DETERMINATION OF BASELINE TITRE OF WIDAL TEST AMONG
HEALTHY POPULATION IN EASTERN PART OF INDIA

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ABSTRACT

Background: Widal test could be the useful tool for the diagnosis of Typhoid fever, provided the results of Widal test are correctly interpreted. Interpretation of Widal test is based on the baseline titre of healthy population of particular geography. Lack of proper knowledge of baseline titre of Widal test in different geographical areas can lead to over diagnosis of typhoid fever leading to mismanagement of patients. Aims: This study was undertaken to evaluate baseline titre of Widal test using quantitative tube Widal test, to determine the cut-off value for positive Widal test in this region, and to correlate the results obtained in quantitative tube Widal test with that of rapid semi-quantitative slide Widal test.

Material and Methods: Sera of 117 apparently healthy blood donors from February 2011 and September 2013 in Blood Bank and Department of Microbiology of Medical College, Kolkata were subjected to quantitative tube and semi-quantitative slide Widal test to know the titre. Results: Highest titre obtained by tube Widal test for TO was 1:160, for TH - 1:320, for AH - 1:20, and for BH - 1:80. Tube Widal titres of ≤1:80 for TO and TH were seen in 113 (96.58%) and 109 (94.01%), TO and TH titres of ≥1:160 were seen in 4(3.42%) and 9 (7.69%) respectively. TH titre of 1:320 was seen in 1(0.85%) and no such high titre was reported in relation to TO. Highest titre obtained by semi-quantitative slide Widal for TO was 1:640, for TH - 1:320, for AH -1:40 and for BH - 1:160. Conclusion: We recommend that TO and TH titre of > 1:320 as diagnostic of typhoid fever and for AH and BH, titres of ≥1:40 and ≥1:160 should be considered diagnostic respectively in our region. Because of high expected false positivity rate of slide Widal test.
even at the higher cut-off titre of 1:640, single slide Widal test appears to have little value in the diagnosis of typhoid fever in this region.

**Key Words**: Baseline titre, Widal test, Enteric fever, Endemic region.

**INTRODUCTION**

Enteric fever is endemic in India and it continues to be one of the major health problem here [1]. Culture of blood is the gold standard for the diagnosis of enteric fever. [2] However, most health care facilities in developing countries do not have ready access to this time consuming diagnostic method [3] as it requires good microbiological laboratory and moreover, patient visits in the hospital during late in the course of the disease and also take antibiotics as self-medication prior to hospital visit. So, in acute febrile illness in endemic typhoid region with ambiguous clinical picture, a rapid, accurate, specific and sensitive test should be used to differentiate typhoidal from non-typhoidal febrile illness.[4] Widal agglutination test as the most common alternative laboratory procedure for the diagnosis of enteric fever [4,5-7] if the results are interpreted correctly. This immunological test is readily available, inexpensive, yet reliable, easy to perform, relatively non-invasive. [8]

The endemicity of the disease is due to the previous immunization against typhoid fever or serious cross-reactivity with other infectious agents [4] such as malaria, and may due to the repeated prior subclinical infections with either of *Escherichia, Shigella, Citrobacter or Proteus* species which shared common O or H antigens with *Salmonella* species. [9,10] So, the majority of the normal healthy individuals carry detectable antibodies and hence the baseline titre of antibodies is normally elevated in healthy individuals in endemic areas. [2] As the titres vary among endemic areas and with time [11], so the present study was conducted to develop a local baseline titre in healthy individuals that will help in correct interpretation of this important test in this area. Though the classical Widal test is the tube agglutination method in most part of India, rapid slide agglutination method that is easy perform and gives quit result, is used to compare the result of slide Widal test with standard recommended tube Widal test

**MATERIAL AND METHODS**

**Selection of subjects**

In this cross-sectional study, a total of 117 subjects attending at Blood Bank of Medical College and Hospital, Kolkata between February 2011 and May 2013, were selected in the
study aged ranging from 21 to 45 years by simple random sampling from 1156 apparently healthy blood donors who have been living in the Kolkata and adjoining region for atleast 5 years. All the donors who were found to be positive for the following screening tests like those for Malaria, Microfilaria, HBsAg and antibodies to HIV, HCV and Treponema pallidum, those who were vaccinated for enteric fever in the preceding three years or individuals with the history of fever of unknown origin in the last 6 months were excluded from the study. Informed consent had been received from each subject.

Collection of samples
Peripheral venous blood from the all the subjects under study were drawn and allowed to coagulate at room temperature for 30–45 min, followed by centrifugation at 2500Xg for 5 min. All serum samples were stored at 4°C and tests were performed as soon as possible.

