Asian Jr. of Microbiol. Biotech. Env. Sc. Vol. 21, No. (4) : 2019 : 980-986

ISSN-0972-3005

STUDY OF VARIOUS LABORATORY METHODS FOR AN EARLY DIAGNOSIS OF ENTERIC FEVER

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(Received 25 February, 2019; accepted 10 May, 2019)

Key words: Early diagnosis, Enteric fever, IgM-ELISA, Nested PCR, Latex agglutination test, Widal test

Abstract – The clinical diagnosis of enteric fever is difficult due to the similarity of symptoms to the other febrile illnesses. Laboratory diagnostic tools currently in use are either not reliable or take time contrary to the requirements. Bacterial culture with its inherent limitation of poor sensitivity is still used as the gold standard, but this facility may not be available in many laboratories more so in endemic areas. Serological tests for detection of antigen and antibody as well as molecular detection test have variable results. Aim and objective: To develop a strategy for rapid and reliable laboratory diagnosis in a suspected case of enteric fever, by detection of anti-Salmonella antibody, especially IgM from patients' serum and antigens as well as *S. typhi* genome from enriched bile broth. The study included patients with fever for maximum duration of ten days. A total number of 117 samples of such patients were subjected to blood culture (BacT/Alert ®PF Culture Bottles) and/or clot culture (in bile broth). Patients' serum was used for antibody detection by IgM-ELISA as well as Widal test and enriched bile broth was used for flagellar H antigens detection by latex agglutination test and bacterial genome detection by nested PCR. Both IgM ELISA and nested PCR methods were specifically designed for detection of *S. typhi* in fection. Latex particles coated with specific *Salmonella* H antibody were usedfor detection of flagellar H antigens from bile broth. Widal test was also performed as a routinely used method in diagnosis of enteric fever. Sensitivity, specificity, positive predictive value and negative predictive value of tests were calculated against blood and/ or clot culture as a gold standard. Keeping in mind the low sensitivity of blood/clot culture, as an alternative strategy, acombination of a battery of three or more tests were evaluated for laboratory confirmation against blood culture. Agreement between the results of more than one test other than blood/clot culture was also evaluated. Excel 2007 and SPSS 15.0 were statistical software used for data analysis of thetests. Out of forty four culture isolates, *S. typhi* was found in 73%, *S. paratyphi* A in 23% and *S. paratyphi* B in 4%. The most common age affected was between 6 to 15 years. In comparison to the blood culture as gold standard, nested PCR was found to be 100% sensitive, with specificity of only 9.6%. Widal test had maximum positive predictive value (46.9%) followed by IgM-ELISA (40.4%). Latex agglutination test showed maximum specificity of 70.45% against gold standard. Blood culture sensitivity was 43.3% with 5.5% negative predictive value when compared with three or more battery tests. Battery of two or three rapid tests like, IgM-ELISA, Latex agglutination and nested PCR can be used as a good strategy for rapid diagnosis of enteric fever. However, nested PCR must be studied further to evaluate it as gold standard for an early diagnosis of enteric fever.

INTRODUCTION

Enteric fever is a potentially multisystem illness, caused by *Salmonella typhi* and *Salmonella paratyphi* A, B and C (Kumar *et al*., 2013). *Salmonella* spp is acquired by feco-oral route making them endemic in developing countries. The burden of the disease in India, as reported by Iyer *et al*., 2005 to 2011 was 112,884 cases with average rate of 26.71 per 100,000 populations (Iyer *et al*., 2014). Febrile illness with high burden needs to be diagnosed and treated early as delayed or inadequate treatment may lead to acute or chronic complication as well as development of a carrier state in around 2-5% of

individuals (Gunn *et al.*, 2014). Bacterial shedding continues for long times, which is risky as the patient becomes a source of infection for others (Caygill, *et al.*, 1994; Huang and Du Pont, 2005). Death in a case of enteric fever may occur from overwhelming infection, but with appropriate and early treatment, prognosis is good and development of complications as well as drug resistance can be avoided.

