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Original Research Article

Baseline titre of Widal amongst healthy blood donors at tertiary care hospital

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ABSTRACT

Background: Indian subcontinent is a hotspot of Typhoid activity with high prevalence rates. The Widal test is one of the commonly used sero-diagnostic test for typhoid fever in developing countries. Lack of proper knowledge of baseline titre of Widal test can lead to over diagnosis of typhoid fever leading to mismanagement of patients. A single cut off value on average titre among healthy individuals needs to be determined. So, the purpose of the present study was to develop recommendations for the interpretation of Widal test results in the local region. The objectives were to determine the baseline Widal titre of study population and to propose titre-values of significance in the diagnosis of enteric fever.

Methods: Sera of 242 apparently healthy blood donors from January 2016 to December 2016 in blood bank and Department of Microbiology, Dr. PDMMC, Amravati, Maharashtra, India were subjected to standard quantitative tube and semi-quantitative slide Widal test to know the titre.

Results: Highest titre obtained by tube Widal test for TO was 1:320, for TH- 1:160, for AH- 1:80, and for BH- 1:80. Tube Widal titres of ≤1:160 for TO were seen in 238 (98.34%) and for TH titre of ≤1:80 were seen in 238 (98.34%), TO and TH titres of ≥1:160 were seen in 24 (9.91%) and 4 (1.65%) respectively. TO titre of 1:320 was seen in 4(1.65%) and TH titre of 1:160 was seen in 4 (1.64%). Highest titre obtained by semi-quantitative slide Widal for TO was 1:640, for TH, AH and BH was 1:160.

Conclusions: We recommend that TO titre of ≥1:320 and TH titre ≥1:160 as diagnostic of typhoid fever and for AH and BH, titres of ≥1:80 should be considered diagnostic respectively in our region. Because of high expected false positivity rate of slide Widal test.

Keywords: Baseline titre, Prevalence, Typhoid fever, Widal test

INTRODUCTION

Enteric fever is endemic in India and it continues to be one of the major health problem here.[1] Culture of blood (and less definitively urine and stool) is the gold standard for the diagnosis of enteric fever.[2] In developing countries, sensitivity of blood culture is lower still as the patient visits in the hospital during late in the course of the disease and also take antibiotics as self-medication or upon unauthorized prescription prior to hospital visit. Culture methods are frequently sub-optimal. Other demerits of the test are its cost and relatively long turnaround time. 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.[3] Widal agglutination test as the most common alternative laboratory procedure for the diagnosis of enteric fever if the results are interpreted correctly.[3-6)] This immunological test is readily available, rapid, inexpensive, yet reliable, easy to perform, relatively non-invasive.[7]

Widal test is a classic serological reaction developed by Georges Fernand Isidore Widal in 1896.[8] It involves the use of bacterial suspensions of *Salmonella typhi* and *Salmonalla paratyphi* "A" and "B", treated to retain only the "O" and "H" antigen. The IgM somatic O antibody appears first, while the IgG flagella H antibody usually develops more slowly but persists for longer.[9-11] The slide test is rapid and is used as a screening procedure. The tube agglutination test serves as a means of confirming the results and clarify erratic or equivocal reactions of the slide test.[12,13] It is essentially an indirect evidence of infection where antibody response rather than the pathogen or its components is detected. But the antibody detected and its titre has to be 'meaningful' for it to be considered as evidence of 'current infection'. The antibody titre of the serum is the highest dilution of the serum that shows an observable reaction with the antigen in the particular test. The recommended definitive interpretation of the Widal test is a 4-fold rise in agglutinins taken 7-10 days apart.[14] Clinically however, this is rarely demonstrated and 2-3-fold rises are commonly seen probably due to the fact that titres are already raised when the patient's serum was first tested.[15,16] Towards the end of first week, titres of either O or H or both may rise to as high as 1:160.[3] The titres rise for the first four weeks followed by fall thereafter. Once elevated above the baseline, the titres remain high for a period of 6 months. In some cases due to severe hypoproteinemia or early administration of antibiotics the titers may not rise at all.[16,17] But generally, the lack of paired sera may lead to erroneous interpretation of test results.[11,18]

