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ABSTRACT

Introduction: Rapid test kit based on immunochromatography test (ICT) in detecting dengue NS-1 antigen for early dengue infection is available in the market. Its availability allows earlier management for dengue infected patient but it remains costly to most people. Recently, Dengue Team of Universitas Gadjah Mada has developed monoclonal antibodies to detect the presence of dengue NS-1 antigen in leucocytes of infected patients based on Streptavidin Biotin Peroxidase Complex (SBPC) immunocytochemistry method.

Objectives: The objective of this study is to determine the validity of the immunochromatography (SD Dengue NS1 Ag) method by determining kappa agreement index between two observers, and to compare the diagnostic performances of ICT and immunocytochemistry methods in detecting dengue NS1 antigen in the blood samples.

Methods: A cross sectional study design is used. This study uses 35 blood plasma remains from a previous study conducted on RT-PCR method. Three drops of blood plasma were added into the well of SD Dengue Duo NS1 and results were read after 15-20 minutes. The diagnostic performances of ICT which defined by sensitivity, specificity, positive predictive value and negative predictive value were calculated and compared to secondary data of immunocytochemistry result from the same blood samples, with reference of RT-PCR as a gold standard. A McNemar’s test was conducted and p value less than 0.05 was considered as significant different.

Result: Detection of dengue infection by using SD Dengue NS1 Ag has strong agreements between two observers with kappa value of 1, and the sensitivity of 50%, specificity of 91%, positive predictive value of 92% and negative predictive value of 45% with reference of RT-PCR as a gold standard. Meanwhile sensitivity and specificity value of the immunocytochemistry test were 88% and 100% respectively, and the positive and negative predictive values were 100,0% and 70,0% respectively with reference of RT-PCR as a gold standard. The immunocytochemistry assay showed overall accuracy of 91,0%.

Conclusion: Immunochromatography (SD Dengue NS1 Ag) method to detect NS-1 antigen has less sensitivity and specificity compared to SBPC immunocytochemistry method.

Keyword: Immunocytochemistry, Immunochromatography, Streptavidin Biotin Peroxidase Complex (SBPC), NS-1 Ag, dengue
INTISARI


Tujuan: Tujuan penelitian ini adalah untuk mengetahui validitas immunochromatography metode (SD Dengue NS-1 Ag) antara dua pengamat (nilai kappa), dan untuk membandingkan kinerja diagnostik antara metode ICT dan IHC dalam mendeteksi dengue NS1 antigen dalam darah sampel.

Metode: penelitian ini menggunakan desain studi cross sectional menggunakan 35 plasma darah yang telah dialisana RT-PCR pada penelitian sebelumnya. Tiga tetes darah plasma ditambahkan ke dalam sumuran dari SD Dengue Duo NS1 dan hasilnya dibaca setelah 15-20 menit. Kinerja diagnostik ICT yang didefinisikan oleh sensitivitas, spesifisitas, nilai prediksi positif dan nilai prediksi negatif dihitung dan dibandingkan dengan data sekunder dari hasil IHC dari sampel darah yang sama, dengan mengacu dari RT-PCR sebagai standar emas. Tes McNemar dilakukan dan nilai p kurang dari 0,05 dianggap sebagai perbedaan yang signifikan.

Hasil: Deteksi infeksi dengue dengan menggunakan SD Dengue NS1 Ag memiliki kesepakatanyang kuat antara dua pengamat dengan nilai kappa 1, akan tetapi memiliki sensitivitas 50%, spesifisitas 91%, nilai prediksi positif 92% dan nilai prediksi negatif 45% dengan RT-PCR sebagai standar emas. Sementara itu uji imunositokimia (IHC) menunjukkan sensitivitas 88% dan nilai spesifisitas 100% dengan nilai prediksi positif 100% dan nilai prediksi negatif 70%.

Simpulan: Metode ICT (SD Dengue NS1 Ag) untuk mendeteksi NS-1 antigen memiliki sensitivitas dan sensitivitas di bawah uji IHC dengan metode SBPC.

Kata kunci: imunositokimia, immunochromatography, Streptavidin Biotin Complex Peroxidase (SBPC), antigen NS-1, demam berdarah dengue

INTRODUCTION

Dengue hemorrhagic fever has been the major burden for the world with more than 100 million people infected yearly. Incidence of dengue has increased in a drastic pattern in the last decade all around the world. Around 2.5 billion people, which is about two-fifth of the world population are at risk of dengue infection. There is a current estimation of 50 million dengue infection occurring worldwide.

