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Original Article

Microbiology Section

# A Comparative Evaluation of Different Diagnostic Modalities in the Diagnosis of Typhoid Fever Using a Composite Reference Standard: A Tertiary Hospital Based Study in Central India

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## ABSTRACT

**Introduction:** Enteric fever, caused by *Salmonella spp.* is a major cause of morbidity and mortality worldwide and endemic in many developing countries including India and other South-East Asian countries. Blood culture is regarded as the gold standard for diagnosis. Currently, the standard serological method is tube agglutination with moderate sensitivity and specificity. Dot blot assay detecting IgM and IgG antibodies to a specific 50kD Outer Membrane Protein (OMP) antigen of *Salmonella spp*. is a simple, reliable, affordable and rapid test which can help in the early diagnosis of typhoid fever.

**Aim:** To systematically evaluate the different diagnostic modalities against a composite reference standard for the better diagnosis of typhoid fever in clinically suspected cases of typhoid fever.

**Materials and Methods:** This cross-sectional, prospective analytical study was carried out at a tertiary care hospital attached to Medical College in central India from November 2011 to June 2013. A total of 163 blood samples, collected aseptically from patients clinically diagnosed of enteric fever, were tested using various component tests like blood culture, Tube Widal and Dot Enzyme Immuno Assay (Dot EIA) for IgG and/or IgM. Composite Reference Standard (CRS) was created for defining the confirmed cases of typhoid fever using the component tests, wherein culture positive and in absence of culture positivity any two component test positive patients were taken as confirmed cases. All the component tests were evaluated against the CRS for sensitivity, specificity, PPV and NPV and their significance in relation to the duration of illness using statistical tests of significance.

**Results:** Blood culture was positive in 16 (9.81%) whereas, Tube Widal, IgM, IgG and IgM+IgG in combination were positive in 88(54%), 58(35.58%), 30 (18.40%) and 75 (46.01%) respectively. Using a two test criteria of CRS framed, a total of 104 patients were considered as confirmed cases. Though specificity of blood culture was 100%, the sensitivity was low with significant detection rate in 1st week of illness. Tube Widal showed a sensitivity of 65.38% and specificity of 89.83% with significant detection rate in 2nd week. Dot blot assay for IgM, IgG and Combined IgM and IgG showed a sensitivity of 71.15%, 65.28% and 51.72% respectively whereas, the specificity was 10.16%, 47.45% and 74.57% respectively with significant detection rate in 2nd and 3rd week of illness.

**Conclusion:** It can be concluded that though blood culture is still the gold standard, Dot blot assay found to have high sensitivity and good specificity might be a practical alternative test for the rapid diagnosis of typhoid fever if interpreted with care particularly using a composite reference standard. Further, it is reliable, simple to perform and rapid; results being available in 1 hour when compared to 48 hours for blood culture and 18 hours for Tube Widal test.

**Keywords:** Dot EIA, *Salmonella enterica*, Widal test

## INTRODUCTION

Enteric Fever is caused by *Salmonella enterica* serotype Typhi and *Salmonella enterica* serotype Paratyphi A, B, or C and continues to be a major public health problem in most developing countries [1-3]. According to recent estimates, 22 million (range 16 million - 33 million) cases occur each year causing 216,000 deaths, predominantly in school-age children and young adults [4]. In recent years, re-emergence of susceptibility to conventional first-line antibiotics and emergence of reduced susceptibility towards fluroquinolones and third-generation cephalosporins amongst *Salmonella spp*. has been a matter of concern in India [5].

Enteric Fever is mostly prevalent in the low or middle-income group people with inadequate sanitation and hygiene, particularly regarding food, water and disposal of human excreta. In such places there are also other causes of febrile illnesses, i.e., malaria, dengue and rickettsiosis as well as environmentally transmitted leptospirosis and melioidosis [6]. During the 1st week of fever these illnesses are not easily distinguished from each other. The lack of cardinal features of enteric fever infection compels the clinicians to depend on diagnostic tests.

The true burden of enteric fever in developing countries is difficult to estimate for two reasons: first, because the clinical picture is mixed with other febrile infections and secondly, due to lack of high standards in diagnostic laboratories of developing countries.

Laboratory diagnosis of enteric fever in developing countries is primarily achieved either by blood culture or bone marrow aspirate culture which are gold standard for diagnosis [7]. Due to invasiveness and technical difficulty of the procedure, one has to rely on serological diagnosis. Unfortunately, neither the Widal test, nor any of the serodiagnostic tests that have since been developed have proven sufficiently sensitive, specific and practical to be of value in areas where this disease is endemic [7].

