Original Article

Comparative Study for Early Diagnosis of Typhoid Fever by Blood Culture, Widal Test and Polymerase Chain Reaction in a Tertiary Hospital, Bangladesh

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Abstract

Background: Typhoid fever is an infectious disease in developing countries like Bangladesh. Laboratory diagnosis of typhoid fever is a major challenge still now. PCR could be a reliable test in the diagnosis and management of typhoid fever. **Objective:** To detect the flagelline gene (*fli C*) of *salmonella typhi* from blood by nested PCR for early and reliable diagnosis of typhoid fever and to compare it with blood culture and Widal test. **Methods:** This was a descriptive type of comparative study conducted among clinically suspected patients of typhoid fever attending the outpatient department of Barind Medical College, Rajshahi, Bangladesh. In this study, 80 clinically suspected patients of typhoid fever were included. From all patients, blood was collected, blood culture, Widal test and nested PCR targeting the flagelline gene (*fli C*) of *Salmonella typhi* were done. **Results:** The positivity rate of PCR and blood culture was 83.7% and 15% respectively. The positivity rate of PCR was significantly higher than blood culture (P< 0.05).With the nested PCR, *S.typhi* DNAs were detected from blood specimens of 82 % patients among the suspected typhoid fever cases on the basis of clinical features with negative cultures. **Conclusion:** PCR technique could be used as a novel diagnostic method of typhoid fever, particularly in culture negative cases in an endemic country like Bangladesh.

Key words: early diagnosis, typhoid fever, nested PCR.

Introduction

Typhoid fever caused by *salmonella typhi,* has aglobal presence. World over 17 million people are affected annually by typhoid fever and resulting in approximately 600000 deaths.[1] While typhoid fever endemicity is geographically widespread, the burden is particularly high in the southern Asia with an incidence rate of 100 to 1000 per 100,00 population.[2] It is endemic in the Indian subcontinent including Bangladesh, South-East and Far–East Asia, the middle East, Africa, central and south America.[3] Its a systemic infection and faeco orally transmitted, thus more infection occurs in an environment with over crowding, poor sanitation and untreated water.[4] The disease may occur in all ages,with the highest incidence found particularly in children.[5]

Since all the sign symptoms of typhoid fever are nonspecific, laboratory based investigations are essential for supporting the diagnosis.[6] The mainstay of laboratory diagnosis of typhoid fever is blood culture.[7] In low income countries, where the majority of typhoid fever cases occur, bacterial culture is not routinely conducted.[8] The widal test has been widely used in low income countries for over a century though it has numerous limitations including poor specificity and a cut off titre that differs according to the endemicity of the disease.[7]

The development of molecular methods for diagnosis of typhoid fever has improved the sensitivity, specificity, quality and availability of diagnosis and treatment.[9] One of the molecular methods, PCR is the most sensitive and specific method for diagnosis of typhoid fever. In typhoid fever, it can be used even in cases where antibiotic therapy has been started or the pathogen load is very low.[10] The nested PCR is superior to conventional methods of PCR.[11] A nested PCR makes the detection more sensitive and is able to detect the presence of even 3-5 bacilli and it has a higher efficacy in detecting the disease than other methods like the widal test, blood and urine culture.[8,12]

In Bangladesh, few studies have been done to diagnose typhoid fever by detecting DNA of salmonella in blood by nested PCR.[13] The present study has designed to evaluate the nested PCR for the diagnosis of suspected cases of typhoid fever targeting the flagellin gene (*fli C*) of *salmonella typhi* from blood. The purpose of this study was to optimize nested PCR in the laboratory and to assess the use of PCR could increase the detection rate of typhoid fever specially in case of culture negative cases due to prior antibiotic intake.

Methods

This descriptive type of comparative study was carried out in outpatient Department of Barind Medical College Hospital, Rajshahi and the laboratory work was done in the Department of Microbiology of Rajshahi Medical College and Barind Medical College, Rajshahi. A total 80 blood samples were collected from patients who were agreed to participate in the study of any age of both sexes irrespective of antibiotic intake. Venous blood was collected from antecubital veinwith sterile disposable syringe and needle after disinfection of the venipuncture site with 70% alcohol followed by 2% tincture of iodine which is allowed to dry for one minute.[14,15] At least 7 ml of blood from each paediatric patient and 10 ml of blood from each adult patient were collected from single venipuncture. After removing the syringe and needle from the venipuncture site the sampling needle was discarded and replaced by a sterile needle. The top of the rubber stoppers of the blood culture bottle were disinfected with 70% alcohol and collected blood were injected immediately into the culture bottle.