Non-feasibility of obtaining the second serum sample from patients makes it practically unhelpful in establishing diagnosis. Hence a single cut-off value is widely used. In the endemic areas, the healthy people may contain antibodies which are capable of reacting up to a variable titre in the Widal test, due to a past exposure, TAB vaccination and cross reacting antigens. Therefore it varies widely from place to place and is referred to as the baseline titre of that area. Furthermore, there are many other conditions such as malaria, brucellosis, dengue fever, chronic liver disease, endocarditis and infections due to other enterobacteriaceae, where the Widal titres may be elevated due to antigenic cross reactions.[19] There are more than 40 cross reacting antigens between *S. typhi* and other enterobacteriaceae.[20] Most of the above conditions are also endemic in typhoid-endemic regions.

Therefore the baseline Widal titres are high in endemic compared to non-endemic areas the level of elevation corresponding to the degree of endemicity and that spiking of titres in febrile illnesses could be due to infections other than typhoid owing to anamnestic responses. The titre-rise in most of these conditions is said to be only transient or nonprogressing compared to that in enteric fever where the rise is sustained and progressive.[17] Due to the multiple reasons given above, both positive as well as negative results of the test may be open to several different interpretations and hence wrong diagnoses. Rapid semi quantitative slide Widal test has replaced conventional tube Widal test in many laboratories. Knowledge of prevalent baseline titres is essential for its interpretation. For the diagnostic purposes a fourfold rise in antibody titer between acute and convalescent phases is considered significant. Therefore, a single cut off value on average titre among healthy individuals needs to be determined. So, the purpose of the present study was to develop recommendations for the interpretation of widal test results in the local region.

METHODS

This was the community based, cross sectional study which was conducted in the department of Microbiology of Dr. Panjabrao alias Bhausaheb Deshmukh Memorial Medical College, Amravati, Maharashtra, India from January 2016 to December 2016.

Selection of subjects

A total of 242 apparently healthy blood donors of both the sexes and age more than 18 years attending the blood bank of Dr. PDMMC, Amravati, Maharashtra, India who didn't have any signs and symptoms of infectious diseases were included in this study. Those who were found positive for the screening test of malaria, microfilaria, HIV, HBsAg, Syphilis, those who were vaccinated for the enteric fever in the preceding 3 years or individual with the history of fever of unknown origin in the last 6 months were excluded from the study. Clearance from the Institutional Ethical Committee was taken.

Collection of sample

Peripheral venous blood from all the subjects under study were collected separately using sterile syringe under all aseptic precautions and allowed to coagulate at room temperature for 30-45min, followed by centrifugation at 2500Xg for 5min. (blood bags were not used to collect the sample). All the samples were stored at 4ºC and test were performed as soon as possible.

Laboratory investigation

Serum sample were 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. Reading of both the test were recorded by two different trained technicians independently. The results were quantitatively defined as titres ranging from 1:20 to 1:640. The result were then tabulated and statistically analysed.

Semi-quantitative slide Widal test

Clean glass slides supplied in the kit were used for the test. 2.5µL (corresponding to 1:640), 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 0.4ml 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 37ºC. 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 statistical analysis was subjected to standard statistical analysis using the Open epi ver. 3 software for windows.

RESULTS

A total of 242 serum samples were analyzed. Out of 242 serum samples, the serum sample from male was 198 (81.8%) and from female were 44 (18.18%) as shown in the Table 1.

Table 1: Age and sex wise distribution of subjects.

