

COMPARISON OF RESULTS OBTAINED BY WIDAL AGGLUTINATION TEST & POLYMERASE CHAIN REACTION AMONG CLINICALLY SUSPECTED TYPHOID FEVER CASES

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ABSTRACT

The diagnosis of typhoid fever currently depends on isolation of Salmonella Typhi from blood. The sensitivity of blood culture is very low due to prior antibiotic treatment which is a common practice in Bangladesh. The sensitivity of blood culture also decreases at later stage of the disease. Widal test is the most utilized test in Bangladesh next to blood culture because it is inexpensive, less invasive. But the result of the test is controversial due to false negative & false positive results in some cases.

In this study, a recently introduced polymerase chain reaction-based technique (which has 100% specificity for S. Typhi) was compared with widal test among 80 clinically suspected typhoid fever cases.

Among 80 cases, the respective figures of positivity for PCR & widal test were 70% & 43.75% respectively.

It can be concluded that PCR based technique is more sensitive & much superior to widal for diagnosis of typhoid fever.

Keywords: Typhoid fever; nested PCR; widal test.

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INTRODUCTION

Typhoid fever, one of the major bacterial infections worldwide, is caused by the human-adapted *S. enterica* serovar Typhi.¹ Typhoid fever is a major public problem in developing countries like Bangladesh. Current estimation shows that total number of typhoid fever episodes was 13.5 million in 2010.² A case of typhoid fever is defined as a person having one or more of the following characteristics- prolonged fever; disturbances of bowel function; headache, malaise and anorexia; rose spots on the chest, abdomen and back.³ Without effective treatment, typhoid fever has a case-fatality rate of 10–30%. This number is reduced to 1–4% in those receiving appropriate therapy¹. Results obtained from the laboratory are important in confirming the clinical diagnosis of typhoid and contribute to the effective management and treatment of typhoid cases.⁴

Isolation of the *S. Typhi* remains the most effective diagnostic procedure in suspected typhoid fever cases

until today. Delayed results obtained with blood culture (2 to 3 days) and decreased Isolation rates due to the widespread practice of early antibiotic administration have limited its practical use in the diagnosis of typhoid fever.⁵

Widal test is simple & inexpensive test which is considered next in the value to blood culture in developing countries. Though widal test gives false positive & false negative results, it is very popular in Bangladesh. Other tests such as Dot EIA, Tubex, ELISA, also has been done but these tests also have some drawbacks. So, efforts have been given to develop more sensitive & specific tests for the diagnosis of typhoid fever.

Recently, nucleic acid amplification tests such as conventional PCR, nested PCR have been explored for detection of specific gene sequence in clinical samples. It can be used in patients having prior antibiotic treatment where blood culture & other tests such as widal, Typhi-dot may become false negative.

This study aimed at comparing the results of widal agglutination test which is invariably done in Bangladesh and polymerase chain reaction among suspected typhoid fever cases.

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MATERIALS AND METHODS

The study was carried out in the Department of Microbiology & Immunology, Bangabandhu Sheikh Mujib Medical University. Serum samples from a total of 80 cases of clinically suspected typhoid fever were collected and subjected to both widal test & PCR in the microbiology laboratory.

Specimen collection:

From each individual included in this study, 4 ml of blood was drawn by vein puncture using disposable syringes. Two ml of blood was placed in plastic disposable tubes, it was left to stand at room temperature (20-25°C) to allow it to clot, then the sera was separated by centrifugation for 5 minutes, and kept in a eppendorf tube and stored at -20°C till examination. Two ml of blood was kept in a EDTA tube for PCR & stored at -20°C until examination. All sera and reagents were allowed to stand at room temperature before use in the test.

Widal Test

Principle of the assay

The widal test measures serum agglutinins against somatic and flagellar antigens. When an antibody combines with a corpuscular antigen (forming part of a cell - e.g. bacteria, or inert part with bound antigen) the cells agglutinate, that means they form clumps (i.e. the clumping of cells such as bacteria in the presence of an antibody. The antibody or other molecule binds multiple particles and joins them, creating a large complex).⁶ Titers greater than 1 in 80 for 'O' and 1 in 160 for 'H' antigens were taken as cut-off tires for seropositivity.

Assay procedure

1. Using a pipette, 20 µl undiluted serum was taken on 3 cm diameter circles on white tile.
2. Using a dropper one drop of appropriate well- shaken antigen suspension (TO, TH) obtained from Biotec Laboratories, UK was added to each serum aliquot.
3. Both were mixed by stirring for a few seconds with a wooden applicator stick.
4. The tile was rotated slowly and agglutination was read at one minute.
5. If agglutination occurred, then double dilution of the serum was done and the test was repeated.