The diagnosis of typhoid was made by using blood culture, Widal tests and nested PCR. Blood was cultured on tryptic soy broth media and subculture was done on MacConkey'sagar media, blood agar media and SS agar media. Salmonella was identified using Triple Sugar Iron medium, citrate utilization test and oxidase test and confirmed by type specific antisera.

Nested PCR of blood:

PCR was done to detect the DNA of *salmonella typhi* from blood.Major steps are:

a) Extraction of DNA from blood sample.

Extraction of DNA from blood samples (1ml) was carried out by modified lysis buffer method. One ml EDTA containing blood was centrifuged by micro-centrifuge at 13000 rpm for 5 minutes. Supernatant was discarded. Then 1 ml 0.2% Triton X-100 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 decanted. One ml 0.2% Triton X-100 was added to the pellet again, vortexed and centrifuged at 13000 rpm for 10 minutes. Then washed with 1 ml nuclease free water, centrifuged for 3 minutes and supernatant discarded. The pellet was suspended in 30 µl nuclease free water. Boiled for 10 minutes ,thencentrifuged for 3 minutes. Supernatant was used as template for PCR.[16]

b) Amplification of flagellin gene specific sequence by using nested PCR- Nested PCR was described by Song et al (1993)[17] and was modified by Frankel et al. in 1994.[18] The following primers will be used for first round PCR to amplify a 458 bp fragment specific for *salmonella typhi*:

ST1 (5=–ACT GCT AAA ACC ACT ACT–3=) and ST2 (5=–TTA ACG CAG TAA AGA GAG–3=)

In nested PCR, the amplified product of the first PCR is used as template. In the second amplification (nested PCR), a second set (ST3 and ST4) of primer was added to the reaction mixture (50 µl).

For nested PCR, oligonucleotides will be used to amplify a 343 bp fragment using the following primers:

ST3 (5=–AGA TGG TAC TGG CGT TGC TC–3=)

ST4 (5=–TGG AGA CTT CGG TCG CGT AG–3=)

c) Electrophoresis and documentation under UV light.

Results

Blood samples were taken from 80 suspected typhoid fever cases irrespective of age and sex for culture, Widal test and PCR.

Table-I. Isolation of *salmonella typhi* by blood culturein different age and sex among the study subjects. (N =80)

Age (years)	Negative for *Salmonella typhi* N(%)	Positive for *Salmonella typhi*			Total N(%)
		Male N(%)	Female N(%)	Total N(%)	
1 – 5	22(81.5)	3(60.0)	2(40.0)	5 (18.5)	27(33.8)
6 – 10	18 (85.7)	1(33.3)	2(66.6)	3 (14.3)	21(26.2)
10 - 18	17 (89.5)	1(50.0)	1(50.0)	2 (10.5)	19(23.8)
> 18	11 (84.6)	2(100.0)	0(0.0)	2 (15.4)	13(16.2)
Total N(%)	68 (85.0)	7(58.3)	5 (41.7)	12 (15.0)	80(100.0)

Table I showed that in age group 1-5 years, 27 (33.8%) were suspected cases and culture yielded growth of *Salmonella typhi* in 5(18.5%) cases of which male & female were 3(60%) and 2 (40%) respectively. Similarly age group 6-10 years had 21 (26.2%) cases and culture yielded growth in 3 (14.28%) cases and male-female cases were 1 (33.3%) and 2 (66.6%). In age group 10-18 years, out of 19 (23.8) cases, culture positive was 2 (10.5%), 1 (50.0%) was male and another 1 (50.0%) was female. Age group >18 years had 13 (16.2%) cases and culture positive was 2 (15.3%) and all were male. Culture positive was predominant in age group 1-5yrs.

Table-II: Detection of salmonella antibodies by Widal Test in different age and sex among the study subjects.

Age (years)	Widal test negative N(%)	Widal test positive (no=31) N (%)			Total N(%)
		Male N(%)	Female N(%)	Total N(%)	
1 – 5	23(85.2)	3(75)	1(25.0)	4(14.8)	27(33.8)
6 – 10	13(61.9)	2(25)	6(75.0)	8 (38.1)	21(26.2)
10 - 18	7(36.8)	7(58.3)	5(41.6)	12 (63.2)	19(23.8)
> 18	6(46.2)	3(42.8)	4(57.1)	7 (53.8)	13(16.2)
Total N(%)	49(61.3)	15(48.3)	16 (51.7)	31 (38.7)	80(100.0)

A total of 80 suspects, Widal test identified 31 cases as positive, among them 15 (48.3%) were males and 16 (51.7%) were females. In 1-5 years age group, widal test was positive in 4 cases out of 27, within them 3 (75%) were male and the rest 1 (25%) was female. Within 6-10 years age group, 8 (38) cases were detected as widal positive out of 21, of which females were predominant (75 %). Of 10-18 years and > 18 years age group, Widal test detected 12 (63.1%) and 7 (53.8%) cases respectively out of 19 and 13 suspects (Table-II).