Sex/Age	Male	Female	Total
18-20	20	08	28
21-29	88	16	104
30-39	58	12	70
40-49	28	08	36
50-60	04	00	04
Total	198	44	242

The sample size of our study was 242 who were screened for the presence of anti-TO, anti-TH, anti-AH, anti- BH agglutinins by Widal tube agglutination test. It was observed that 29.75% (72 out of 242), 56.19% (136 out of 242), 71.9% (174 out of 242) and 85.95% (208 out of 242) subjects showed no agglutination for TO, TH, AH and BH antibodies respectively as showed in Table 2.

Table 2: Distribution of Positive and Negative samples for agglutination in Widal test.

Widal reactivity	Frequency (percentage)			
	Anti-TO	Anti-TH	Anti-AH	Anti-BH
Positive (≥ 1:20)	170 (70.24%)	106 (43.80%)	68 (28.09%)	34 (14.04%)
Negative (≤ 1:20)	72 (29.75%)	136 (56.19%)	174 (71.9%)	208 (85.95%)

Table No. 3: Distribution of samples with antibody titre of Tube Widal and Slide Widal test against different serotypes among 242 healthy blood donors.

Types of Widal test	1:20	1:40	1:80	1:160	1:320	1:640	<1:20
Antibody against O antigen of *Salmonella typhi*							
Tube test	24 (9.91%)	46 (19%)	76 (31.4%)	20 (8.26%)	04 (1.65%)	0(0)	72 (29.75%)
Slide test	11 (4.54)	47 (19.42%)	80 (33.05%)	23 (9.5%)	07 (2.89%)	04 (1.65%)	70 (28.92%)
Antibody against H antigen of *Salmonella typhi*							
Tube test	10 (4.13%)	52 (21.48%)	40 (16.52%)	04 (1.65%)	0 (0)	0(0)	136 (56.19%)
Slide test	0 (0)	60 (24.79%)	46 (19%)	06 (2.47%)	0 (0)	0 (0)	130 (53.71%)
Antibody against H antigen of *Salmonella paratyphi A*							
Tube test	26 (10.74%)	30 (12.39%)	12 (4.95%)	0 (0)	0 (0)	0 (0)	174 (71.9%)
Slide test	20 (8.26%)	36 (14.87%)	14 (5.78%)	03 (1.23%)	0 (0)	0 (0)	169 (69.83%)
Antibody against H antigen of *Salmonella Paratyphi B*							
Tube test	18 (7.43%)	14 (5.78%)	02 (0.82%)	0 (0)	0 (0)	0 (0)	208 (85.95%)
Slide test	10 (4.13%)	22 (9.09%)	07 (2.89%)	04 (1.65%)	0 (0)	0 (0)	199 (82.23%)

In the Table 3 it was observed that, 98.34% (238 out of 242) cases of study population showed TO titre of ≤1:160 whereas 98.34% (238out of 242) cases were positive for TH with titre ≤1:80. TO titre of 1:320 was observed in 1.65% (4 out of 242) cases no such high titre was observed in relation to TH. All the cases showed AH and BH titre of ≤1:80 by tube Widal test.

Table 4: Comparison of titre of tube Widal with semi-quantitative slide Widal test.

	Tube Widal test	Slide Widal test
TO titre		
1:20	24 (9.91)	11 (4.54)
≥1:80	100 (41.32)	114 (47.10)
≥1:160	24 (9.91)	34 (14.04)
1:320	04 (1.65)	07 (2.89)
1:640	0	04 (1.65)
TH titre		
1:20	10 (4.13)	0
≥1:80	44 (18.18)	52 (21.48)
1:160	04 (1.65)	06 (2.47)
AH titre		
≥1:40	42 (17.35)	53 (21.9)
1:160	0	03 (1.23)
BH titre		
≥1:40	16 (6.61)	29 (11.98)
1:160	0	04 (1.65)

