Widal slide agglutination test using antigens from locally prevalent Salmonella typhi for diagnosis of typhoid fever in children

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ABSTRACT

A laboratory study to assess the diagnostic value of Widal slide agglutination test (SAT) using antigens from 5 locally prevalent phage types of Salmonella typhi, was carried out on 44 sera from typhoid fever patients with a positive blood culture, and 44 sera from nontyphoid febrile patients aged less than 12 years. The patients entered into the study were inpatients of the Infectious Diseases Ward, Pediatric Department of the Dr. Soetomo Hospital and outpatients of Gotong Royong Clinics in Surabaya during January 2001 until March 2002. The cutoff value of the above mentioned Widal SAT in children was assessed as a titre of 1/40 for O, H, PA agglutinin, and 1/80 for PB agglutinin. The single O agglutinin titre of ≥ 1/80 or combined O and H agglutinin titres ≥ 1/80, were found to be justifiable for establishment of the diagnosis of the disease. The results of the study revealed that the Widal SAT is an eligible tool to detect typhoid fever in children with diagnostic sensitivity as high as 86.3%, specificity 84.1%, a positive predictive value 84.4%, a negative predictive value of 86.0%, and a diagnostic efficiency as high as 85.2%. Based on this study, it can be concluded that the Widal SAT has a high diagnostic value as a screening test for the diagnosis of typhoid fever in children.

Keywords: Typhoid fever, Widal slide agglutination test, locally prevalent Salmonella typhi

Uji Widal lempeng (slide) lokal dengan antigen Salmonella typhi jenis faga lokal untuk diagnosis demam tifoid pada anak

ABSTRAK

Suatu penelitian laboratorium untuk menilai keandalan dan kepraktisan (nilai diagnostik) uji Widal lempeng lokal (SAT) menggunakan 5 jenis faga Salmonella typhi sebagai antigen, telah dilakukan pada 44 penderita demam tifoid anak (< 12 tahun) dengan kultur darah positif S. typhi dan 44 penderita demam nontifoid. Sera yang diikutkan dalam penelitian ini diperoleh dari penderita rawat inap di ruang menular anak RSUD Dr. Soetomo dan penderita rawat jalan di klinik Gotong Royong Surabaya selama kurun waktu Januari 2001 - Maret 2002. Pada sera penderita dilakukan uji Widal lempeng dengan prosedur seperti yang direkomendasikan pabrik. Ambang atas titer rujukan uji Widal SAT untuk anak umur < 12 tahun adalah 1/40 untuk titer aglutinin O, H, PA, dan 1/80 untuk titer aglutinin PB. Kriteria diagnosis demam tifoid uji Widal SAT dalam penelitian ini yaitu bila titer aglutinin O saja ≥ 1/80 atau O dan H ≥ 1/80. Hasil penelitian menunjukkan bahwa uji Widal SAT merupakan suatu uji laboratorium yang memenuhi syarat untuk mendeteksi demam tifoid pada penderita anak dengan sensitivitas diagnostik sebesar 86.3%, spesifisitas 84.1%, nilai ramal positif 84.4%, nilai ramal negatif 86.0%, dan efisiensi diagnostik 85.2%. Berdasarkan penelitian ini, dapat disimpulkan bahwa uji Widal SAT merupakan sarana diagnostik laboratorium yang andal untuk menunjang diagnosis penyakit demam tifoid pada anak.

Kata kunci: Uji Widal lempeng lokal, demam tifoid, antigen S. typhi prevalen lokal
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% off all typhoid cases in Indonesia are found in this special group, a community which mainly consists of school children. The Health Department of 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 individuals in the population. The national case fatality rate of hospitalized patients was found to be 2-5%. Resistance to anti-typhoid drugs also tend to be on the increase.

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 antimicrobial 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.

