

Development of Inhouse Immunoblot Method for Detection of Anti Salmonella Antibody and Comparison with Widal Test

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Enteric fever has emerged as an important infectious disease in the early 19th century. This study is carried out in search of an accurate diagnostic method enabling direct observation of antibody binding to antigen profiles, avoiding problems with non-specific antibody binding. To develop an inhouse immunoblot method for detection of anti salmonella antibody against antigens like LPS and *S.typhi* culture filtrates and compare with Widal in search of an accurate diagnostic assay for typhoid fever. Cross-sectional comparative study for a period of 1 year in a tertiary care hospital. 100 cases having clinical suspicion of typhoid fever and 40 controls (20 healthy persons and 20 non- typhoidal febrile patients) were studied. Subjects were investigated by blood culture, Widal test and Immunoblot and the results were compared. SPSS version 20 was used to analyze the results. Out of 100 cases 58(58%) were positive by widal and 74(74%) by immunoblot. Sensitivity and Specificity was found to be 58%, 85% for widal and 74%, 95% for Immunoblot respectively. Positive and negative predictive values were found to be 90.63%, 44.74% for widal and 97.37%, 59.38% for Immunoblot. Immunoblotting procedure incorporating *Salmonella typhi* culture filtrates is found to be more sensitive than the Widal agglutination assay for providing evidence of infection with *Salmonella typhi*.

Keywords: Immunoblot, Typhoid fever, Widal agglutination test.

Enteric fever is an important infectious disease which is endemic in India¹. Untreated typhoid fever may be fatal hence early diagnosis of enteric fever is essential for an effective management². Culture and Widal tests are the routine conventional tests. Although attempts are made to standardize Widal test³, it is of diagnostic value only in second week of illness⁴ and usually done with paired sera⁵. SDS PAGE (Sodium dodecyl sulfate Polyacrylamide gel electrophoresis) Immunoblotting proved to be one of the highly efficient techniques for detection of Salmonella antibodies⁶. Hence, it was determined to compare Widal test with Immunoblot test results for accurate and early diagnosis of typhoid fever.

MATERIALS AND METHODS

It is a cross-sectional comparative study done for a period of 1 year. Appropriate history, clinical findings and laboratory records were collected and recorded for all cases and were analyzed using SPSS Version 20. Study population includes 100 clinically suspected typhoid fever cases (irrespective of age and sex) were selected on the basis of following criteria of Fever for > 3 days, with no obvious focus of infection, Abdominal discomfort, constipation or loose motion, Coated tongue, toxic look, Hepatomegaly, splenomegaly, Relative bradycardia, rose spot etc⁷ and 40 controls comprising 20 healthy and 20 non typhoidal febrile illness cases.

Blood culture was done by conventional method. Suspected colonies were identified as *Salmonella typhi* by Grams stain, motility test and

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by biochemical tests and confirmed with slide agglutination test with high titer sera. Widal test was done by Tube agglutination using various dilutions for detection of agglutinins (O and H) and Significant titer was taken as 1:80 & above for O antibodies (TO) and 1:160 & above for H antibodies (TH) ².

For SDS-PAGE /Immunoblotting LPS obtained was a commercial preparation (Sigma chemical Co) and a standard strain of *Salmonella typhi* obtained from King Institute, Guindy, Chennai, was used for extraction of culture filtrate protein and compared with strains obtained from our culture. The bacteria was further characterized and their identity was reconfirmed. Culture filtrates of *Salmonella typhi* was grown in minimal salt medium (Sauton's medium) and subjected to Millipore membrane filtration⁸.

SDS-PAGE and immunoblotting was performed using a mini gel system. Initially various dilutions of LPS & *S.typhi* culture filtrate were tried before standardizing for electrophoresis along with various dilutions of human sera for blotting. Then 30 microgram of LPS or 15µl of *S.typhi* culture filtrate and 15µl standard negative control were loaded per lane of a gel comprising a 4.5% stacking gel and a 12.0% resolving gel. Following electrophoresis, of LPS and *S.typhi* culture filtrate, Immunoblotting and reaction with human sera were done. For immunoblotting, profiles were transferred onto nitrocellulose membranes and blocked with 5% skimmed milk. Then they were allowed to react with human sera for 3hrs on a rocker at room temperature and bound antibodies detected with a Rabbit anti- human IgG-HRP conjugate. Antibody-antibody-conjugate complexes were detected with an enzyme substrate comprising 3,3'-Diaminobenzidine.