Extraction of DNA from blood sample:

DNA extraction from blood was done according to the procedure done by Nagarajan *et al.* 2009.⁷ One ml EDTA containing blood was centrifuged by micro centrifuge at 13000 rpm for 5 minutes. Supernatant was discarded. Then one ml 0.2% Triton X-100 (Sigma,

USA) was added to the pellet. The mixture was vortexed, incubated at room temperature for 10 minutes and centrifuged at 13000 rpm for 10 minutes. Supernatant was discarded. One ml 0.2% Triton X was added to the pellet again, vortexed and centrifuged at 13000 rpm for 10 minutes. Then washed with 1 ml nuclease free water, centrifuged at 13000 rpm for 10 minutes & supernatant discarded. The pellet was resuspended 30µl nuclease free water. Boiled for 10 minutes at 99°C then centrifuged for 3 minutes. Supernatant was used as template for PCR.

Contamination Precautions

DNA extraction and PCRs were performed in separate rooms, using different sets of pipettes and tips. For further safety, all the reagents used in the PCR were aliquoted, so that an aliquot was used only once and then discarded.

Primer

Primers which target the flagellin gene of *Salmonella typhi* which is designed by Song *et al.* 1993⁸ were synthesized on Jena Bioscience, Germany. Oligonucleotides ST1 (5'- ACT GCT AAA ACC ACT ACT-3') and ST2 (5'- TTAACGCAGTAAAGAGAG-3'), which were used for regular PCR to amplify a 458 bp fragment. For nested PCR, oligonucleotides ST3 (5'- ACT GCT AAA ACC ACT ACT -3') and ST4 (5'- TGGAGACTTCGGTCGCGTAG-3') were used to amplify a 343 bp fragment.

PCR Conditions

For regular PCR, 10 µL master buffer contained PCR buffer, MgCl₂, deoxy nucleoside triphosphate obtained from Texas Biogene Inc. USA, 1.5 µl of each primer, 0.2 µl of *Taq* polymease, 2 µL of template are taken in a 0.2 ml PCR tube. Using a thermal cycler (Applied biosystem 2720), the reaction mixture was subjected to 40 cycles of 1 minute each at 94°C, 55°C and 72°C, followed by heating at 72°C for 10 minutes. For nested PCR, conditions were the same, except that a 1:5 dilution of amplified product was used as template.

Detection of PCR Products

A reaction mixture of 10 µL was fractionated electrophoretically in 2% agarose gel containing 0.5 µg of ethidium bromide per ml. A positive control representing diluted suspension containing DNA (1:200) from isolate of *S. typhi*, and a negative control without any DNA were also included in each lot.

RESULTS

Table I shows distribution of sex among the suspected typhoid fever cases. Among 80 cases, male were 52 (65%) & female were 28 (35%). Male & female ratio was 1.85: 1.

Table - I

Distribution of suspected typhoid fever cases according to gender.

Sex	Number of suspected fever cases	Percentage
Female	28	35
Male	52	65
Total	80	100

Table II shows age range of the study population. The results of the study showed that the age ranged between (3-48) years. Age distribution of 80 cases showed that majority of the cases (21.25%) belonged to the age group of 1-5 years followed by (17.5%) to the age group of 6-10 years group which was not statistically significant ($P>0.05$).

Table II

Distribution of the suspected typhoid fever cases according to age

Age groups	Number of suspected typhoid fever case	Percentage
1-5	17	21.25
6-10	14	17.5
11-15	6	7.5
16-20	11	13.75
21-25	7	8.75
26-30	12	15
> 30	13	16.25
Total	80	100

Table III shows results of PCR & widal test performed for the diagnosis of typhoid fever. Among the study population, 56 (70%) cases were positive for *S. Typhi* by PCR & 35 (43.75%) cases were widal positive. The results of widal test in this study are presented in (table – IV). A total of 18, 13, 4 out of 80 cases had significant O agglutination titer value (1:80, 1:160, 1:320 respectively) & therefore regarded as positive. On the other hand, a total of 9, 7, 15, 4 out of 80 cases had significant H agglutination titer value (1:160, 1:320, 1:640, 1:1280 respectively) & regarded as positive.

Table III

Results of PCR & widal test performed for the diagnosis of PCR

Name of the tests	Results of the test	
	Positive (%)	Negative (%)
PCR	56 (70)	24(30)
Widal test	35 (43.75)	45 (56.25)

Table IV

Anti S Typhi O & H antibody titers in serum of suspected typhoid fever cases detected by widal test

Widal test result	No. of suspected typhoid fever cases	Percentage
1: 80	18	51.4
Antibody titers to S Typhi O 1: 160	13	37.1
1:320	4	11.4
1:640	0	0
Antibody titers to S Typhi H 1:80	9	25.7
1:160	7	20
1:320	15	42.8

Comparison between PCR & widal test for the diagnosis of typhoid fever are presented in (Table-V).

Among the study population, 29 (36.3%) cases were both test (PCR & widal) positive & 18 (22.5%) cases were both test negative. Six cases were widal positive but negative by PCR. Twenty seven cases were PCR positive but widal negative.

