THE WIDAL SLIDE AGGLUTINATION TEST (SAT) USING ANTIGEN FROM LOCALLY PREVALENT Salmonella typhi AS A DIAGNOSTIC TOOL FOR TYPHOID FEVER

Indro Handojo, SP Edijanto, Endang Retnowati, Sophia Yudaprawira Salim

ABSTRACT

A laboratory study to assess the diagnostic value of Widal slide agglutination test (SAT) using antigens from 5 locally prevalent phage-types of S. typhi, was carried out on 85 sera, comprising 45 sera from typhoid fever adult patients with a positive blood culture, and 40 sera from non-typhoid febrile patients. The patients entered into the study were inpatients of the Tropical Disease Ward of the Dr. Soetomo Hospital and outpatients of Gotong Royong and Waluyo Jati Clinics in Surabaya during January 2001-January 2002. All sera were tested by Mekar Jaya Diagnostika Widal Slide Agglutination Test (SAT) using a mixture of 5 phage-types of locally prevalent S. typhi as the antigens. To obtain the optimal dilution for the determination of the antibody titre in the sera, a list of the ratio of serum and phosphate buffer saline solution (PBS) was used as recommended by the manufacturer. Incubation was carried out at room temperature for only 5 minutes. The results of the test were read with the naked eye above a 10 Watt neon light. The cut off value of the above mentioned Widal slide agglutination test in adults was assessed as a titre of 1/80 for O and H agglutinin, 1/40 for PA agglutinin and 1/160 for PB agglutinin. The single O agglutinin titre of ≥ 1/160 or combined O and H agglutinin titres of ≥1/160 or a fourfold increase of the agglutinin titres within 5 – 7 days, were found to be justifiable for the establishment of the diagnosis of the disease using the Widal SAT-MJD. The results of the study revealed that the Widal SAT-MJD is an eligible tool to detect typhoid fever in adults with a diagnostic sensitivity as high as 82.22%, a diagnostic specificity as high as 82.5%, a diagnostic efficiency as high as 82.35%, a diagnostic positive predictive value as high as 84.09% and a negative predictive value of 80.49%. From the practical point of view, the application of this Widal slide agglutination test can be considered as very practicable, as the incubation period is less than 5 minutes and the cost is far from expensive. Based on the data obtained in this study, it can be concluded that the Widal SAT-MJD has a high diagnostic value and is very practicable to be applied as a screening test for the diagnosis of typhoid fever.

Keywords: typhoid fever, Widal slide agglutination test- locally prevalent S. typhi

INTRODUCTION

Typhoid fever is an endemic infectious disease, which continues to be a serious public health problem in Indonesia. The incidence rate of typhoid fever in Indonesia is still high, especially in the age group of 3 to 19 years i.e 78% of all typhoid cases in Indonesia are found in this special group, a community which mainly consists of school children (Simanjuntak, 1990). The Health Department of the Republic of Indonesia reported that the incidence rate of the disease increased from 9.2 in 1990 to 15.4 in 1994 per 10,000 at the population (Muliawan, 2000). The national case fatality rate of hospitalized patients was found to be 2-5% (Widodo, 1999). Resistance to anti-typhoid drugs also tends to be on the increase (Prihatini, 1982).

Immuno-vaccination using anti-typhoid vaccine as a strategy to control the disease in Indonesia, appeared to be of no avail and environmental sanitation has to be considered as disappointing. Thus, case finding and contact tracing followed by adequate anti-microbial treatment appear to be the mainstay in the fight against the disease in Indonesia. For the purpose of case finding, a reliable, practicable and cheap diagnostic tool is of crucial importance (Handojo, 2000).

Confirmation of the diagnosis of typhoid fever can only be made through isolation of S. typhi in blood or bone marrow (Tsang, 1992). This type of laboratory test, though very specific, is still far from satisfactory, as case- yield based on the finding of positive isolates varies from 40%-78% (Prihatini, 1982; Baron, 1990; Tsang, 1992). The relatively low degree of practicability of the blood culture constitutes the other side of the coin. The PCR (Polymerase Chain Reaction) test, though very sensitive and specific, remains to be an expensive laboratory test with a low degree of practicability.

To date, conventional Widal test is still widely used to approach the diagnosis of typhoid fever because it can be easily carried out. Another advantage is that the test is considered a very inexpensive test. However, the
sensitivity as well as specificity of the conventional Widal test remains doubtful (Verdugo – Rodrigues et al, 1983; Bidasari, 1990; Moehario et al, 1995), especially for endemic areas such as in Indonesia.