Double band formation at 37 kDa was observed in controls and looked for in test sera also. This signifies a definite positive. No double band formation at this region was taken as a negative result. The blotting of LPS did not show up well hence it was not appreciated well on the membrane. Further reconstitution may be required.

RESULTS

The present study was conducted among 140 subjects. 100 were clinically suspected case

of typhoid fever and 40 were age and sex matched healthy and sick controls. Out of 100 cases, 16 were culture positive for *Salmonella typhi* and 58 were widal positive and rest of the 32 were clinically diagnosed typhoid fever but blood culture and widal test was negative.

Out of 100 clinically diagnosed typhoid fever cases 16(16%) were blood culture positive for *Salmonella typhi*, 58(58%) were widal test positive and 74(74%) were immunoblot positive (Table-1). The Immunoblot was found to be highly significant than widal test among clinically diagnosed typhoid fever. (p value < 0.001).

Among the 100 clinically suspected typhoid fever cases 32 cases were both blood culture and widal test negative of which 08 cases were Immunoblot positive. Immunoblot was positive in 14(87.5%) out of 16 culture positive typhoid cases. 58(100.00%) Immunoblot were positive among 58 widal positive typhoid cases. The test was also positive in 2(10%) for Immunoblot out of 20 febrile controls. None of the healthy controls were positive by Immunoblot (Fig 1).

The widal test was positive in 06(37.50%) out of 16 blood culture positive cases and in 52(61.90%) out of 84 culture negative typhoid cases. Out of 16 blood culture positive typhoid cases 14(87.5%) were Immunoblot positive .

Out of 84 culture negative typhoid cases, 60(71.40%) were positive for Immunoblot and only 52(61.90%) for widal. Among the non typhoidal febrile illness cases 02(10%) were Immunoblot positive (02 were *Salmonella paratyphoid A* cases & the other two had respiratory and urinary tract infection).

Of the healthy control subjects none were positive for Immunoblot but 02(10%) were positive for widal test (Fig 1). The immunoblot was more significant in both culture positive and culture negative cases than widal test. Sensitivity and specificity of Immunoblot among typhoid cases was calculated as 74.00% and 95.00% . Widal test was positive in 58(58.00%) out of 100 typhoid cases. In 40 controls 06(15%) were positive and 34(85.00%) were negative. Accordingly sensitivity and specificity was calculated as 58.00% and 85.00% respectively.

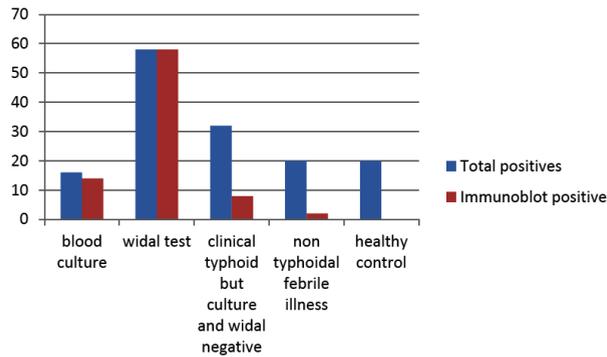


Fig. 1. Categorywise results of Immunoblot Test

SDS PAGE TYPING OF *S.typhi* LYSATES

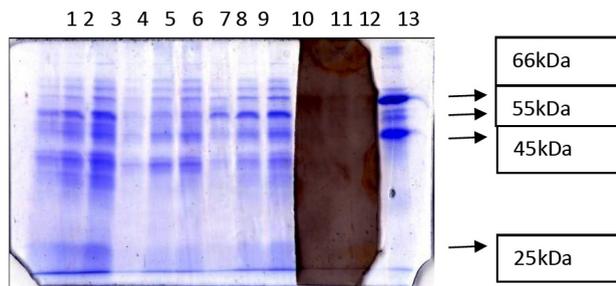


Fig. 2. Ponsseau blue staining: Lane 1: *S.typhi* standard strain - 5µl. Lane 2: *S.typhi* standard strain- 10µl. Lane 3: *S.typhi* standard strain- 15µl. Lane 4: Negative control strain - 5µl. Lane 5: Negative control strain - 10µl. Lane 6: Negative control strain - 15µl. Lane 7: *S.typhi* strain- 5µl. Lane 8: *S.typhi* strain- 10µl. Lane 9: *S.typhi* strain- 15µl. Lane 10 : LPS - 15µg. Silver staining : Lane 11 : LPS – 10µg. Lane 12 : LPS – 5µg. Lane 13: Molecular weight marker – (BSA- 5µg, OVA- 5µg, IgG- 5µg)