The challenge in any diagnostic studies is to reach to a correct final diagnosis in all participants. Usually a single error-free reference test, known as a gold standard, is used to determine the final diagnosis and estimate the accuracy of the test under evaluation. In diseases

where there is no failsafe method for diagnosis, a Composite Reference Standard (CRS) is used to make a final diagnosis based on the results of two or more diagnostic tests known as component tests. Patients are subjected to same component tests which are combined and interpreted in a fixed way for all patients which reflects the presence or absence of the target disease [8-13].

Other than culture and tube agglutination test, a dot blot assay detecting IgM and IgG antibodies against *Salmonella enterica* serovar Typhi can help in early diagnosis using a composite reference. In this context, the present study was undertaken for comparative evaluation of the diagnostic tests using composite reference standard (CRS), for the early and rapid diagnosis of enteric fever.

## MATERIALS AND METHODS

This cross-sectional, prospective analytical study was carried out in the Department of Microbiology, People's College of Medical Sciences and Research Centre, Bhopal from November 2011 to June 2013 after obtaining permission from the Institutional Ethics Committee. A total of 163 patients of any age and either sex of which 89 were males and rest 74 were females, showing signs and symptoms suggestive of enteric fever were enrolled for the study whereas all repeat cases and patient's having history of prior antibiotic treatment and/or typhoid immunization in the recent past were excluded. After obtaining the informed written consent, patient's details were noted down according to the case record form, including their chief complaints, duration of illness, past history of similar illness and vaccination.

Blood samples were collected under aseptic precautions, 5ml from children and 10ml from adults respectively and inoculated into Brain Heart Infusion Broth with SPS from HiMedia India [14]. Another 2 to 3ml blood was collected in sterile plain tube and allowed to clot till the serum separated. Serum was stored at -20°C in small aliquots, properly labelled and used for both Widal test and Dot blot assay. Samples thus, collected were processed for microbiological examination using standard procedures [15,16].

Blood culture bottles were incubated aerobically at 37°C. Sub-cultures were made on Blood agar and MacConkey agar on every alternate day till the 7$^{th}$day. The growth of *Salmonella* was identified as per standard protocol and confirmed by standard biochemical tests followed by sero-agglutination test using antisera [15]. Antimicrobial susceptibility testing was done for all isolates using Kirby Bauer disc diffusion method.

The Widal test was performed by tube method and it was considered positive when a titre of equal to or more than 160 was observed as the prevailing titre in the region was 120 [15]. Dot blot assay based on the principle of Enzyme Immuno Assay (EIA) was carried out and interpreted using Typhipoint (AB Diagnostics, India) as per manufacturer's instructions.

To increase the specificity, a composite reference standard was created and two test criteria was adopted wherein patients with any two component test positivity were considered as confirmed case of enteric fever. By this criteria, all the culture positive cases and in the absence of culture positivity, patients with any two (2) tests positive out of four (4) serodiagnostic tests i.e. a). Tube Widal; b) IgM; c) IgG; d) Both IgM and IgG that have been carried out were considered as confirmed cases as per CRS [8-13].

Comparative evaluation of different diagnostic modalities used was done against the composite reference standard using various test of statistical significance. We have also evaluated component test used in reference to duration of illness and evaluated them for sensitivity, specificity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV).

All the data was maintained in Microsoft Office Excel and was analysed using test of proportions as well as test of significance like Pearson's Chi-Square test and paired t-test.

## RESULTS

A total of 163 clinically suspected patients of enteric fever were evaluated by different modalities for laboratory diagnosis of enteric fever. A male predominance of 89/163 (54.60%) was observed, as compared to that of 74/163 (45.40%) in females. Most of the patients presented in the active age group of more than 10-20 years.

Blood culture was positive in only 16 out of 163 clinically suspected cases of enteric fever of which 14 were *Salmonella enterica* serotype Typhi and 2 were *Salmonella enterica* serotype Paratyphi A.

A total of 104 patients out of 163 (63.80%) clinically suspected cases of typhoid fever were found to be confirmed cases using composite reference standard while the remaining 59 (36.19) fell into the category of "Reference Standard negative" group [Table/Fig-1].

Along with the culture, which is a gold standard, determination of Dot blot assay for IgM and IgM+IgG in combination was found to be significantly associated with the predictive diagnostic outcome with p-value of <0.05 [Table/Fig-2].