In the past, diagnosis of dengue hemorrhagic fever is solely based on the clinical symptoms as stated in the criteria set by WHO without further virology confirmation as they were time consuming, thus leading to late diagnosis. Recently, the presence of rapid test kit using immunochromatography method in detecting dengue NS-1 antigen for early dengue infection is available in the market to allow earlier management for dengue infected patient but it still remain costly to most people. To respond this situation, Dengue Team of Universitas Gadjah Mada has developed monoclonal antibodies to detect the presence of dengue NS-1 antigen in leukocytes of infected patient. SBPC immunocytochemistry method to test NS-1 antigen showed 94% sensitivity and 90% specificity when compared to RT-PCR.

New diagnostic test with high sensitivity and specificity will benefit in early management of dengue hemorrhagic fever.

The main objective of the research is to compare the accuracy of diagnostic test of SD Dengue NS-1 Ag (a component of SD Dengue Duo rapid test kit-immunochromatography) with the SBPC Immunocytochemistry method on single sera in detecting NS1 antigen. Other objectives are: (1) to evaluate of the reliability and applicability of both the methods in detecting dengue virus in sub-urban...
setting of Yogyakarta, (2) to evaluate the sensitivity of immunochromatography (SD Dengue NS1 Ag) method and SBPC immunocytochemistry method in detecting NS-1 antigen in different days of fever, and (3) to determine the validity of immunochromatography (SD Dengue NS1 Ag) method by determining kappa agreement index between two observers.

MATERIALS AND METHODS

Sample used in this study was collected in the previous study. Thirty five samples from febrile patients regardless of any diseases, suffering from fever for 1 to 7 days who visited Panembahan Senopati District Hospital in Bantul during January to March 2010 were used in this study. Those samples had been tested with SBPC immunocytochemistry and RT-PCR and were stored in deep freezer at Department of Parasitology, Faculty of Medicine, Universitas Gadjah Mada.

The Dengue Duo Rapid Test Kit contains Dengue NS-1 Ag and Dengue IgG/IgM Combo Device, 10 ìL of capillary pipette and disposable dropper. In the strip included, Gold Conjugates serve as the main component [composing of mouse monoclonal anti-dengue NS1-Gold Colloid (0.27±0.05 ìg)], test line (as main component) contains mouse monoclonal anti-dengue NS1 (0.72±0.14ìg) and the control line (as main component) contains goat anti-mouse IgG (0.72±0.14 ìg).

The test device is removed from the foil pouch and place on a flat, dry surface before 3 drops of blood plasma (about 100ùL) is added into the sample well (S). The test begins to work with the purple color moving across the result window in the center of the test device. The test result is interpreted at 15-20 minutes. A positive result will not change once it has been established at 15-20 minutes. However, in order to prevent any incorrect result, the test result should not be interpreted after 20 minutes.

The results of the rapid test kit ware indicated by the presence of color line at the control line and test line. Positive result is indicated by presence of both control line and test line. Negative result is indicated by presence of only control line. However, result is interpreted as invalid if the control line does not appear as the control line serves as procedural control.

![Positive — Negative](image)

Figure 1. Result Interpretation of SD Dengue NS1 Ag component of SD Dengue Duo rapid test kit (Standard Diagnostics Inc, 2010).

The validity and reliability of the measurement between two observers were evaluated based on kappa values according to Landis and Koch.

Sensitivity, specificity, positive predictive value and negative predictive value from the SD Dengue NS1 Ag component of SD Dengue Duo rapid test kit and both the SBPC immunocytochemistry method will be calculated with RT-PCR as the gold standard. The performance were measured based on Hermann formula.

RESULTS AND DISCUSSIONS

Thirty five plasma samples were tested using immunochromatography test (SD Dengue Duo NS1)
and SBPC immunocytochemistry method. RT-PCR was used as the gold standard reference. Using Immunochromatography test, thirteen samples showed positive and twenty two were negative results. PCR test confirmed 12 true positive and 1 false positive results out of thirteen plasma that diagnosed as positive result by immunochromatography test and confirmed 12 false negative and 10 true negative results out of 22 patients that tested negative by immunochromatography test (Table 1).