An accurate diagnosis on clinical grounds alone is difficult and more so in endemic areas. Currently, the laboratory diagnosis of enteric fever is performed by isolating *S typhi* and *S. Paratyphi* from a clinical sample or by detection of rising antibody titer by Widal test against the lipopolysaccharide antigen (O) and flagellar antigens (H,AH,BH). Widal test with all its limitations of variable sensitivity and specificity is the most widely used test in routine diagnosis and blood culture is still considered as the gold standard, but not useful for early diagnosis of enteric fever (Wain and Hosoglu, 2008). Bacterial culture facility for definitive diagnosis of enteric fever may not be available and is expensive for people with low socioeconomic status. Amount of blood collected, time of collection and exposure to antibiotics influence the outcome of blood culture. Clot culture is expected to be better over whole blood culture as the bacteria is expected to be concentrated and inhibitory substances present in the serum are removed (Watson, 1954) and the separated serum can also be used for further investigation.

Rapid diagnosis of enteric fever is important to eliminate infection, prevent outbreak, reduce carrier and to decrease antibiotic resistance. An early diagnosis of enteric fever is possible by laboratory methods that can detect antigen and/ or bacterial genome with or without amplification. Antibody detection, especially IgM by Enzyme-linked immunosorbent assays (ELISA) has also been considered as an alternative approach for the rapid diagnosis of enteric fever, based on detection of anti-LPS antibodies and has been reported to be more sensitive as well as more specific than the Widal test (Gopalakrishnan, *et al*., 2002; JE Sippel *et al*., 1987). Nested PCR specific for *S.entericaserovartyphi* can be used even when antibiotics have been started with high specificity and sensitivity (Frankel, 1994; Hatta and Smits, 2007; Khan *et al*., 2012). In this study we have made an effort to evaluate various methods available for an early detection of antibodies, antigen and/or genome, of *S.typhi* and/or *Salmonella paratyphi*.

MATERIALS AND METHODS

This cross-sectional study, duly approved by Human Research Ethics Committee (HREC) was conducted at a tertiary care hospital and teaching institute from July 2014 to April 2016. During the study period, 117 blood culture specimens were received and processed for diagnosis of enteric fever in the microbiology section of central diagnostic laboratory (CDL). 5 to 10 mL of peripheral blood sample was collected aseptically for culture and inoculated into 30 mL BacT/Alert ®PF Culture Bottles. For serology and clot culture 2.0 to 3.0 mL of peripheral blood was collected and inoculated separately into 4.0 mL BD Vacutainer plain tube. Automated BacT/ALERT Microbial Detection System and Vitek2 compact system was used for isolation, identification and antimicrobial sensitivity of organism.

Clot culture (Mathew and Jobin, 2013; Rockhill, *et al*., 1980; Watson, 1955)

Under aseptic condition,2.0 to 3.0 mL of peripheral blood was collected into 4.0 mL BD Vacutainer plain tube from the patient suspected of enteric fever. Serum was used for serological testing and blood clot was transferred directly into McCarty bottle containing bile broth base (HiMedia) with streptokinase and glass beads to enhance the clot lysis. Bile broth culture was incubated at 37°C for five to six hours and then centrifuged in a cold centrifuge at 3000 rpm for 15 minutes. This modification was done for reducing the incubation time of clot culture and concentration of organisms. One drop of centrifuged deposit was platted on solid media (MacConkey agar) for bacterial isolation while part of the deposited sample was used for antigen and genome detection. The remaining part of deposits was re-suspended with 10 mL of fresh bile broth, incubated at 37 °C and subcultured on MacConkey agar after 48, and 72 hours interval.

Hi *Salmonella*™ Latex test Kit, an antigen detection test (Himedia) where latex particles are coated with polyvalent antibodies against *Salmonella species* was used to detect flagellar H antigens directly from clinical samples. This simple and rapid test is originally made for detection of H antigens from pure colonies.