Table V

Comparison between PCR & widal test for diagnosis of typhoid fever among the study population

Results of PCR	Results of widal test		Total (%)
	Positive (%)	Negative (%)	
Positive	29 (36.3)	27(33.8)	56(70)
Negative	6(7.5)	18 (22.5)	24 (30)
Total	35 (43.8)	45 (56.3)	80(100)

DISCUSSION

Typhoid fever is a major health problem causing significant morbidity & mortality in developing country till today due to poor sanitary condition. The study shows that majority (52%) of the suspected typhoid fever patients was male & the male female ratio was 1.82. Both sex can be affected by typhoid fever. This findings correlate with the findings reported in several studies in Bangladesh by where (55%)⁹ and (54%)¹⁰ typhoid fever patients were male. The higher frequency of infection with typhoid fever among males may be attributed to socio-community nature of Bangladeshi people which makes men undergone the responsibility of working and eventually are in great contact with the pathogens rather than the women. High percentage of infection (21.25%) occurs in the age group of 1-5 years which is similar with the findings of some studies done in Bangladesh.^{9,10} Infection can occur at any age but the results of several studies showed that the prevalence of typhoid fever in children of under 5 years were much higher than other age group.¹¹

The diagnosis of typhoid fever on the basis of clinical feature is difficult because they are also present in other febrile illness. Widal test is the most utilized test which is used in Bangladesh. Single-tube widal test was applied in this study. Originally widal test was recommended using paired sera, 1-2 weeks apart & demonstrating four-fold or greater rise of antibody titer. However, in typhoid fever, a rise in titer between acute and convalescent sera is not always demonstrable even in blood culture confirmed cases, owing to the natural history of the infection, prior antibiotic administration or late presentation to the hospital. Patient management decisions cannot be put off for the results of convalescent phase sera and for all practical purposes, a treatment decision must be made on the basis of a single tube widal test.¹²

There is no consensus in literatures which shows guideline about the diagnostic criteria for interpreting widal test. Widal test interpretation in endemic areas like Bangladesh is difficult because majority of healthy individual has detectable antibodies. There is controversy about the predictive value of O & H antibodies for diagnosis of enteric fever.¹³ Several studies show that a raised O agglutinin is of slightly greater diagnostic value than a raised H agglutinin because this antibody decline early after acute infection.^{13,1} In this study the high titres (>1/80 for *Salmonella typhi* "O" and >1/160 for *salmonella typhi* "H" antigens) in the Widal test performed on single acute-phase sera was considered as significant and diagnostic. According to this criteria, 35 (43.75%) cases were widal positive.

Another diagnostic method for diagnosing typhoid fever cases such as nested PCR has been recently introduced. Several studies showed that PCR technique offers highly specific, sensitive and reasonably quick diagnostic

modality.^{8,15} Even 1-5 bacteria/ml can be detected with absolute specificity within 1-2 days.¹³ We decided to check this theoretical promise of PCR in the actual situation and compare it with widal test.

We used flagellin gene to detect *S. Typhi* by PCR, because flagellin gene of *S. Typhi* has unique nucleotide sequences in hypervariable region VI which are different from those sequence in other strains of *Salmonella*.⁸ The result shows that 56 (70%) suspected cases were positive for *S. Typhi* by PCR. The results of this study agreed with the results of similar study done by Haque et al. 1999 who reported that a significant difference has been noticed between widal test and PCR for detection of typhoid fever.¹⁶ Additionally, Prakash et al. 2005 has found that a considerable difference among widal and PCR for investigating typhoid fever, also found that PCR was not only relatively rapid but also more accurate than traditional method (widal test).¹⁷

This study showed that 6 (7.5%) cases were widal positive but PCR negative. Among these 6 cases, 2 were diagnosed as dengue fever, 1 was diagnosed as viral hepatitis later. This positive widal test might be due to cross reaction by non-typhoidal antigens in some organisms (malaria, dengue, miliary tuberculosis, endocarditis, chronic liver disease, brucellosis, etc).⁶

In this study, 27 (33.8%) cases were PCR positive but widal negative. Possible hypotheses put forward to explain this phenomenon are prior use of antibiotics, the existence of less immunogenic strains of *S. Typhi*, reduced immunity in some patients from severe nutritional hypoproteinaemia.¹⁸ Without PCR, these cases would have been missed.

CONCLUSION

Typhoid fever is endemic in Bangladesh, so a local titer is present in healthy population which should be reviewed yearly. Although, widal test is a popular test in Bangladesh, it can give false negative or false positive results which lead us to a wrong diagnosis resulting initiation of inappropriate treatment. As a result, the chance of development of multidrug resistant strains increase which increases the cost of treatment & patient's sufferings. PCR can overcome the drawbacks of widal test as it is positive in cases where widal test is false negative. It is negative in cases where widal is false positive & can prevent the initiation of unnecessary treatment. So, it can be concluded that PCR is accurate, precise, objective and well suitable for routine determination of typhoid fever.

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