Using Immunocytochemistry (SBPC NS1), twenty one samples showed positive results and fourteen were negative. PCR test confirmed all 21 samples that diagnosed as positive result by Immunocytochemistry (SBPC NS1) and confirmed 3 false negative and 11 true negative out of 14 patients that tested negative by immunocytochemistry test (Table 2).

The samples were used to evaluate the performance of the immunocytochemistry assay in the thick blood smear in terms of sensitivity, specificity, positive predictive value, negative predictive value and the overall accuracy for the detection of dengue antigen in the cytoplasm of...
leucocytes, compared to the secondary data that had been obtained by RT-PCR method as a gold standard. The sensitivity and specificity value of the assay were 88% and 100% respectively. The positive and negative predictive values of the assay were 100,0% and 70,0% respectively. The immunocytochemistry assay showed overall accuracy of 91,0%.

Table 3. Comparison of sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of immunochromatography (SD Dengue NS1 Ag) and immunocytochemistry (SBPC) method in detecting dengue NS-1 antigen with RT-PCR as gold standard.

<table>
<thead>
<tr>
<th></th>
<th>IMMUNOCHROMATOGRAPHY</th>
<th>IMMUNOCYTOCHEMISTRY</th>
</tr>
</thead>
<tbody>
<tr>
<td>SENSITIVITY</td>
<td>0.9</td>
<td>0.98</td>
</tr>
<tr>
<td>SPECIFICITY</td>
<td>0.91</td>
<td>1</td>
</tr>
<tr>
<td>PPV</td>
<td>0.92</td>
<td>1</td>
</tr>
<tr>
<td>NPV</td>
<td>0.45</td>
<td>0.79</td>
</tr>
</tbody>
</table>

Table 3 showed that immunochromatography test (ICT) is less sensitive and specific for detection dengue NS-1 antigen compared to the immunocytochemistry assays. It also showed that the positive and negative predictive values were lower than immunocytochemistry assay. However, no significant statistical differentiation is found based on McNemar test (P=0.77; > 0.05) as shown in Table 4.

Table 4. Tabulation of Immunocytochemistry (SBPC NS1) Result against Immunochromatography (SD Dengue NS1 Ag) Result.

<table>
<thead>
<tr>
<th></th>
<th>IMMUNOCYTOCHEMISTRY</th>
<th>IMMUNOCHROMATOGRAPHY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>POSITIVE</td>
<td>NEGATIVE</td>
</tr>
<tr>
<td>IMMUNOCHROMATOGRAPHY</td>
<td>9</td>
<td>4</td>
</tr>
<tr>
<td>POSITIVE</td>
<td>12</td>
<td>10</td>
</tr>
<tr>
<td>TOTAL</td>
<td>21</td>
<td>14</td>
</tr>
</tbody>
</table>

*McNemar Test: p-value=0.77 (p > 0.05)

Table 5. Comparison of Sensitivity of Immunochromatography (SD Dengue NS1 Ag) and Immunocytochemistry (SBPC NS1) Method in Detecting NS-1 antigen on Different Day of Fever with RT-PCR as reference.

<table>
<thead>
<tr>
<th>DAY OF FEVER</th>
<th>ICT</th>
<th>IMMUNOCYTOCHEMISTRY</th>
<th>RT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>POS</td>
<td>%</td>
<td>NEG</td>
<td>%</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>1</td>
<td>10.10</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
<td>25</td>
<td>11</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>20.83</td>
<td>4</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>8.33</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>n</td>
<td>n</td>
<td>7</td>
</tr>
<tr>
<td>TOTAL</td>
<td>13</td>
<td>54.17</td>
<td>22</td>
</tr>
</tbody>
</table>

Note: ICT = immunochromatography test. POS= Positive. NEG= Negative
Table 5 showed that dengue NS1 antigen was detected in the plasma of patient in the 4th day of fever to the 6th day of fever based on immunochromatography test (ICT), but it was detected in the 1st day of fever to the 7th day of fever based on the immunocytochemistry assay in the thick blood smear and RT-PCR in the whole blood of patient.

Table 6 showed the strong agreement between two observers to detect dengue NS1 antigen in the sera of patient.