Table –III Diagnosis typhoid fever by PCR in different age and sex among the study subjects. (N=80)

Age (years)	PCR positive (n=67)			PCR Negative (n=13) N (%)	Total N(%)
	Male N(%)	Female N(%)	Total N(%)		
1 – 5	10 (45.5)	12(54.5)	22 (81.5)	5 (18.5)	27(33.8)
6 – 10	11(61.2)	7(38.8)	18 (85.7)	3(14.3)	21(26.2)
10 - 18	6 (35.3)	11(64.7)	17 (89.5)	2(10.5)	19(23.8)
> 18	7 (70.0)	3(30.0)	10 76.9)	3(23.1)	13(16.2)
Total N(%)	34(50.7)	33(49.3)	67 (83.7)	13 (16.3)	80(100.0)

PCR detected total 67 (83.7%) cases as positive within 80 suspects. Detected male and female cases are almost equal (50.7% vs 49.3%). Males were 10 (45.5%) and females were 12 (54.5%) in 1-5 years age group. In 6-10 years age group, males are 11(61.2%) and females were 7 (38.8%). PCR detected 17 and 10 cases within 19 and 13 suspects respectively in 10-18 and >18 years age group (Table-III).

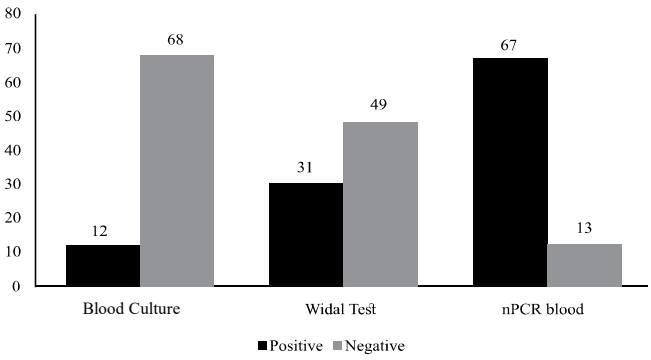


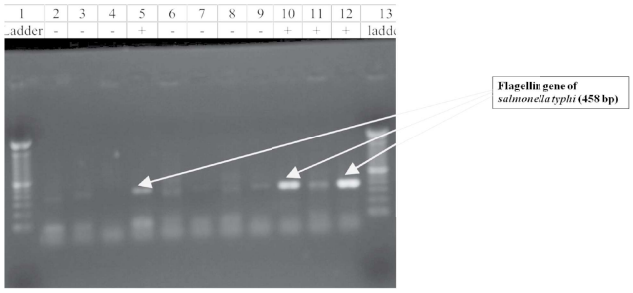
Figure 1: Comparison of blood culture, Widal test and nPCR among clinically suspected typhoid fever (N=80).

Among 80 suspected cases, blood culture had yieldedgrowth of Salmonella typhi in 12 (15%) cases,Widal test was positive in 31(31.7%) cases and nPCRwas positive in 67(83.7%) cases. nPCR is superior over blood culture and Widal test.

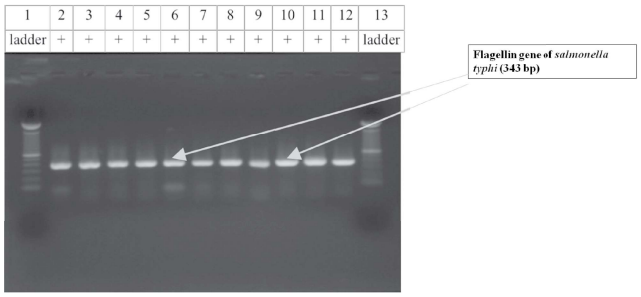
Table IV: Diagnosis of clinically suspected typhoid fever cases by blood culture, widal test and nPCR of different duration of fever (N=80).