S. typhi isolated from a patient was used as a positive control for genome detection and for

internal quality control for DNA extraction,.

Enriched clot culture were preserved at -20°C before use for Nested Polymerase Chain Reaction (NPCR). Nested PCR and IgM-ELISA were tested for *S. typhi* strains only.

Nested PCR protocol:

The primers used in the process were-

- First set of primers;
  - ST1: (5′-TATGCCGCTACATATGATGAG -3′)
  - ST2 (5′-TTAACGCAGTAAAGAGAG-3′)
- Second set of primers;
  - ST3: (5′-ACTGCTAAAACCACTACT-3′)
  - ST4 (5′-TGGAGACTTCGGTTGCGTAG-3′)

Primers targeting the flagellin gene (FliC) of *S.typhi* were used, verified by Xcelris™ Labs Limited, an Albert company, in Gujarat, India. Oligonucleotides ST1 and ST2 were used for regular PCR to amplify a 497 bp fragment, corresponding to nucleotides 1024-1044 and 1504-1521, respectively, in the flagellin gene of *S.typhi*. For nested PCR oligonucleotides ST3 and ST4 were used to amplify a 366 bp fragment corresponding to nucleotide 1060-1077 and 1407-1426. Master Mix was prepared in Eppendorf tube and reaction was carried in PCR tube (20 µL/reaction tube). The reaction mixture was subjected to 34 cycles; for denaturation for 30 seconds each at 95 °C, annealed at 52 °C for 30 seconds and elongation at 72 °C for 45 seconds. For nested PCR, all steps of amplification were similar to the first round, except that annealing was done at 68 °C for 75 seconds. After preparing a reaction system all the reaction tubes were kept in Bio-Rad thermo cycler and were allowed to run for the following programmed time; 95 °C for 10 minutes one cycle, 95 °C for 30 seconds for 34 cycles, 52 °C for 30 seconds for first set of primer and 68 °C for 75 seconds in second set of primer (nested PCR). 72 °C for 45 seconds, repeat the cycle from step 2 for 34 times in first set primer and 38 times in the second set of primer (nested PCR) for 72 °C for 10 Minutes and 4 °C for 10 Minutes.

After completion of the thermo cycles, 5 µL from each reaction tube was loaded in 2% agarose gel containing EtBr for 35 minutes at 100V. PCR products were visualized under UV transilluminator of Bio-Rad gel doc system. Results for FliCflagellin gene of *S.typhi* were directly interpreted from electrophoresis of nested PCR. The product of 366 base pair (bp) was compared with the control in electrophoresis. 100 bp DNA ladder was loaded along with sample to compare fragment size.

Widal tube and slide test (Tydal®, Tulip, diagnostic (P) LTD) were performed to detect anti-O, H, AH and BH antibodies in patient serum with antigen suspension. For samples found positive by slide tests, Widal tube test was performed to detect titre and the results were reported after 24 hours.

Indirect micro well-ELISA (Typhiwell, Anand Brothers, Pvt.Ltd) was used to detect IgM antibody raised against *S.typhi* (anti-lipopolysaccharide O antigen). Serum diluents containing sorbent to remove human IgG interference and rheumatoid factor were added to wells coated with purified *S.typhi* antigen.

For quality control, each batch of suspected samples was processed with positive and negative controls provided by the company. Known positive as well as negative samples were also used for Internal Quality Control (IQC).

Patient's data was collected from medical record department at research location, as per onset of illness starting from 1st to 10th days only. Cross sectional study was aimed for early detection of enteric fever. To find out the performance of the tests, their sensitivity, specificity, positive predictive value and negative predictive value against blood culture and/ or clot culture as gold standard was calculated. Due to lower sensitivity of gold

Table 1a. Performance analysis of Latex agglutination test for S. *typhi and* S. *paratyphi* antigen detection in comparison to blood culture and/or clot culture for suspected cases within 10 days of history of pyrexia.