Blood Culture positivity was found to be highest during the 1$^{st}$ week of illness with 15/16 (93.75%). Majority of patients i.e., 59/88 (67.04%) and 23/88 (26.13%) were positive for Tube Widal test in 2$^{nd}$ & 3$^{rd}$ week of illness. IgM & IgM+IgG in combination was found to be useful for diagnosis in 1$^{st}$ and 2$^{nd}$ week with positive outcome of 29/58 (50%) & 26/58 (44.82%) for IgM and 16/75 (21.33 %) and 49/75 (65.33%) for IgM+IgG, thus detecting more than 90 % of the cases. [Table/Fig-3]. Of the component tests used Blood culture followed by tube Widal and Dot blot assay for IgM+IgG with specificity of 100%, 89.83 % and 74.57% respectively were found to be useful diagnostic tools [Table/Fig-4].

| Test | Confirmed Cases |
|---|---|
| Blood Culture Positive | 16 |
| Tube Widal + IgM Positive | 14 |
| Tube Widal + IgG Positive | 20 |
| Tube Widal + IgM + IgG Positive | 34 |
| IgM + IgG Positive | 20 |
| Total | 104 |

**[Table/Fig-1]:** Confirmed Cases of Enteric fever taken as Reference Standard

| Test | Reference Standard Positive | Reference Standard Negative | Total | Chi square value | p-value** | Interpretation |
|---|---|---|---|---|---|---|
| Culture | 16 | 0 | 16 | 10.06 | 0.0015 | Highly Significant |
| Tube Widal | 68 | 06 | 74 | 46.29 | 1.02 | Not Significant |
| IgM | 68 | 53 | 127 | 7.63 | 0.0057 | Highly Significant |
| IgG | 74 | 31 | 99 | 2.603 | 0.1066 | Not Significant |
| IgM + IgG | 54 | 15 | 69 | 10.82 | 0.001 | Significant |

**[Table/Fig-2]:** Comparative evaluation of different test with Reference Standard.
** p-value- p<0.05= Significant association, p<0.001= Highly significant association.

| Duration of illness (weeks) | No of Patient | Blood Culture Positive | Tube Widal Positive | Dot Blot Assay | | |
|---|---|---|---|---|---|---|
| | | | | IgM Positive | IgG Positive | Both IgM + IgG Positive |
| 1$^{st}$ | 52 | 15 (93.75) | 6 (6.81) | 29 (50.00) | 7 (23.33) | 16 (21.33) |
| 2$^{nd}$ | 88 | 1 (6.25) | 59 (67.04) | 26 (44.82) | 13 (43.33) | 49 (65.33) |
| 3$^{rd}$ | 23 | 0 (0.00) | 23 (26.13) | 3 (5.17) | 10 (33.33) | 10 (13.33) |
| Total | 163 | 16 | 88 | 58 | 30 | 75 |

**[Table/Fig-3]:** Correlation of component tests for diagnostic utility with duration of illness in suspected cases of enteric fever
Figures in the bracket indicate % out of total positive tests.

| Tests | Sensitivity | Specificity | PPV | NPV |
|---|---|---|---|---|
| Blood Culture | 15.38% | 100% | 100% | 40.13% |
| Tube Widal | 65.38% | 89.83% | 91.89% | 59.55% |
| Dot Blot Assay IgM | 71.15% | 10.16% | 58.26% | 16.66% |
| Dot Blot Assay IgG | 65.38% | 47.45% | 68.68% | 43.75% |
| Dot Blot Assay IgM+IgG | 51.92% | 74.57% | 78.26% | 46.80% |

**[Table/Fig-4]:** Comparision of various diagnostic methods with sensitivity, specificity, and predictive values.
PPV : Positive predictive value, NPV : Negative predictive value

## DISCUSSION

Enteric fever continues to be a major public health problem in India affecting all age groups and both sexes [3]. In the present study, a clear male predominance of 89/163 (54.60%) was observed, as compared to that of 74/163 (45.40%) in females. Our findings were in accordance with the study done by Singhal L who reported a male predominance of 67% with a male-to-female ratio of 2:1 [5]. Similar findings were found in a study done in India by Maheshwari V and Ochiai et al., in which the active age group of 5 to 15 years was found to be predominantly involved [17,18].

Out of the 163 clinically suspected cases of enteric fever, 16/163 (9.81%) were positive by blood culture. The low rate of isolation may be due to prior antibacterial therapy, late presentation by patients and difficulties in obtaining large volume of blood for cultures. In the present study culture showed specificity and positive predictive value (PPV) of 100% with a negative predictive value (NPV) of 40.13% and sensitivity of only 15.38%. Our culture sensitivity was in accordance with findings of Jesudasson and Sivakumar with the isolation rate of only 6.92% whereas Baragundi M *et al*., reported *Salmonella* isolation rate of 17.9% [19,20].