Laboratory investigation
The serum was subjected to semi-quantitative slide and quantitative tube Widal test using standardized suspension of S. enterica serotype Typhi ‘O’ and ‘H’ and S. enterica serotype Paratyphi A ‘H’ and S. enterica serotype Paratyphi B ‘H’ antigen purchased from Span diagnostic test reagents on the same day to know the titre. Readings of both the tests were recorded by two different trained technicians independently. The results were quantitatively defined as titres ranging from 1:20 to 1:640. The results were then tabulated and statistically analysed.

Semi-quantitative Slide Widal test
Clean glass slides supplied in the kit were used for the test. 5μl (corresponding to the titre of 1:320), 10μl (corresponding to the titre of 1:160), 20μl (corresponding to the titre of 1:80), 40μl (corresponding to the titre of 1:40) and 80μl (corresponding to the titre of 1:20) of undiluted serum were dispensed in respective circles using calibrated micropipette. One drop of appropriate antigen suspension was added to each circle and mixed using separate stick and rotated for one minute to take the readings. The whole process was followed as recommended by the manufacturer. Highest dilution of the serum showing minimum of 50% agglutination was taken as titre. Quality control was done using the positive polyspecific control of the same dilutions as the test sample. Normal saline was used for a negative control.
Quantitative tube Widal test

The tube agglutination test was carried out with 0.4 ml of two fold serially diluted patient’s sera (dilutions from 1:20 to 1:640) in 0.9% normal saline was tested by adding an equal amount of antigen. A negative control was included in each batch of the tests. The results were interpreted after overnight incubation (16-20 hours) of the samples at 37°C. The results were analyzed. The baseline titre for the O, H, AH and the BH agglutinins was the highest titre which was shown by any of the study samples.

Statistical analysis

The data for biochemical analysis was subjected to standard statistical analysis using the Statistical Package for Social Science (SPSS) 11.5 software for windows.

RESULTS

A total of 117 serum samples were analyzed. Out of 117 serum samples, the serum sample from male was 53 (45.3%) and from female were 64 (54.7%) as shown in the Table 1.

Table 1: Personal profile and clinical details of the subjects

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>117</td>
</tr>
<tr>
<td>Age (Years)</td>
<td>29 ± 7.92</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>53 (45.3)</td>
</tr>
<tr>
<td>Female</td>
<td>64 (54.7)</td>
</tr>
</tbody>
</table>

Value are mean ± SD; n = number of cases; Data are expressed as numbers (group percentages in parentheses)

Tube Widal test -

In the table 2 it was observed that 40.17% (47 out of 117) and 33.33% (39 out of 117) of the subjects showed no agglutination for TO and TH antibodies respectively by tube Widal test, 93.16% (109 out of 117) and 90.6% (106 out of 117) showed no agglutination for AH and BH antibodies respectively.

96.58% (113 out of 117) of study population showed TO titre of ≤1:80 whereas 92.31% (108 out of 117) were positive for TH less ≤1:80.
TO and TH titre of $\geq 1:160$ was observed in 3.42% (4 out of 117) and 7.69% (9 out of 117) respectively. TH titre of 1:320 was noted in 1(0.85%) case and no such high titre was observed in relation to TO.

All the cases showed AH titre of $\leq 1:20$ whereas BH titre of all was noted of $\leq 1:80$.

**Table 2: Distribution of samples with antibody titre of Tube and slide Widal test against different serotypes among 117 healthy blood donors**

<table>
<thead>
<tr>
<th>Types of Widal test</th>
<th>Antibody titres against O antigen of <em>Salmonella typhi</em></th>
<th>Antibody titres against H antigen of <em>Salmonella typhi</em></th>
<th>Antibody titres against H antigen of <em>Salmonella paratyphi A</em></th>
<th>Antibody titres against H antigen of <em>Salmonella paratyphi B</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Tube test</td>
<td>47 (40.17)</td>
<td>33 (28.2)</td>
<td>21 (17.95)</td>
<td>12 (10.26)</td>
</tr>
<tr>
<td>Slide test</td>
<td>45 (38.46)</td>
<td>8 (6.84)</td>
<td>20 (18.8)</td>
<td>33 (28.2)</td>
</tr>
<tr>
<td></td>
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<tr>
<td></td>
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</tr>
</tbody>
</table>

Values are mean ± SD; Data are expressed as numbers (group percentages in parentheses)