Test evaluated	Results of Blood Culture/Clot Culture			Total
		Positive	Negative	
Latex agglutination	Positive	3	13	16
	Negative	14	31	45
Total		17	44	61

Table 1b. Test value

Sensitivity	Specificity	PPV	NPV
17.6%	70.45%	18.75%	68.8%

standard, alternatively, an agreement of a battery of test was also evaluated against other tests. MS Excel sheet 2007 and SPSS 15.0 software was used for data analysis.

RESULTS

Out of forty four culture isolates, *S. typhiwas* found in 73%, *S.paratyphi* A in 23% and *S. paratyphi* B 4%. Enteric fever suspected cases were received maximum from the pediatrics 42% (N=49) and medicine 38% (N=45) departments.

The majority of cases i.e. 28% (N=33) were in the age group of 6 to 15 years while the minimum, i.e. 9% (N=7) was in the age group of 36-45 years. Male to female ratio was 1.4:1 with 58% (N=68) male and 42 % (N=49) females in the study.

Various tests were compared with gold standard (blood and /clot culture). Sensitivity, specificity, positive and negative predictive values were determined to evaluate the performance of various tests and are given in following tables:

As seen in above table, significant number of cases (17) were blood culture negative but were identified as positive by any of the three tests in above combination. This signifies limitation of blood culture when used as gold standard. Blood culture though has a 100% specificity and positive predictive value but it's sensitivity (43.3%) and negative predictive value (5.5%) are low.

DISCUSSION

To reduce the mortality rate and elimination of infection by *Salmonella* spp, especially in an endemic area, reliable and rapid laboratory diagnosis is necessary. An early diagnosis of enteric fever by using available methods such as blood culture, IgM-ELISA, Latex agglutination test and nested PCR, is not satisfactory due to variations in their sensitivity and specificity. Blood culture though considered as the gold standard, has its own limitations (Sultana *et al.*, 2016). In this study we have tried to compare the performance of different tests available for early

Table 2a. Performance analysis of IgM-ELISA test for *S.typhi* antibody detection in comparison to Blood Culture and/or clot culture for suspected cases within 10 days of history of pyrexia.

Test evaluated		Results of Blood Culture/Clot Culture		Total
		Positive	Negative	
Results of ELISA IgM*	Positive	19	28	47
	Negative	6	38	44
Total		25	66	91

(*) method target S.*typhi* only, while S.*paratyphi* was excluded.

Table 2b. Test value

Sensitivity	Specificity	PPV	NPV
76.0%	57.57%	40.4%	86.36%

Table 3a. Nested PCR for *S.typhi* gene detection in comparison to Blood Culture and/or clot culture for suspected cases within 10 days of history of pyrexia.

Test evaluated		Results of Blood Culture/Clot Culture		Total
		Positive	Negative	
Nested PCR*	Positive	26	47	73
	Negative	0	5	5
Total		26	52	78

(*) method target S. *typhi*only, while S. *paratyphi* was excluded.

Table 3b. Test value

Sensitivity	Specificity	PPV	NPV
100.0% 9.6%	35.6%	100.0%	

Table 4a. Widal test for S. *typhi* and S. *paratyphi* antibody detection in comparison to Blood Culture and/or clot culture for suspected cases within 10 days of history of pyrexia.