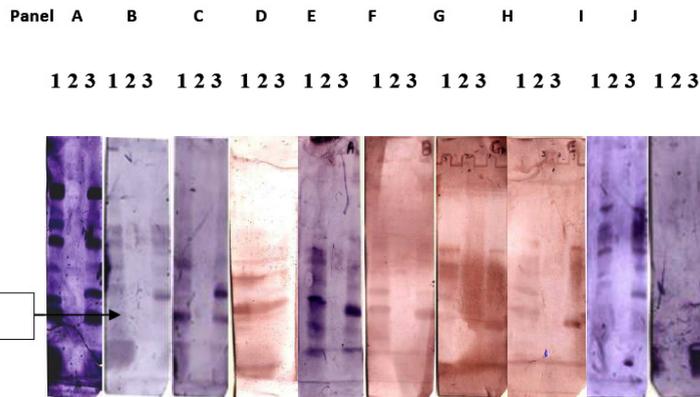


Fig. 3. 10panels, 3 lanes each, as follows: Lane 1: *S.typhi* cells-15 µl. Lane 2: LPS (Sigma)-15 µg. Lane 3: Control cells of another gram negative bacteria-15 µl. Panel A – Positive control sera. Panel B – Negative control sera. Panel C – Patients sample (positive). Panel D - Patients sample (positive). Panel E - Patients sample (positive). Panel F – Patients sample (positive). Panel G – Patients sample (negative). Panel H - Patients sample (negative). Panel I - Patients sample (negative). Panel J - Patients sample (negative)

DISCUSSION

Blood culture and Widal test still prevail as the common tests for diagnosing typhoid fever, with culture being the gold standard. Culture is also helpful for the antibiotic susceptibility testing. However, Blood culture has got a limited utility due to low sensitivity and delay in obtaining the results. Also, facilities for doing culture is not available in many places. It has been reported that sensitivity of Culture is lesser in children compared to adults in diagnosing the infection⁹.

Widal test is a very useful test in endemic areas where culture facilities are limited. The test helps to differentiate typhoid fever from other illnesses. Though Widal is widely used, it has many set-backs, like being non-specific, not standardized properly, difficult to interpret the results¹⁰. Widal test is based on agglutination of patient's antibodies to Salmonella antigens. Moreover, sharing of O and H antigens by other Salmonella serotypes and other members of Enterobacteriaceae makes the role of Widal test even more controversial in diagnosing enteric fever¹¹. Discrepancies in results between different laboratories and within laboratories when the antigen is prepared from different sources is one of the major drawback in Widal test¹².

Widal test may give false positive results in non typhoidal Salmonella infections¹³. On the other hand Immunoblot enables direct observation of antibody binding to antigen profiles. Keeping this in mind this study was carried out in search of an appropriate replacement for Widal test.

Table 1. Category of cases

Category of Cases	No of Individuals
Blood culture positive	16
Widal positive	58
Immunoblot positive	74
Clinically suspected typhoid fever but both culture and widal negative	32

The disease affected all ages, however most of the cases 36% of the study were in the age group of 1-5 years. These findings correlates with the observations made by Saha *et al*¹⁴ who found that children between 2-3years of age were the most susceptible age group (35.6%). Almost similar study done by Sinha *et al*¹⁵ showed 44% children were aged under 5 years, may be due to absence of immunity obtained from mother's milk or they may not drink potable water which is usually used in rural areas¹⁴. In our study, among 100 clinically suspected typhoid cases 63% were male and 37% were female. This finding was similar with Roxas & Mendoza¹⁶ who reported 56% male and 44% female. Another study done by Butler *et al*¹⁷ also showed that infection rate is slightly higher in male, maybe because of increased exposure of male to contaminated food and water outside the home.

In our study, among clinically suspected 100 enteric fever cases 32 cases were both blood culture and Widal test negative. But out of these 32 cases, 8 cases were Immunoblot positive. In the present study Widal test was carried out in a group of patients and controls. In the current study, Widal test is found to be positive in 58% of cases, which is comparable with various other studies by Sherwal BL *et al.* (57%)¹⁸ and Bhutta ZA *et al* (54%)⁵. The cut off value of Widal test was considered as 1:80 for TO and 1:160 for TH¹⁹. Although Widal test usually become positive from second week, in this study out of 16 culture positive typhoid patients 06 (37.77%) had an initial TO titer >80 and TH>160 in the first week of illness. Almost same results were obtained by Hatta *et al* 2002²⁰ where 33 (47.8%) showed Widal positive in the first week of illness. Another study done by Shukla *et al*, 1997²¹ showed 44.2% in the earlier period of illness from suspected patients. This may be due to the increased immunity in a population with constant exposure to *Salmonella typhi* and other Salmonellae²². This is important as paired sera is usually not collected and sent to laboratory and also correlates to the