On the other hand, other investigators (Suwahyo, 1979; Setyawati, 1997) who made use of antigens obtained from S. typhi that were locally prevalent, showed significantly (p<0.05) higher diagnostic sensitivities and specificities when compared to other Widal tests using antigen materials obtained from imported strains or from other strains or phage-types of S. typhi which were not locally prevalent.

Recently, in Indonesia many laboratories use imported Widal slide agglutination tests to establish the diagnosis of typhoid fever. However, many clinicians are not satisfied with the frequent findings of false positive as well as false negative results. The conventional Widal tube test, which makes use of locally prevalent antigen, also gives unsatisfactory results. Poor standardization of the antigens and improper use of examinations without prior determination of the cut off value in the related endemic areas needed for establishing the diagnosis of the disease may account for the findings of the abovementioned unsatisfactory results.

The above mentioned shortcoming opened the way for a further study with the aim of evaluating the diagnostic value of Widal Slide Agglutination Test (SAT) which, makes use of a mixture (in equal proportions) of 5 locally prevalent phage-types of S. typhi as the antigens, standardized carefully by Mekar Jaya Diagnostika Research Laboratory in Indonesia, using cut off values which have been determined from 129 healthy individuals in endemic areas in Indonesia.

### MATERIALS AND METHODS

This laboratory study was performed on sera obtained from 85 adult patients, comprising 45 patients with typhoid fever (positive blood culture for S. typhi) and 40 non-typhoid febrile patients (negative blood, urine and stool culture for S. typhi) who attended the outpatient Gotong Royong and Waluyo Jati Clinics and inpatients who were hospitalized in the Tropical Disease Ward of the Dr. Soetomo Hospital in Surabaya.

In the list of non-typhoid diseases with fever, 13 patients with malaria (positive blood smear), 17 patients with dengue fever (primary as well as secondary) and 10 patients with urinary tract infection were entered.

The population under study consisted of adults (male and female) who were older than 15 years at the time of entrance into the study, showing fever for more than 7 days and had signed the informed consent. None of the patients were under treatment with corticosteroids or other immunosuppressive drugs during the previous month, and did not suffer from diseases that could interfere with the generation of the humoral immune response. Besides this, they did not suffer from malnutrition.

Sera obtained from the population under study were tested with the Widal Slide Agglutination Test (SAT) produced by Mekar Jaya Diagnostika (MJD) Research Laboratory, following the instructions given by the manufacturer of the kit. The antigen used for the Widal Slide Agglutination Test (SAT-MJD) is a mixture of an equal quantity of antigens obtained from 5 different phage-types of S. typhi that are locally prevalent. To obtain antibody titre in the test sera, optimal serum dilution has to be carried out. A list of the ratio of serum and phosphate buffer saline solution (PBS) made available by the manufacturer of the test kit, can be seen in table 1.

<table>
<thead>
<tr>
<th>Titre</th>
<th>Ratio of Serum</th>
<th>Ratio of Phosphate Buffer Saline</th>
<th>Ratio of Antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/ 20</td>
<td>10 µL</td>
<td>30 µL</td>
<td>40 µL</td>
</tr>
<tr>
<td>1/ 40</td>
<td>7 µL</td>
<td>34 µL</td>
<td>40 µL</td>
</tr>
<tr>
<td>1/ 80</td>
<td>5 µL</td>
<td>35 µL</td>
<td>40 µL</td>
</tr>
<tr>
<td>1/160</td>
<td>4 µL</td>
<td>36 µL</td>
<td>40 µL</td>
</tr>
<tr>
<td>1/320</td>
<td>3 µL</td>
<td>38 µL</td>
<td>40 µL</td>
</tr>
</tbody>
</table>
WIDAL SAT-MJD Test Procedure

In this study Widal SAT-MJD was performed on an object glass with a concavity at its center. For the O and H agglutinins, serum dilution begins with the titer of 1:40, for paratyphi B (PB) agglutinin serum dilution begins with the titer of 1:80, and for paratyphi A (PA) agglutinin serum dilution begins with the titer of 1:20. The diluted sera as shown in table 1, were mixed with 40 µl of antigen suspension (O, H, PA, PB) using an applicator, and the object glass was afterwards rotated gently for 5 minutes at room temperature. The result of the test (agglutination) was read with the naked eye above a 10 Watt neon light or with the aid of sunrays near a window. Each slide was read by 3 laboratory technicians and the reported end results were those approved by at least 2 of the 3 readers. However, if the Widal SAT-MJD showed a negative result of examination, the test has to be terminated, and the result reported as negative.