Test evaluated		Results of Blood Culture/Clot Culture		Total
		Positive	Negative	
Results of Widal test	Positive	23	26	49
	Negative	7	43	50
Total		30	69	99

Table 4b. Test value

Sensitivity	Specificity	PPV	NPV
76.6%	62.3%	46.9%	86.0%

Table 5a. Performance of blood and/clot culturecompared to pooled results of other methods

Test evaluated	Nested PCR/IgM-ELISA/Latex agglutination/Widal tests			Total
		At least three tests Positive	All Negative	
Blood and/clot Culture	Positive	13	0	13
	Negative	17	1	18
Total		30	1	31

diagnosis of enteric fever along with the demography of the cases enrolled. Majority of the cases in our study were in the age groups of 6 to 15 years (28%, N=33), which is similar to the study conducted by AFM Sattar *et al.* (2016). They concluded that typhoid fever is most common in the above age group (Sattar *et al.,* 2016). Male (58%) were more in comparison to female (42%) in our study, giving a M:F ratio of 1.4:1 which is similar to findings by Prajapati *et al* who found a M:F ratio of 1.1:1 with 53.2% male and 46.8% female (Prajapati *et al.,* 2008).

Among the culture positive cases, *S.typhi* was the main species isolated in 73% cases (n=32) followed by *S. paratyphi* A in 23% (n=10) and *S. paratyphi* B in 4% (n=2) in our study. Similar distribution has been found in a study conducted in Lagos, Nigeria and Ispat hospital, Rourkela, India where they isolated S.typhi in 67.3% and 79% cases whereas *S. paratyphi* in 16.8% and 21% cases respectively (Bhattacharya *et al.,* 2011; Kabir'O *et al.,* 2000).

Blood culture though considered as the gold standard has poor sensitivity (43.3%) as has been found by us and other investigators (Guerra-Caceres *et al.,* 1979). Bone marrow culture (95% positivity) though has a higher sensitivity in comparison to blood culture (only 43.3% positivity) (Guerra-Caceres *et al.,* 1979), its use as a gold standard, is difficult due to painful technique and it may not be pertinent as a routine diagnostic test for enteric fever.

This makes it necessary to evaluate the performance of other more sensitive and user friendly tests either alone or in combination with each other and develop a strategy for early diagnosis of enteric fever. When using blood culture as gold standard, IgM antibodies detection by ELISA was found to have sensitivity of 76.0% and specificity of 57.57% in our study, which was 64% and 95% respectively in a study conducted by Sippel *et al.* (1989).

The sensitivity, specificity, positive predictive value and negative predictive value of Widal test in our study was 76.6%, 62.3%, 46.9% and 86.% respectively compared to study conducted by Andualem *et al*. (2014) where they found sensitivity, specificity, positive predictive value and negative predictive value to be 71.4%, 68.44%, 5.7% and 98.9 respectively (Andualem *et al*., 2014).

Latex agglutination test is recommended for the confirmatory identification of presumptive *Salmonella* colonies from selective agar plates and it has been found to have 100% specificity and 98.4% sensitivity (Bouvet and Jeanjean, 1992).

We detected *Salmonella* antigen directly from the enriched clot culture fluid and found it to have 70.45% specificity and 17.6% sensitivity when compared with blood culture. No such study is found in literature to compare the performance.

Nested PCR is considered to be highly sensitive

test and we had 100% sensitivity and negative predictive value when compared with blood culture but specificity of the test was poor (9.6%) with positive predictive value of 35.6%. Sensitivity and negative predictive value of the nested PCR was 100% while specificity as well as positive predictive value was 76.9% in the study conducted by Khan *et al.* (2012)

For evaluation of any test, the gold standard is required to be the one with highest specificity and sensitivity but single blood culture, as a gold standard test for diagnosis of enteric fever is not suitable for the purpose. We evaluated three battery tests with the performance of blood culture and found that blood culture has a sensitivity of only 43.3% with a negative predictive value of 5.5%. Seventeen cases in our study were positive in at least three different tests while blood culture was negative which indicates the limitation of blood culture as gold standard test. With availability of more sensitive and specific test other methods like PCR need to be considered as gold standard and evaluated.(Prakash, *et al.,* 2005)

CONCLUSION

Findings of our study reveal that battery of three rapid tests like IgM-ELISA, Latex agglutination and PCR can be used as good strategy for rapid diagnosis of enteric fever. However nested PCR can be evaluated further as gold standard for an early diagnosis of enteric fever.

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