Table 2. Comparison of results among Widal and Immunoblot

Test	Sensitivity	Specificity	PPV	NPV
Immunoblot	74.00%	95.00%	97.37%	59.38%
Widal	58.00%	85.00%	90.63%	44.74%

concept that specimens collected in the first week of illness are little diagnostic use.

The incidence of false negative Widal test among the bacteriologically proven cases of this study was 10(62.50%). This finding was higher than other studies which showed false negatives in Bangladesh as 11.3%²³, in Iran as 24%²⁴ and 6.9% in Malaysian populations²⁵, Possible hypothesis put forward to explain this phenomenon was, prior use of antibiotics, the existence of less immunogenic strains of *S.typhi* and reduced immunity from severe nutritional hypo proteinemia²⁴. Out of 20 non-typhoidal febrile cases 4(20%) showed high titer in the widal test, Which is similar to the findings of Duthie & French²⁶ with 23% false positive result. Handjoet *al*²⁷ found 7% false positivity for Widal test in non typhoidal cases. But Pang & Puthuchear²⁸ found only 3% false positivity. This may be attributed to the fact that they may be infected by *Salmonella typhi* in the past and may show a non-specific raise now²⁸.

Among 20 healthy controls 2(10%) cases were positive for TH (titer>160) this was similar to Saha *et al*¹, which showed 4.3% false positives out of 300 healthy Bangladeshi children who had a TH titers of >160. These raised TH titer among healthy controls may be due to previous exposure to *Salmonella typhi* as typhoid is endemic in our region¹.

With Immunoblotting our study showed a positivity of 74% out of 100 clinically suspected case of typhoid fever, whereas widal showed only 58% positivity. The sera from 14 culture-positive patients gave strong immunoblot reactions with the *Salmonella typhi* culture filtrates, confirming that patients mount a humoral antibody response to the antigens during infection with this organism²⁹. The sensitivity and specificity of Immunoblot in the present study was 74.00% and 95.00% respectively. This was relatively high when compared to that of widal which showed a sensitivity of 58.00% and specificity of 85.00%. Hence the Immunoassay described here provides a sensitive means of detecting antibodies to soluble proteins of *Salmonella typhi* with a positive predictive value of 97.37% and negative predictive value of 54.55%. The double band formed at 37kDa of the *S.typhi* culture filtrates was found to be specific for Salmonella, as it did not appear in the negative

control strain of the other Gram negative bacilli as well as in the negative control sera. Hence it was found to be highly specific.

The LPS obtained commercially did not show up initially in SDS-PAGE using Coomassive brilliant blue. Then it was stained by silver solution which showed up at 15 & 30µg. But did not appear in blotting. This may be attributed to the fact that it must have been further diluted and the molecular weight reduced for the protein band to appear on the gel. Further on during the processing it appeared faintly in certain cases where we did an extended blocking of overnight. Hence all these might be taken up for future studies. Limitations of the study were, it was quite expensive than widal, difficult to maintain the stability of reagents and requires skilled personnel to perform. This study is in accordance with the previous studies, which reports that SDS PAGE along with immunoblotting is reported to be more sensitive than WIDAL assay for detecting the LPS and flagellar Salmonella antigens. Immunoblot provides sensitive means of detecting the serum

CONCLUSION

Thus this study shows that the immunoblotting procedure incorporating *Salmonella typhi* culture filtrates is more sensitive than the established Widal agglutination assay for providing evidence of infection with *Salmonella typhi*. Immunoblot technique has been simplified extensively in our study. Further, in contrast to Widal test which requires 2 days, Immunoblot can be done in few hours. After analyzing the findings of the present study it was concluded that though blood culture is gold standard for diagnosis of typhoid fever along with rising titer of Widal test, Typhoid IgM / IgG and Immunoblot with their higher sensitivity and specificity might actually be a practical alternative test for diagnosis of enteric fever. Even though bacteriology remains the gold standard, serology can be a good adjunct and make valuable additions in diagnosis of Salmonella infection. From our study, we conclude that Immunoblotting technique can be an effective alternative to Widal test in the diagnosis of typhoid fever.

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