Acute dengue virus infection is important to be detected earlier through a laboratory examination to provide appropriate management and early public health control of dengue outbreak. At present, the three basic methods used by most laboratory for diagnosing dengue virus infection are virus isolation and identification, detection of viral genomic sequence by nucleic acid amplification assay (RT-PCR) and detection of dengue virus-specific IgM antibodies by IgM- capture enzyme-linked immunosorbent assay (MAC-ELISA) and/or rapid dengue immunochromatographic test for dengue specific IgM (DIT). Virus isolation and characterization was considered as the gold standard of laboratory diagnosis of acute dengue virus infection, it is however expensive and time consuming in detection as it requires at least 6-10 days for the virus to replicate in tissue culture cells or laboratory mosquitoes (adult or larvae). The current gold standard laboratory diagnosis is by reverse transcriptase-polymerase chain reaction (RT-PCR)³.

This method is also an expensive method and is not widely available in most hospital diagnostic laboratories. Assay of anti-dengue specific IgM is dependent on the time taken for infected person’s immune response to produce IgM antibodies against dengue virus antigens. Hence, both DIT and MAC-ELISA do not provide accurate information on early diagnosis of acute dengue because IgM is commonly firstly detected only on day 4-5 of illness in most cases. Besides, single serological detection of IgM merely indicate recent dengue virus infection and it should not be interpreted as a diagnosis of an acute infection without a paired second serum sample. Hence, a rapid test kit is particularly useful in providing early diagnosis of acute dengue virus infection⁷.

The main advantage of using SD dengue duo rapid test kit is the time taken for the procedures to be carried out and the result interpretation is less than half an hour. Besides, it is a combination of both NS-1 antigen detection and differential IgG/IgM antibodies to dengue virus to human blood detection. NS-1 antigen is generally detected during Day 1 and up to Day 9 after onset of fever. Detection of NS-1 is however inhibited if anti-NS1 antibodies

<table>
<thead>
<tr>
<th>OBSERVER</th>
<th>POSITIVE</th>
<th>NEGATIVE</th>
<th>TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>OBSERVER 1</td>
<td>POSITIVE</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>OBSERVER 1</td>
<td>NEGATIVE</td>
<td>0</td>
<td>22</td>
</tr>
<tr>
<td>TOTAL</td>
<td>13</td>
<td>22</td>
<td>35</td>
</tr>
</tbody>
</table>

OBSERVED AGREEMENT (Po) = (13 + 22)/35 = 1.00, CHANCE AGREEMENT (Pe) = [(13/35) * (13/35)] + [(22/35) * (22/35)] - [(0.37 * 0.37) + (0.62 * 0.62 - 0.1369 - 0.3844) = 0.3213, KAPPA VALUE = (1 - 0.5213)/(1 - 0.5213) = 1
are present. IgM, as mentioned above become detectable by Day 3 to Day 5 after onset of illness in primary dengue and by Day 1 to Day 2 after onset of illness in secondary infections. 

However, in this study, only SD Dengue NS1 Ag component of the SD Dengue Duo rapid test kit is being evaluated and compared with SBPC immunocytochemistry method in diagnosis of dengue infections. NS-1 antigen detection in SD Dengue NS1 Ag rapid test kit has a sensitivity of 50%, which is significantly lower as compared to recent study. On the other hand, immunocytochemistry (SBPCNS1) has a sensitivity of 88% and specificity of 100%, in which it compares well with recent study. 

During testing with McNemar test, the result showed that the probability of change in sensitivity in both tests was not significant. Therefore, there is no significant tendency of change in terms of sensitivity that may occur. The result proves that the sensitivity of both SBPC immunocytochemistry and immunochromatographic methods using SD Dengue NS1 Ag component of SD Dengue Duo rapid test kit has insignificant tendency to changes by chance. 

In the comparison of result in terms of day of fever of patient, it was observed that the highest sensitivity of SD Dengue NS1 Ag is at day 5 days, where the result is significantly good as 5 out of 8 positive samples were detected as positive and the accuracy compared to the total positive sample is 20.83%. Sample taken from patient suffer from fever for 4 days has highest percentage of positive detection with accuracy of 25% of the total positive sample. This does not show the sensitivity of test is highest at day 4 due to the total positive sample at day 4 of fever is 12, but the number of sample detected as positive is only 6. Thus, the accuracy at day 4 as compared with RT-PCR is only 50%. 

For SBPC immunocytochemistry method, the highest sensitivity and specificity is on day 4 where all positive samples were detected as positive and has accuracy of 50% of total positive samples. All negative samples were also detected as negative with accuracy of 45.45% of total negative sample. On day 5, the sensitivity of SBPC immunocytochemistry is similar to SD Dengue NS1 Ag where 5 out of 8 positive samples were detected as positive and the accuracy is 20.83% when compared to total positive sample. 