In the Table 4 it was found that the larger number of study population showed higher titre when tested by semi-quantitative slide Widal test. Titre of ≥1:80 for TO was seen in 41.32% (100 out of 242) cases of tube agglutination test in contrast to 47.10% (114 out of 242) cases in semi-quantitative slide test ($p<0.0001$). Titre of ≥1:160 for TO was seen in 19.91% (24 out of 242) cases in tube Widal test as compared to 14.04 % (34 out of 242) cases in semi-quantitative slide Widal test. ($p<0.0001$). 1.65% (4 out of 242) cases showed titre of ≥1:320 in tube Widal test whereas 4.54% (11 out of 242) cases showed this titre in semi quantitative slide Widal test in relation to TO. None of the cases showed titre of 1:640 in tube Widal test whereas 1.64% (4 out of 242) cases showed this titre in semi quantitative slide Widal test in relation to TO. The titre of ≥1:80 for TH was observed in 18.18% (44 out of 242) cases by tube Widal test in contrast to 21.48% (52 out of 242) cases by semi-quantitative slide Widal test. Titre of ≥1:160 for TH was observed in 1.65% (4 out of 242) cases by tube Widal test compare to 2.47% (6 out of 242) cases in semi-quantitative slide Widal test. Titre of ≥1:40 for AH was seen in 17.35% (42 out of 242) cases in tube Widal test whereas semi-quantitative slide Widal test reported 21.90% (53 out of 242) cases.

None of the cases showed titre of 1:160 in tube Widal test whereas 1.23% (3 out of 242) cases showed this titre in semi quantitative slide Widal test in relation to AH. Titre of ≥1:40 for BH was observed in 6.61% (16 out of 242) cases of tube Widal test compared to 11.98% (29 out of 242) cases reported by semi-quantitative Widal test. Titre of 1:160 for BH was reported in 1.65% (4 out of 242) 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 24 (9.91%) compared to 11 (4.54%) by semi-quantitative slide test. The difference was being highly significant ($p<0.0001$). Likewise the number of subject showing titre of 1:20 for TH when tested by tube Widal test was 10 (4.13%) compared to just none by semi quantitative slide Widal test.

DISCUSSION

It is ironical that the best practices of diagnosis are observed in regions of the world where enteric fever is far less prevalent and in countries where the disease is rampant and where such practices are more acutely needed, it often goes undiagnosed or over-diagnosed. The isolation of various strains of *Salmonella enterica subspecies enterica* from blood remains the gold standard for the diagnosis of enteric/typhoid fever.

However, in the modern era, there is an alarming upsurge in the empirical use of broad spectrum antibiotics, the practice of self-medication and the lack of proper timing for the specimen collection that attributes to the reduced productivity of the blood culture technique. Also, in the developing countries, such as the Indian subcontinent, many clinics and hospitals do not have a ready access to the blood culture method, thus making the Widal tube agglutination test the most common alternative laboratory procedure for the diagnosis of enteric fever. The serological diagnosis relies classically on the demonstration of the rising titre of the antibodies in paired samples, 10 to 14 days apart.

In typhoid fever, however, such a rise is not always demonstrable, even in the blood culture confirmed cases. This situation may occur because of the acute phase sample which is obtained late in the natural history of the disease, because of the high levels of the background antibody in a region of endemicity or because in some individuals, the antibody response is blunted by the early administration of an antibiotic.[21] Furthermore, the patient treatment cannot wait for long. For practical purposes, the treatment decision must be made on the basis of the results which are obtained with a single acute phase sample.[20] The cut off titre in a particular population depends on the background level of the typhoid antibodies and the level of the typhoid vaccination, which may vary with time.[22] Since these antibody titre vary with age and geographical area so the present study was aimed to determine the baseline titre of different antibodies of enteric fever in normal population in study region.[23]