In the Table 3 it was found that the Larger number of study population showed higher titre when tested by semi-quantitative slide Widal test. Titre of $\geq 1:80$ for TO was seen in 13.68% (16 out of 117) cases of tube agglutination test in contrast to 32.48% (38 out of 117) cases in semi-quantitative slide test. (p<0.001). Titre of $\geq 1:160$ for TO was seen in 3.42% (4 out of 117) cases in tube Widal test as compared to 9.4% (11 out of 117) cases in semi-quantitative slide Widal test. (p<0.05). None of the cases showed titre of 1:320 in tube Widal test whereas 2.56% (3 out of 117) cases showed this titre in semi quantitative slide Widal test in relation to
TO. The titre of ≥1:80 for TH was observed in 21.37% (25 out of 117) cases by tube Widal test in contrast to 28.2% (33 out of 117) cases by semi-quantitative slide Widal test. Titre of ≥1:160 for TH was observed in 7.69% (9 out of 117) cases by tube Widal test compare to 11.11% (13 out of 117) cases in semi-quantitative slide Widal test. Titre of 1:320 for TH was seen in 0.85% (1 out of 117) and 1.71% (2 out of 117) cases of tube and semi-quantitative slide Widal test respectively. Titre of ≥1:40 for AH was not seen in any of the case in tube Widal test whereas semi-quantitative slide Widal test reported 3.42% (4 out of 117) cases. Titre of ≥1:40 for BH was observed in 4.27% (5 out of 117) cases of tube Widal test compared to 10.26% (12 out of 117) cases reported by semi-quantitative Widal test. Titre of 1:160 for BH was reported in 0.85% (1 out of 117) case by semi-quantitative tube test whereas tube Widal test has reported none. In contrast to the higher titres which were found more commonly when tested with semi-quantitative slide test the number of subjects tested positive for lower titre of 1:20 were significantly lower when compared to the tube test. The number of study population showing titre of 1:20 for TO when tested by tube Widal test was 33(28.2%) compared to 8 (6.84%) by semi-quantitative slide test. The difference was being significant (p<0.001). Likewise the number of subject showing titre of 1:20 for TH when tested by tube widal test was 24(20.51%) compared to just 11(9.4%) by semi quantitative slide Widal test (p<0.001).

Table 3: Comparison of Titre of Tube Widal with Semi-quantitative Slide Widal Test

<table>
<thead>
<tr>
<th></th>
<th>Tube Widal test</th>
<th>Slide Widal test</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TO titres</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:20</td>
<td>33 (28.2)</td>
<td>8 (6.84)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>≥1:80</td>
<td>16 (13.68)</td>
<td>(32.48)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>≥1:160</td>
<td>4 (3.42)</td>
<td>11 (9.4)</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>1:320</td>
<td>0 (0)</td>
<td>3 (2.56)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>1:640</td>
<td>0 (0)</td>
<td>1 (0.85)</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td><strong>TH titres</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:20</td>
<td>24 (20.51)</td>
<td>11 (9.4)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>≥1:80</td>
<td>25 (21.37)</td>
<td>33 (28.2)</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>≥1:160</td>
<td>9 (7.69)</td>
<td>13 (11.11)</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>1:320</td>
<td>1 (0.85)</td>
<td>2 (1.71)</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td><strong>AH titres</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥1:40</td>
<td>0 (0)</td>
<td>4 (3.42)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td><strong>BH titres</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥1:40</td>
<td>5 (4.27)</td>
<td>12 (10.26)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>1:160</td>
<td>0 (0)</td>
<td>1 (0.85)</td>
<td>&lt; 0.05</td>
</tr>
</tbody>
</table>
DISCUSSION

Widal test, widely used diagnostic test for enteric fever in developing countries, has been an exclusive choice either due to the non-availability of blood culture or other reasons such as cost, technical demands and time consumption. [12] But the majority of the normal healthy individual in endemic region also carry detectable antibodies due to the repeated prior exposure with low inoculums of typhoid bacilli, the knowledge of baseline titre is important for using the Widal test as diagnostic tool for enteric fever in endemic area [13]. Since these antibody titres vary with age and geographical area [14] so the present study was aimed to determine the baseline titre of different antibodies of enteric fever in normal population in study region.