Day 1, 2, 3, 6 and 7 of fever are however not able to be evaluated well as the samples are less than 3 on those day. Therefore, it does not reflect the actual accuracy on those days because the small number of sample on those day will lead to bias of interpretation. 

In a previous review, it was stated where the NS1 antigen detection decreases with increase of days of fever as the time progress, patient will develop antibody against NS-1 antigen. NS-1 antigen detection will also decrease when patient is encountering secondary infection as IgG in patient body will rapidly increase upon second exposure to dengue virus and it will form pre-existing virus-IgG immunocomplexes in the serum of patient. This leads to lower concentration of NS1 antigen present in secondary infection and hence directly compromising sensitivity of test by detection of NS1 antigen.

Previous study supported our finding that NS1 antigen detection decreases with increase day of fever. The sample size is also significantly larger than that of this study in evaluating the sensitivity of NS1 antigen detection at different day of fever. 

From the data that was obtained in this study, it could be seen that the main advantage of SBPC immunocytochemistry is the high sensitivity, specificity, positive predictive value and negative predictive value as compared to SD Dengue NS1 Ag. Despite its high diagnostic significance, SBPC immunocytochemistry is a very complex method in detection of NS1 antigen as the reagents used in this method are not able to be prepared earlier. They can only be prepared during the period of testing. Besides, expertise is required to observe the color change of monocytes and lymphocytes.
Immunochromatography (SD Dengue NS1 Ag), on the other hand, is simple to be conducted as the sample that can be used are serum, plasma or whole blood. No training is required to perform this rapid test because only drops of sample were added into the sample well and interpretation is based on the presence or absence of color line in the kit. The total time needed to perform this test is also very short, approximately only 20 minutes is required to obtain the result of detection.

The major limitation in the research conducted is the sample used in the experiment is stored in freezer at the temperature of -80 °C for duration of almost 2 years. There were no previous study regarding the stability of NS-1 antigen in serum at such temperature, thus there is a possibility where the duration of storage may influence the structure of NS1 antigen, leading to low sensitivity. The second limitation is the indication for usage of frozen specimen is not clearly stated. The kit instruction only mentioned that frozen specimen should be brought to room temperature prior to use but there was no clear instruction or indication on the optimal temperature that should be achieved in the specimen before tested. The next is the small sample size that was used in this study. The samples that were tested were only enough to evaluate the sensitivity as compared to RT-PCR and its tendency to changes as compared to SBPC immunocytochemistry. The sensitivity on different days of fever is however not able to be established as the samples on fever day 1, 2, 3, 6 and 7 are subjected to interpretation bias because the samples tested were limited in number.

The forth limitation in the data of SBPC immunocytochemistry and RT-PCR are based on previous studies conducted with the same sample used. The procedures of SBPC immunocytochemistry and RT-PCR were not conducted in this research. The last limitation is the study only evaluate the sensitivity and specificity of SD Dengue NS1 Ag and it does not reflect the actual sensitivity and specificity of the SD Dengue Duo rapid test kit as the IgG/IgM component of the SD Duo rapid test kit was not evaluated.
CONCLUSION

This study concludes that immunochromatographic method of NS1 detection using SD Dengue NS1 Ag yields significantly lower sensitivity as compared to SBPC immunocytochemistry method. Evaluation of sensitivity of SD Dengue NS1 Ag and SBPC immunocytochemistry on different day of fever is not able to be performed due to small sample size.

SUGGESTIONS

The combination use of both components in the SD Dengue Duo rapid test kit is needed to diagnose dengue virus infection more accurately as the sensitivity and specificity increases when both IgG/IgM component and NS-1 component were used. Larger sample size is needed in the next study to evaluate the sensitivity of test kit in different day of fever in patient as minimal sample size will not able to yield significant result. Further evaluation of NS1 antigen stability in frozen plasma is needed. Current study does not provide sufficient data to support the assumption of low temperature influence to stability of NS1 antigen. Streptavidin Biotin Peroxidase Complex Immunocytochemistry method in diagnosing acute dengue virus infection has huge potential for future use as it has very high diagnostic value significance and the method is more cost-efficient as compared to other commercial diagnostic tools available in the market.

REFERENCES

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