Baseline titre by quantitative tube Widal test

The highest titre reported by quantitative Tube Widal test was found to be 1:320 for the TO and 1:160 for the TH of *Salmonella typhi* whereas the highest titre for *Salmonella paratyphi* A (AH) and B (BH) were found to be 1:80 for each. 238 out of 242 (98.34%) of AHDs have shown the TO titre of ≤1:160 and 238 out of 242 (98.34%) of AHDs have shown TH titre of ≤1:80. 230 out of 242 (95.04%) and 240 out of 242 (99.17%) of AHDs have shown AH and BH titre of ≤1:40. Collard et al offered an interesting proposal for arriving at the so called significant Widal titers for a population. They proposed the value that the titer of agglutinins to be considered of significance should be such as would not be expected in more than 5% of normal population.[27] It follows from this premise that, the highest value found in the remaining 95% of the studied healthy population can still be taken as baseline titer. Hence the baseline titre for TO and TH in our study is ≤1:160 and ≤1:80 respectively. Also the baseline titre for AH and BH in our study is ≤1:40 for each. Taking this into consideration we recommend TO Titre of >1:160 (≥1:320) , TH titre of >1:80 (≥1:160) and also AH and BH titre of >1:40 (≥1:80) for each is consider as diagnostic (titre above the baseline) for S. Typhi, paratyphi A and B respectively in this region. In this study TO and TH titre of ≥1:160 has been observed in 9.9 % (24 out of 242) and 1.65 % (4 out of 242) 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.[6] Bhadur et al have documented that TO and TH titre of ≥160 among 3.7% and 9.3 % of 107 healthy blood donors from Raichur, Karnataka, India.[24] In contrast to these studies some studies from India, Punia JN 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.[1] 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, low standard of living and lack of medical facilities.[25,27] It is 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 and is less likely to be affected by antibiotic use when compared to nonendemic areas and hence the endemicity of typhoid in these places.[6] 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.

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 1:160 respectively, whereas the highest titre for *Salmonella paratyphi* A (AH) and B (BH) were found to be 1:160 for each. 238 out of 242 (98.34%) of AHDs have shown the TO titre of ≤1:320 and 236 out of 242 (97.52%) of AHDs have shown TH titre of ≤1:80. 239 out of 242 (98.76%) and 238 out of 242 (98.34%) of AHDs have shown AH and BH titre of ≤1:80. Hence the baseline titre for TO and TH in our study is ≤1:160 and ≤1:80 respectively. Also the baseline titre for AH and BH in present study is ≤1:40 for each. Taking this into consideration we recommend TO Titre of >1:160 (≥1:320) , TH titre of >1:80 (≥1:160) ,AH titre of >1:80 (≥1:160) and BH titre of >1:40 (≥1:80) is consider as diagnostic (titre above the baseline) for *S. typhi*, *paratyphi* A and B respectively in this region as indicated by tube Widal test above, 7.02% (17 out of 242) cases would be falsely diagnosed as typhoid cases as compared to 3.30% (8 out of 242) cases by tube Widal test. 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 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.[28]

Another study by Mussa A et al 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.[29] In India, one study by Bahadur et al have found that titre of 1:320 for TO and TH in 2 (1.86%) and 2 (1.86) of 107 apparently healthy blood donors in the region of Raichur, Karnataka, India.[24] Based on another study in north Kornataka, India, Madhusudhan et al India have found that antibody titre of 1:40 for TO, 1:80 for TH and 1:40 for AH antigen considered as baseline titre in this region.[30] 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.[28,31,32]

Proper hygiene and sanitation are the keys to a low prevalence of enteric fever in the developed countries, which can result in a low antibody titre.[33]

CONCLUSION

A substantial number of apparently healthy blood donors in this region have shown titre of 1:160 for TO and 1:80 for TH by tube Widal test, hence we recommend titre of ≥1:320 for TO and titre of ≥1:160 for TH as diagnostic of typhoid fever. For AH and BH, titre of ≥1:80 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.

Several studies have highlighted the limitations of using the Widal serological test in the laboratory diagnosis of Salmonella, the worst being its non-specificity. Despite this fact, considering the low cost and the absence of comparatively cheap tests, the Widal tube agglutination test is likely to remain the test of choice in many developing countries, as of ours, provided a baseline antibody titre of healthy individual in the population, is known.

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