Each run of examination must be accompanied by positive and negative control sera (enclosed in the kit). For the performance of the Widal SAT-MJD, these control sera were diluted twofold of the cut off value of the test. The positive and negative control sera should give a positive and negative result. If there is a discrepancy in the result of the control sera, this run of examination has to be repeated.

As stated in the leaflet enclosed in the kit, the cut off value of the Widal SAT-MJD in adults as based on the test results of 129 healthy individuals in East Java was determined as the following:

1. for O agglutinin, corresponding with the titre of 1:80
2. for H agglutinin, corresponding with the titre of 1:80
3. for PA agglutinin, corresponding with the titre of 1:40
4. for PB agglutinin, corresponding with the titre of 1:160

Blood culture in bile-broth media, urine culture and stool culture were done according to the standard procedures of the Microbiology Division, Department of Clinical Pathology, Dr. Soetomo Hospital in Surabaya. The diagnostic criterion of the Widal SAT-MJD test for typhoid fever used in this study (Setyawati, 1997) were as follows:

1. If the titre of O agglutinin or O and H agglutinin was/were equal or higher than 2 times their cut off value (≥ 1:160).
2. If within an interval of 5 – 7 days, there was a fourfold increase of the agglutinin titres.

In the group of patients with non-typhoid fever, the result of the Widal SAT-MJD was considered as false positive if the titre of O agglutinin, O and H agglutinins, PA agglutinin or PB agglutinin were equal or higher than 2 times their cut off values. The diagnostic value of Widal SAT-MJD was assessed based on the determination of the diagnostic sensitivity, the diagnostic specificity, the diagnostic predictive value, and the negative predictive value. A positive result of blood culture served as the gold standard for the confirmation of typhoid fever in this study.

RESULTS

The results of the study were summarized in table 2.

Table 2. The Results of Widal SAT-MJD in 45 Patients with Typhoid Fever and 40 patients with Non – Typhoid Fever.

<table>
<thead>
<tr>
<th>Type of the Disease</th>
<th>Na</th>
<th>Results of Widal Sat-Mjd</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Positive</td>
</tr>
<tr>
<td>Typhoid Fever</td>
<td>45</td>
<td>37</td>
</tr>
<tr>
<td>Non-Typhoid Fever</td>
<td>40</td>
<td>7</td>
</tr>
</tbody>
</table>

NA : Number of patients (typhoid as well as non-typhoid)
NB : Number of patients with positive Widal SAT-MJD
NC : Number of patients with negative Widal SAT-MJD

As shown in table 2, out of the 45 patients with typhoid fever admitted to this study, 37 patients (82.22%) showed positive Widal SAT-MJD. The diagnostic sensitivity of the Widal slide agglutination test produced by Mekar Jaya Diagnostika Research Laboratory in this study was found to be 82.22%.
Out of the 8 typhoid fever patients showing false negative Widal SAT-MJD, 2 patients (4.44%) showed only positive results of the H agglutinin (titres of 1/320), and thus cannot be considered as having typhoid fever when based on the diagnostic criterion of Widal SAT for typhoid fever.

In the group of nontyphoid fever patients, 33 of the 40 patients (82.5%) had a negative Widal SAT-MJD. The diagnostic specificity of the Widal SAT-MJD in this study was thus 82.5%. In this study, out of the 7 patients with nontyphoid fever showing a false positive Widal SAT-MJD , 5 patients (2 with dengue hemorrhagic fever, 2 with urinary tract infection and 1 with malaria) showed false positive O agglutinin or O and H agglutinins (with a titre ≥ 2 times the cut off value), 2 patients (one with malaria and another one with urinary tract infection) showed false positive results of the PA agglutinin but none of the patients showed false positive results of the PB agglutinin.

In this study, the diagnostic efficiency of the Widal SAT-MJD was thus 82.35%, the diagnostic predictive value was 84.09% and the negative predictive value was 80.49%.