Baseline titre by Tube Widal test-

The highest titre reported by quantitative tube Widal test was found to be 1:160 for the TO and 1:320 for the TH of Salmonella typhi whereas the highest titre for Salmonella paratyphi A (AH) and B (BH) were found to be 1:20 and 1:80 respectively. 113 out of 117 (96.58%) and 109 out of 117 (93.16%) of AHDs have shown TO and TH titre of ≤1:80. Hence the baseline titre for TO and TH in our study was ≤1:80. Taking this into consideration if TO and TH titre of >1:80 (≥1:160) is considered as diagnostic (titre above the baseline). 3.42% cases tested for TO and 7.69% cases tested for TH would give false positive Widal test at this presumptive titre in our area. To address this high false positive rates we recommend a titre of >1:160 (≥1:320) for TO and TH as diagnostic of typhoid fever. This would reduce the false positive rate from 11% to 0.85%. Similarly we recommend AH and BH titre of >1:20 (≥1:40) and >1:80 (≥1:160) as diagnostic titre for paratyphoid A and B respectively in this region. In this study TO and TH titre of ≥1:160 has been observed in 3.42 % (4 out of 117) and 7.69 % (9 out of 117) respectively. Pang T et al (1983) have reported TO and TH titre of ≥1: 160 among 5% and 2% of 300 normal subjects from Malaysia [7]. Bhadur et al. (2013) have documented that TO and TH titre of ≥ 160 among 3.7% and 9.3 % of 107 healthy blood donors from Raichur, Karnataka, India. [15] In contrast to these studies some studies from India, Punia JN (2003) [1] have shown TO titre of >1: 160 in none of the 490 and 255 normal subjects tested and TH titre of >1: 160 only in 0.6% (3 out of 490) and 1.5% (4 out of 255) respectively. This wide variation in titre of antibodies in different endemic places signifies the importance of evaluating the local titre and interpreting the results of Widal test accordingly. This variation may be the result of difference in safe water supply and sanitary conditions [16], low standard of living and lack of medical facilities.[17] It is also probable
that in endemic areas where the population is permanently sensitized the antigens of salmonella species due to constant exposure, the response to infection is more rapid, reaching higher levels & is less likely to be affected by antibiotic use when compared to nonendemic areas [7] and hence the endemicity of typhoid in these places. Another observation was that lower antibody titre against AH and BH antigens highlighted the lower endemicity of Paratyphi infection compared with typhi infection and/or low antibody response against paratyphi infection. In this study subjects have shown higher titre for BH antibodies compared to AH, this is in contrast to other recent studies from India [1,18]. Although Salmonella paratyphi A is reported to be second most common cause of enteric fever and its incidence is increasing in endemic countries like India and Thailand [19]. There are occasional reports of Salmonella paratyphi B infection from our country [20]. Hence, possible endemicity of Salmonella paratyphi B organism has to be ruled out in our region by application of proper identification methods of enteric bacilli.

**Baseline titre by semi-quantitative slide Widal test**

The highest titre for TO and TH obtained using semi-quantitative slide Widal test were 1:640 and 1:320 respectively. 113 out of 117 (96.58%) and 109 out of 117 (93.16%) of AHDs have shown TO and TH titre of ≤1:80. Hence the baseline titre for TO and TH in our study was ≤1:80. Taking this into consideration if TO and TH titre of >1:80 (≥1:160) is considered as diagnostic (titre above the baseline). Taking TO and TH titre of >1:80 (i.e. ≥1:160) as presumptive diagnostic titre (i.e. titre above the baseline) as indicated by the tube Widal test above, 20.51% (24 out of 117 cases) would be falsely diagnosed as typhoid cases as compared to 11.11% (13 out of 117 cases) by tube Widal test. Six (5.13%) study population showed titre of ≥1:320 for TO and TH. Because of this higher titre and high expected false positivity rates even at higher cut off titre of 1:640, slide Widal test in area endemic for typhoid fever provides minimal if any, diagnostic assistance. If at all used, the cut off titre for tube Widal test cannot be applied to the slide Widal for declaring positive test. Teddy C, et al. (2010) has reported 31(15.5%) blood donors with antibody titre of 320 and 9(4.5%) donors with the antibody titre of 640 against S. typhi (D) antigen in the study consisting of 200 blood donors [21].

Another study by Mussa A et al (2011) have reported titre of 1:320 for TO and TH in 7(8.7%) and 11(13.7%) of 80 healthy individuals from endemic area of Iraq [22]. In India, one study by Bhadur et al. (2013) have found that titre of 1:320 for TO and TH in 2 (1.86%)
and 2 (1.86) of 107 apparently healthy blood donars in the region of Raichur, Karnataka.[14] Based on another study in north Kornataka, Madhusudhan et al India have found that antibody titre of 1:40 for TO, 1:80 for TH & 1:40 for AH antigen considered as baseline titre in this region. [23] Further large scale studies using titre of more than 1:640 may be required to address the issue of cut off titre in slide Widal test. Many studies which have used slide Widal test for evaluation of endemic titre have reported higher endemic titre compared to studies which have used the tube Widal test [21,24,25].

CONCLUSION
A substantial number of healthy subjects in this region have shown highest titre of 1:160 for TO and 1:320 for TH by tube Widal test, hence we recommend titre of ≥ 1:640 as diagnostic of typhoid fever. For AH and BH, titre of ≥1:40 and ≥1:160 should be considered diagnostic respectively. Titre observed in semi quantitative slide Widal test were significantly higher than those seen in tube Widal test hence the cut off titre used for tube Widal test cannot be used for slide Widal test. Because of high expected false positivity of single slide Widal test even at the higher cut-off titre of 1:640, slide Widal test appears to have little value in diagnosis of typhoid fever in our region. Further studies are needed to evaluate slide Widal titre of more than 1:640 in healthy individuals and proven typhoid cases which will help to know the actual cut off titre of slide Widal test in this region.

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