Confirmation of the diagnosis of typhoid fever can only be made through isolation of Salmonella typhi in blood or bone marrow. 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% - 80%. The relatively low degree of practicability of the blood culture constitutes the other side of the coin. The polymerase chain reaction (PCR) 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 advantages is that the test is considered a very inexpensive test. However, the sensitivity as well as specificity of the conventional Widal test remains doubtful, especially for endemic areas such as in Indonesia. On the other hand, other investigators 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 test 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 test (SAT) 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 examination without prior determination of cutoff value in the related endemic areas needed for establishing the diagnosis of the disease may account for the findings of the above mentioned unsatisfactory results.

The chance to get sub clinical infection with S. typhi (less than 10^5/ml) is higher in endemic areas such as in Indonesia compared with that of individuals living in the non endemic areas because the chance to consume contaminated food (S. typhi in sub clinical dose) is higher in these endemic areas. This chance is greater in adult individuals living in endemic areas compared to children because the habit to have their meal outside their house in small non hygienic food stalls is more frequent in adults compared with children less than 12 years of age. This is proved by the fact that the cutoff titres of the Widal SAT produced by Mekar Jaya Diagnostika (MJD) in children of less than 12 years old are lower than that of adults as shown in the leaflet enclosed in the kit. The above mentioned problems led to the performance of this study with the aim to evaluate the diagnostic value of the Widal SAT which makes use of a mixture (in equal proportion) of 5 locally prevalent phage types of S. typhi as the antigens in children, and standardized carefully by MJD Research Laboratory in Indonesia, using cutoff values which have been determined from 129 healthy individuals in endemic areas in Indonesia.
MATERIALS AND METHODS

Subjects

This laboratory study was performed on sera from 88 patients aged less than 12 years, comprising 44 patients with typhoid fever (positive blood culture for *S. typhi*) and 44 nontyphoid febrile patients (negative blood, culture for *S. typhi*) who attended the outpatient Gotong Royong Clinic and inpatients who were hospitalized in the infectious disease ward, Pediatric Department of the Dr. Soetomo Hospital in Surabaya during January 2001 until March 2002. In the list of nontyphoid diseases with fever, 14 patients with malaria (positive blood smear), 24 patients with dengue hemorrhagic fever, 2 patients with mumps, 2 patients with diptheria, 2 patients with urinary tract infection and 1 patient with bronchitis, were entered.

The population under study consisted of children (male and female) who were under 12 years at the time of entrance into the study, showing fever 7 days or more and the parents had signed the informed consent. None of the patients were under treatment with corticosteroids or other immunosuppressive drugs during 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.

Sample Procedures

Sera obtained from the population under study were tested with the Widal SAT produced by MJD Research Laboratory, following the instructions given by the manufacturer of the kit. The antigen use for Widal 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 the 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 (printed in the leaflet enclosed in the kit).

**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, H and parathyphi A (PA) agglutinins serum dilution begins with the titre of 1:20, and for paratyphi B (PB) serum dilution begins with the titre of 1:40. The diluted sera as shown in Table 1, were mixed with 40 µl of antigen suspension (O, H, PA and 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, this control sera were diluted twofold of the cutoff of the test. The positive and negative control sera should give a positive and negative result. If

<table>
<thead>
<tr>
<th>Equivalent to the titre of</th>
<th>Serum</th>
<th>Phosphate buffer saline</th>
<th>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>
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 cutoff value of the Widal SAT-MJD in children less than 12 years old were as follows:

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

Blood culture in bile broth media, urine culture and stool culture were done according to the standard procedure of the Microbiology Division, Department of Clinical Pathology, Dr. Soetomo Hospital in Surabaya. For blood culture, 5 ml of blood sample was inserted into a bottle containing 50 ml bile broth media (1:10). After it was well shaken, the bottle was incubated at 37°C for 18-24 hours. To examine the bacterial growth, the bile broth was subcultured into SS agar plate and which was then incubated at 37°C for 18-24 hours. If no bacteria were found in the SS agar plate, the process of subculturing was repeated on the 5th, 7th, and 14th days of bile broth inoculation. If there was any bacterial growth, the suspected bacterial isolate was identified to confirm that the growing colony was S. typhi. The identification tests included biochemical test (Triple Sugar Iron, Lysine Indol Motility, Simon’s citrate), serological test, and Gram staining. The urine and stool culture used the same method of S. typhi detection and identification as the blood culture but the bile broth media was not used. The urine sample was centrifuged 2500 rpm for 5 minutes, and then the sediment was inoculated in the SS agar plate. For the stool culture, the stool was directly inoculated to the SS agar plate from the Carry and Blair transport media. The urine and the stool cultures performed in this study were used to detect healthy carriers of S. typhi among the nontyphoid fever patients, which were then excluded from the study. The diagnostic criterion of the Widal SAT-MJD test for typhoid fever used in this study were as follows:

1. if the titre of O agglutinin was ≥ 1/80 or titre of O and H agglutinins were ≥ 1/80.
2. if within an interval of 5-7 days, there was a fourfold increase of the agglutinin titre.

In the group of patients with nontyphoid fever, the result of the Widal SAT-MJD was considered as false positive if O agglutinin, O and H agglutinins, PA agglutinin or PB agglutinin were equal or higher than 2 times their cutoff values. A positive result of blood culture served as the gold standard for the confirmation of typhoid fever in this study. The diagnostic value of Widal SAT-MJD was assessed based on the determination of the diagnostic sensitivity, the diagnostic specificity, the diagnostic positive predictive value, and the diagnostic negative predictive value. The following formulas were used to determine the above mentioned diagnostic values, \((15-17)\):

\[
\text{Diagnostic Sensitivity} = \frac{TP}{TP + FN}
\]

\[
\text{Diagnostic Specificity} = \frac{TN}{TN + FN}
\]

\[
\text{Diagnostic Efficiency} = \frac{TP + TN}{N}
\]

\[
\text{Diagnostic Positive Predictive Value} = \frac{TP}{TP + FP}
\]

\[
\text{Diagnostic Negative Predictive Value} = \frac{TN}{TN + FN}
\]

Note: TP: true positive; FN: false negative; TN: true negative; FP: false positive; N: number of individuals

**RESULTS**

As shown in Table 2, out of the 44 patients with typhoid fever admitted to this study, 38 patients (86.4%) showed positive Widal SAT-MJD. The diagnostic sensitivity of the Widal SAT produced by MJD Research Laboratory in this study was found to be 86.4%.
against *S. typhi* was still at an initial stage and that the levels of these antibodies were relatively low compared with those found during the second week.\(^5\)\(^-\)\(^21\) If these patients were examined during the midstage of the disease, i.e. in the second week of the disease, it can be anticipated that the diagnostic sensitivity of this Widal SAT-MJD would be higher than that obtained in this study.

However, 5 (11.4%) out of the 6 patients suffering from typhoid fever with false negative Widal SAT-MJD (O agglutinin), showed positive results for H agglutinin (titre \(\geq 1/80\)), and thus can not be considered as having typhoid fever when based on the diagnostic criterion of Widal SAT-MJD for typhoid fever. It is enticing to speculate that these 5 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 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 maintained for a longer period of time (\(\pm 2\) years) compared to the O agglutinin (\(\pm 5\) months) implies the possibility that the concentration of H agglutinin in the sera of some patients can increase to a titre above its cutoff value within a shorter period of time than the O agglutinin following manifestation of the disease after secondary infection.

<table>
<thead>
<tr>
<th>Results of Widal SAT-MJD</th>
<th>Type of the diseases</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Typhoid fever</td>
<td>Non typhoid fever</td>
</tr>
<tr>
<td>Positive</td>
<td>38</td>
<td>7</td>
</tr>
<tr>
<td>Negative</td>
<td>6</td>
<td>37</td>
</tr>
<tr>
<td>Total</td>
<td>44</td>
<td>44</td>
</tr>
</tbody>
</table>

In the group of nontyphoid fever patients, 37 of the 44 patients (84.1%) had a negative Widal SAT-MJD. The diagnostic specificity of Widal SAT-MJD in this study was 84.1%. In this study, 7 patients (15.9%) with nontyphoid fever showing a false positive Widal SAT-MJD (5 patients with dengue hemorrhagic fever and 2 patients with malaria) had false positive O agglutinin or O and H agglutinins (with titre \(\geq 1/80\)).