**DISCUSSION**

The results of this study revealed that the diagnostic value of Widal SAT produced by Mekar Jaya Diagnostika (MJD) Research Laboratory, in accordance with the criterion of Handojo (1988) was classified as high (80 – 95%). The antigen of the Widal SAT-MJD was believed to be the main factor accountable for the high diagnostic sensitivity and specificity of the test. The mixture (in equal quantity) of antigens derived from 5 different phage-types of *S. typhi*, produced a broad spectrum antigen for the Widal SAT-MJD, giving rise to the achievement of a higher degree of diagnostic sensitivity, while the locally prevalent phage-typhes of *S. typhi* used in this test are presumably accountable for the achievement of a high degree of diagnostic specificity of the antigen for the Widal slide agglutination test (SAT).

As stated above, patients with typhoid fever admitted to this study had positive blood culture of *S. typhi*, which indicates that they were still in the early stage of the disease (the first week). It could therefore be expected that antibody production against *S. typhi* was still at an initial stage and the levels of these antibodies were relatively low compared with those found during the second week (Hisch et al, 1985; Tandra, 1986).

If these patients were examined during the mid-stage of the disease, i.e. in the second week of the disease, it can be anticipated that the diagnostic sensitivity of the Widal SAT-MJD would be higher than that obtained in this study.

However, 2 out of the 8 patients suffering from typhoid fever with false negative Widal SAT-MJD (O agglutinin), had positive results for H agglutinin. It is enticing to speculate that these 2 patients have been exposed to a low dose (subclinical) of infection with *S. typhi*, thereby resulting in the generation of memory cells to the H antigen of *S. typhi* (T-cell dependent). Thus, if these patients fell ill due to a secondary infection with *S. typhi*, the immune response generated against the invading *S. typhi* is inherent to the pattern of a secondary immune response.

It is worth to note that the O antigen or lipopolysaccharide belongs to the T-cell independent antigen which has the ability to stimulate directly B-lymphocytes without the help of T- lymphocytes for the production of O agglutinin. It goes without saying that in primary infection with *S. typhi*, O agglutinin will be produced earlier than H agglutinin, resulting in a higher titre of the O agglutinin when compared with the titre of H agglutinin in the first week of illness.

On the other hand, in secondary infection with *S. typhi*, the production of O agglutinin and H agglutinin took place in approximately the same rate on account of the presence of memory cells to the H antigen of *S. typhi* in these patients.

The fact, that the titre of H agglutinin in the sera of individuals infected with a subclinical dose of *S. typhi* can be maintained for a longer period of time (± 2 years) compared to the O agglutinin (± 5 months) implies the possibility that the concentration of H agglutinin in the sera of some patients can increase to a titre above its cut off value within a shorter period of time than the O agglutinin following manifestation of the disease after secondary infection. The use of locally prevalent phage – types of *S. typhi* as the antigen of Widal SAT-MJD is believed to be accountable for the high degree of diagnostic specificity (82.5%) of this test.

It is a common knowledge that the affinity of the antigen for homologous antibody is significantly higher than for the heterologous antibody. Thus, as the antigens used in this test are derived from 5 different phage-types of locally prevalent strains of *S. typhi*, it can be anticipated that the degree of diagnostic sensitivity as well as of diagnostic specificity of the test...
must be high. Evidence supporting this hypothesis was reported by Suwahyo (1979) and by Setyawati (1997) who had obtained significantly higher (p < 0.05) diagnostic sensitivity and specificity of the Widal test which made use of antigens obtained from locally prevalent phage – types of S. typhi when compared to other Widal tests using antigens which was obtained from imported strains.

The method of keeping the stock of S. typhi for the production of antigens for the Widal SAT-MJD also plays an important role in the maintenance of quality and the stability of the antigen. According to the manufacturer of the kit, the stock of S. typhi for the production of antigens for the Widal SAT-MJD is kept at a temperature where genetic mutation of S. typhi could possibly be prevented. It is important to note that genetic mutation can easily occur in S. typhi if it is not properly stored; this may decrease the sensitivity as well as the specificity of the Widal test.

According to Thong (1995), the genetic diversity rate between the strains of S. typhi from Indonesia, Malaysia and Thailand was approximately 15%. In Indonesia, it is presumed that there are 5 to 6 phage types of S. typhi with a genetic diversity rate as high as 40% (Thong, 1995). The high mobility of the population in Indonesia was observed during the previous two decades; this enhanced the similarity of the different phage- types of S. typhi prevalent in several endemic areas in Indonesia.