In present study 52 (31.90%) patients were found harbouring the infection during their first week of illness wheras 88(53.98%) and 23(14.11%) patients were found in their second & third week of illness. When blood culture was compared with duration of illness it was found that out of 52 blood cultures processed during first week 15(28.85%) were blood culture positive and 37 (71.15%) were blood culture negative. During second week only 1(01.13%) was found to be positive out of 88 blood cultures processed and remaining i.e 87 (98.87 %) were negative. During third week all 23(100%) blood cultures showed no growth of organisms [Table/Fig-3]. Similar observation was reported by Baragundi M et al., [20].

In developing countries for over a century, Widal test has been used for diagnosing typhoid fever but it has been reported to have a low specificity, sensitivity and positive predictive value [7]. In the present study, Widal test was positive in 88/163 (54%) of the patients. Sensitivity of tube Widal test was 11.53% in the first week of illness and it was increased to 67.04% in the second week of illness and it was maximum in the third week of illness (100%). This is consistent with earlier reports [7] [Table/Fig-3].

The proportion of positivity in cases of tube Widal was significantly higher in second and third week as compared to first week (z=7.269, p <0.001). Usually O antibodies appear on 6$^{th}$ to 8$^{th}$ and H antibodies on 10$^{th}$ to 12$^{th}$ day after the onset of the disease which accounts for the same [15].

In the present study, tube Widal had a specificity of 89.83% with PPV of 91.89% and showing sensitivity of 65.38% and NPV of 59.55% [Table/Fig-4]. This corroborates well with data from a study in Turkey by Willke A et al., using a cut-off of >1/200 for the O antigen test performed on acute-phase serum gave a sensitivity of 52% and a specificity of 88% with a PPV of 76% and NPV of 71% [21].

Determination of presence of IgM and/or IgG antibodies against *Salmonella* in serum sample of patients is new, inexpensive and reliable sero diagnostic test available commercially and has been reported to have good sensitivity and specificity [7]. Our study shows an overall IgM positivity of 58 (35.58%), IgG positivity of 30 (18.40%) and IgM and IgG combined positivity of 75 (46.01%).

The proportion of positive cases in cases of IgM only was significantly higher in the first week as compared to the second and the third week (z=3.502, p<0.001). In the present study IgM showed a sensitivity of 71.15% with PPV of 58.26% and NPV of 16.66% with a specificity of only 10.16%.

The proportion of positive cases in cases of IgG only was significantly higher in the third week as compared to the first and the second week (z=3.058, p<0.001). In present study, IgG showed a Sensitivity of 65.38% with PPV of 68.68% and NPV of 43.75% with a specificity of 47.45% [Table/Fig-4].

The proportion of positive cases of both IgM and IgG was significantly higher in the second week as compared to the first and the third week (z=2.506, p=0.012). In the present study, the sensitivity and specificity of dot blot assay (IgM and IgG) was 51.92% and 74.57% respectively. A specificity of 74.57% is comparable to the studies from Indian sub-continents who have reported specificity of 77-87% [22,23].

Varied specificity ranging from 10.16% to 89.83% in tube Widal, IgM, IgG and both IgM and IgG in the "Reference standard negative" was observed and it may be most likely because of anamnestic reaction.

## LIMITATION

Combining multiple tests to define a target disease status rather than using a single imperfect test is a transparent and reproducible method for dealing with the common problem of not having the gold standard for the same. CRS helps us to reduce the amount of such bias but cannot completely eliminate it as it is unlikely that a combination of imperfect tests will have a composite standard with 100 % sensitivity and specificity. Instead of categorizing the patients in diseased and non-diseased, multiple disease categories can also be defined according to the degree of certainty of disease. Large scale, multi-centric studies involving various component tests defining the stages of degrees of certainty need to be carried out to strengthen such findings.

## CONCLUSION

In diseases where there is no single perfect reference standard, CRS provides a reliable and transparent alternative for evaluation. It can be concluded that though blood culture is still the gold standard, dot blot assay is found to have a good specificity and high sensitivity and if interpreted with care, might prove to be a practical alternative test for the rapid diagnosis of typhoid fever. Further, it is, simple, reliable to perform and rapid; results being available in 1 hour when compared to 18 hours for tube Widal test and 48 hours for blood culture.

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