitions were performed 3 times. The samples with positive results showed more number of mosquitoes that live more than 3-5 mosquito indicating the higher virus titers.

This study found the false-positives result of SBPC immunocytochemistry due to several factors such as the presence of non-specific color reaction, which is caused by the presence of endogenous peroxidase produced by tissues and cells normal[28]. Another factor that can cause a false positive is a non-specific binding occurs between streptavidin with biotin endogenous components contained in cell[28,29]. In this study, we make an effort to minimize the reaction by adding peroxidase blocking solution to eliminate endogenous peroxidase activity, but have not been able to remove endogenous peroxidase. In immunocytochemistry test, the less clean washing factors also affect the occurence of false positive results. However, false positive results

still can be distinguished by the positive results of dengue virus infection.

During this time, SBPC immunocytochemistry test has been developed to detect dengue virus in various organs such as the head squash mosquitoes, abdominal squash, paraffin tissue or on bufficoat also in human blood smears. On the other hand, detection of dengue virus in C6/36 cells is one of the important parts in the development of a monoclonal antibody as a diagnostic tool.

SBPC immunocytochemistry examination can be used as one of the methods of detection of Dengue virus in cell culture. This method proved to have high sensitivity and specificity for detecting antigen of DENV 1, 2, 3, 4 in C6/36 cells. Immunocytochemistry method has the advantage since it uses light microscope and does not require special skills, relatively simple, inexpensive and can be done in a simple laboratory.

CONCLUSION

There was WDSSB5 monoclonal antibody specific of Dengue virus identified. WDSSB5 monoclonal antibody belonged to class IgG and subclass IgG1 and can be applied to detect Dengue virus originating from serum of patients positively carrying Dengue virus inoculated in C6/36 cells using SPBC immunocytochemistry method.

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