Duration of illness (days)	Blood culture N(%)/n	Widal test N(%)/n	nPCR blood N(%)/n
< 6 (n=34)	7 (20.6)	0(0.0)	31 (91.2)
6 – 9 (n=17)	3 (17.6)	6 (35.2)	14 (82.3)
> 9 (n=29)	2 (6.8)	25 (86.2)	22 (75.8)

Table IV showed that the diagnosis typhoid fever by 3 diagnostic stools. In <6 days of fever blood culture was positive in 7(20.5%), Widal test was negative and nPCR was positive in 31 (91.1%) cases out of 34 samples. Similarly in 6-9 days induration of fever blood culture was positive in 3 (17.6%), Widal test was positive in 6 (35.2%) cases and nPCR was positive in 14 (82.3%) among 17 sample. In > 9 days blood culture was positive in 2(6.8%),Widal test was positive 25 (86.2%) and nPCR was positive in 22 (75.8%) out of 29 samples. So nPCR is superior over blood culture and Widal test throughout all the duration of fever.



PhotographI: Flagellin gene of Salmonella typhiafter first round of nested PCR test.



Photograph II: Flagellin gene of *Salmonella typhi after* **second round of nested PCR test.**

Photograph 1 and 2 shows amplicons of *salmonella typhi* specific flagelline gene sequence after 1st and 2nd round of nPCR test from blood sample (bp is 458 and 343 respectively).

Discussion

In the present study, 80 clinically suspected cases of typhoid fever was selected. The disease affected all ages, however in this study, 27(33.7%) were in the age group of 1-5 years followed by 21(26.2%) were 6 to 10 years This findings correlates with the observation made by Saha and his associates who found that 35.6% were in the group 2-3 years.[19] Almost similar study done by

Brooks et al. (2005)[20] and they observed that the prevalence of typhoid fever in children under 5 years were much higher than other age group.[20] The aged group < 5 years were more prone to typhoid fever due to a lack of immunity transferred through mothers milk.[19]

In present study, among 80 clinically suspected typhoid cases, blood culture was positive for 12 (15%) cases of which 7 (58.3%) were male and 5 (41.6%) were female. Another study done by Butler *et al.* (1991)[21] showed that a slightly higher incidence occurs in male than female.[21] They explain this higher incidence of male is due to consumption of contaminated food and water outside the home.

In this study blood culture, Widal test and nested PCR were also done. The nested PCR showed 67 (83.7%) cases were positive, Widal test was positive in 31 (38.7%) cases where as blood culture was positive 12 (15%) cases out of 80 cases. All of the culture positive cases and among 68 (85%) culture negative cases 55 (80.8%) were positive by PCR. Almost similar study was done in Delhi, India where they found 20 (100%) culture positive and 5 (75%) of culture negative cases positive by PCR.[4] In this study blood culture yielded growth of S. *typhi* in 12 (15%) cases. Similar finding was also reported by Begum *et al. (200)*[22] in Bangladesh, where they isolate 14% S. *typhi.* Similarly Sahael, *et al. (2003)*[19] in Kolkata, India reported that an isolation of 21.1% S. *typhi.*[19] Hossain (2001)[23] from Bangladesh reported that an isolation rate of S. *typhi* was 16.67%. The relative low sensitivity of the blood culture in diagnosing typhoid fever may be due to indiscriminate use of antibiotics and difficulties in obtaining adequate volume of blood for culture from children.[24] In first week of fever blood culture is very specific but poor sensitive due to indiscriminate use of antibiotics, type of culture medium used, length of incubation and variations of bacteremia.[25]

In the present study PCR was 67(88.7%) and blood culture was positive in 12(15.3%.) The nested PCR need only 1 ml of blood whereas blood culture need up to 10 ml of blood. So nested PCR method may be an alternative tool for confirm clinical diagnosis of typhoid fever where PCR facilities are available as it is costly.

Conclusion

It can be concluded that typhoid fever still remain an endemic disease in this region. All the signs and symptoms of the disease are nonspecific.It is common with other acute febrile illnesses. So a definitive diagnosis of typhoid fever is required for treatment, prevent transmission and reduce thecomplications. Although blood culture is gold standard though it detect a very few cases and also give false negative results. Detection of antibody by Widal test is easier, less time consuming but failure to detect in the early stages of disease and also required paired samples for giving significant result. But patients are not available for second sample due to medication or partial recovery. On the other hand PCR method is much superior over other methods as it has high sensitivity and specificity. PCR machine is extensive, require specialized skilled person and cannot be made available everywhere, especially in developing countries. It can be made available only in reference centers for utilizations by other healthcare facilities following referral system. So it is strongly recommended to take necessary steps for setup and start PCR at least in the tertiary care hospitals in Bangladesh.

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