In this study, the diagnostic efficiency of the Widal SAT-MJD was thus 85.2%, the positive diagnostic predictive value was 84.4% and the negative predictive value was 86.0%.

**DISCUSSION**

The results of this study revealed that the diagnostic value of Widal SAT-MJD produced by MJD Research Laboratory, in accordance with the criterion of Handojo (1988)\(^{18}\) 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 higher degree of diagnostic sensitivity, while the locally prevalent phage types of *S. typhi* used in this test are presumably accountable for high degree of diagnostic specificity of the Widal SAT. As stated above, patients with typhoid fever admitted to this study had positive blood culture of *S. typhi*, which indicates that they were usually 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 that the levels of these antibodies were relatively low compared with those found during the second week.\(^5\)\(^-\)\(^21\) If these patients were examined during the midstage of the disease, i.e. in the second week of the disease, it can be anticipated that the diagnostic sensitivity of this Widal SAT-MJD would be higher than that obtained in this study.
The use of locally phage types of *S. typhi* as the antigen of Widal SAT-MJD is believed to be accountable for the high degree of diagnostic specificity (84.1%) of this test. It is 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 strain of *S. typhi*, it can be anticipated that the degree of the diagnostic sensitivity as well as of diagnostic specificity of the test must be high. Evidence supporting this hypothesis was reported by Suwahyo (1979)(13) and by Setyawati (1997)(14) 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 test 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 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 prevalent. 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 specificity of the Widal test. According to Thong (1996),(22) the genetic diversity rate between the strains of *S. typhi* from Indonesia, and Malaysia was approximately 15%. In Indonesia, it is presumed that there are 5 to 6 phage types of *S. typhi* with a genetic diversity as high as 40%.

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 cutoff value of the Widal SAT 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 SAT enclosed in the test kit, the cutoff 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. The determination of this cutoff value was based on examination of healthy population in the country where the kit was produced.

Therefore, the mentioned cutoff value is not always eligible to be used in Indonesia since the human leucocyte antigen (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 test using imported Widal SAT (Soewandojo E, personal communication). Some investigators reported that the Widal SAT using locally prevalent *S. typhi* and *S. paratyphi* as the antigens had a significantly higher degree of diagnostic values compared with Shield,(14) Murex,(23) and Omega.(24) In this study, the diagnostic specificity of the Widal SAT-MJD can be classified as high (84.1%). The 7 patients (15.9%) with nontyphoid fever showing false positive results of the Widal SAT-MJD, had false positive results for the O and H agglutinins. This implies that patients had a true false positive result for typhoid fever. It is worth to note that out of the 7 patients suffering from nontyphoid fever with a false positive result, 5 patients (11.4%) 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-cell activation may occur. 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 cutoff value. It has to be kept in mind that the dengue hemorrhagic fever is a disease that has to be differentiated from typhoid fever, but both diseases may occur concomitantly.
However, only 5 among 23 patients (21.7%) with dengue hemorrhagic fever reported here, showed false positive results. Out of the 14 patients under study suffering from malaria (with positive plasmodium in their blood), only 2 patients (14.3%) had false positive result for typhoid fever. In this study, there is still a possibility of combined infection with S. typhi and any other cause of nontyphoid fever, although all patients with nontyphoid fever showed a negative blood culture for typhoid fever. The degree of positivity of blood culture in typhoid fever is often low. Thus when the blood culture is positive, it can establish the diagnosis but when it is negative it can not exclude the diagnosis of typhoid fever. 

From the practical point of view, the Widal SAT-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 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 SAT produced by MJD 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 inexpensive screening test for the detection of typhoid fever in children.

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