Determination of the cut off value of the Widal slide agglutination test in an endemic area in countries like Indonesia, which has a significant influence on the degree of the reliability of the Widal test is another point to be given due consideration. As stated in the leaflet of the imported Widal slide agglutination test enclosed in the test kit, the cut off value of the above mentioned test is fixed at a titre of 1:80 for the O, H, PA as well as for the PB agglutinin. This cut off value was determined based on examination of healthy population in the country where the kit was produced.

The aforementioned cut off value is not always eligible to be used in Indonesia since the HLA of the Indonesian people differs from the HLA of the nation of the country where the kit was made. Besides this, the strains or phage – types of S. typhi used to produce the antigens of the imported test kit are not always the same as those prevalent in Indonesia. Moreover, the degree of endemicity of typhoid fever in Indonesia is not the same as that of the country where the imported test kit is made. This finding was furthermore strengthened by the fact that a lot of clinicians were unsatisfied with the results of tests using imported Widal slide agglutination test.

In this study the diagnostic specificity of The Widal SAT-MJD can be classified as high (82.5%). Out of the 7 patients suffering from non-typhoid fever with false positive Widal SAT-MJD, 5 patients had false positive results for the O or for the O and H agglutinins. This implies that only 5 patients (12.5%) had a true false positive result for typhoid fever. The remaining 2 patients (5%) had false positive results for the paratyphoid A fever.

It is worth to note that out of the 5 patients suffering from non-typhoid fever with a false positive test result, 2 patients (5%) suffered from dengue hemorrhagic fever. The rationale behind this finding must be based on the fact that in viral infections such as dengue hemorrhagic fever, polyclonal B- cells activation may occur (Pang, 1996). Infection with a subclinical dose of S. typhi has the capacity to stimulate B lymphocytes or plasma cells, activated by dengue virus, to produce O agglutinin or O and H agglutinins to a titre above the cut off value. It has to be kept in mind that dengue hemorrhagic fever is a disease that has to be differentiated from typhoid fever, but both diseases may occur concomitantly. However, only 2 among the 17 patients (5.9%) with dengue hemorrhagic fever reported here, showed false positive results.

Out of the 10 patients under study suffering from urinary tract infection (with positive urine culture for E.coli), 2 patients (20%) showed false positive result for typhoid fever and 1 patient (10%) for paratyphoid fever. The antigen of the Widal SAT test using LPS (Lipopolysaccharide), consisting of O polysaccharide, core and lipid A. The O polysaccharide is species specific but the core and lipid A have the same structure as the most Gram negative bacteria. Thus, the LPS of antigen in the Widal SAT has a possibility to cross react with E.coli (Muliawan, 1999; Sarasombath 1998). Out of the 13 patients under study suffering from malaria (with positive plasmodium in their blood), only 1 patient (7.7 %) had false positive result for typhoid fever and 1 patient (7.7%) for agglutinin PA.

In this study, there is still a possibility of double infection between typhoid fever or paratyphoid fever with any other cause of non-typhoid fever, although all patients with non-typhoid fever showed a negative blood culture for typhoid and paratyphoid fever. The positivity of blood culture for typhoid fever is often low, so when the blood culture is positive, it can establish the diagnosis but when negative it cannot...
exclude the diagnosis. (Juwono, 1996; Pangalila, 1999).

From the practical point of view, the Widal slide agglutination test MJD is a test with a high degree of eligibility. A refrigerator is still needed to keep the antigen of the Widal SAT-MJD at 4°C. In this test, no special pipette or micropipette (as has to be used in the imported Widal test) is required to dilute the sera of the patients. For the purpose of serum dilution a very simple pipette is enclosed in the kit of Widal SAT-MJD. Object glasses used to mix the antigens and diluted serum are also enclosed in the test kit. For repeated use, these object glasses can be washed.

The incubation period for this test is also very short i.e. not longer than 5 minutes. For reading the result of the test, no special equipment is required. Reading takes place with the aid of sunrays penetrating through a glass window or by placing the test material 15 cm above a 10 Watt neon light.

The low cost of the Widal SAT-MJD is an added advantage of the test, which is presumably half the cost of the imported Widal test. Analysis of data obtained during the performance of the study reported here indicate that the Widal slide agglutination test produced by Mekar Jaya Diagnostika Research Laboratory using a mixture (in equal quantity) of antigens derived from 5 different locally prevalent phage-types of S. typhi, is an eligible and cheap screening test for the detection of typhoid fever.

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