Diagnosis of Typhoid Fever: Evaluating nPCR as Diagnostic Tool using Blood and Urine Sample

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ABSTRACT

Introduction: *Typhoid fever caused by Salmonella Typhi is a major health problem and endemic in developing countries like Bangladesh. This study was aimed to detect the flagelline gene (fli C) of salmonella typhi from blood and urine sample by nested PCR (nPCR) for early diagnosis of typhoid fever in clinically suspected patients.* ***Methods:*** *This descriptive study was conducted among clinically suspected patients of typhoid fever attending the outpatient department of medicine and paediatrics of Barind Medical College Hospital, Rajshahi, Bangladesh from July, 2021 to June, 2022. Eighty (n-80) clinically suspected patients of typhoid fever were included according to selection criteria. The blood samples were collected for culture and widely test. Blood and urine samples were selected for nPCR targeting the flagelline gene (fli C) of Salmonella typhi.* ***Results:*** *Among 80 suspected cases of typhoid fever, majority were male within 10 years of age. Blood culture and Widal test were positive in 12(15%) and 31 (38.75%) of cases respectively. Nested PCR of blood and urine detected 67(83.75%) and 62 (77.5%) cases as positive respectively of typhoid fever.* ***Conclusion:*** *The nPCR of blood and urine targeting the flagelline gene (fli C) of Salmonella typhi can be a very useful diagnostic tool of clinically suspected, culture negative cases of typhoid fever.*

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INTRODUCTION

Typhoid fever caused by salmonella typhi is an important global health issue. Worldwide, 17 million people are affected and approximately 0.6 million were died annually by typhoid fever.[1] The burden is particularly high in the southern Asia.[2] It is endemic in the Indian sub-continent, South-East and Far-East Asia, the middle East, Africa, central and South America.[3] It is a systemic infection and feco-orally transmitted, thus most infections occur in an environment with overcrowding, 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 signs and symptoms of typhoid fever are non specific, a definite diagnosis of the disease depending on the clinical presentation alone is very difficult.[6] The mainstay of laboratory diagnosis of typhoid fever is blood culture, which is considered as gold standard.[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 low specificity and a cut off titre that differs according to the endemicity of the disease.[7] The clinical presentation of typhoid fever is similar with other febrile illness, especially during the first weeks of infection and Widal test becomes positive during the second week of infection.[3]The development of molecular methods for diagnosis of typhoid fever has improved the sensitivity, specificity, quality and availability of diagnosis and treatment.[9] 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] and is able to detect the presence of very few bacilli even 3–5 in number.[8] Furthermore, the utilization of alternative specimen types, such as urine, in addition to blood, has been proposed to improve the diagnostic field, particularly in cases where bacteremia is intermittent or low grade.[12]

In Bangladesh, few studies have been done to diagnose typhoid fever by detecting DNA of salmonella in blood by nested PCR.[13] But nPCR in urine has not yet been done in this locality. This study was designed to evaluate the nPCR for the diagnosis of suspected cases of typhoid fever targeting the flagellin gene (fli C) of salmonella typhi from blood and urine samples.

METHODS

This descriptive study was carried out in the Institute of Biological Sciences (IBSc), University of Rajshahi. A total 80 clinically suspected patients of typhoid fever attending the outpatient department of medicine and paediatrics of Barind Medical College Hospital, Rajshahi, Bangladesh were enrolled for this study during July, 2021 to June, 2022. Blood and urine samples were collected irrespective of age, sex and antibiotic intake. Ethical committee of University of Rajshahi, Bangladesh approved the study protocol (ERC no.09(17)/320/IAMEBBC/IBSc). Blood culture and Widal test were done on all blood sample and nPCR was done on both blood and urine sample. Blood culture was done by conventional method on tryptic soy broth media and subculture was on MacConkey's agar media, blood agar media and Salmonella shigella agar media. Salmonella was identified using Triple Sugar Iron medium, citrate utilization test and oxidase test and confirmed by type specific antisera. Widal test was performed by slide method and a dilution of >1:80 was considered suggestive of typhoid fever.

Nested PCR protocol for blood and urine:

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

a) Extraction of DNA from blood and urine sample.

Extraction of DNA from blood samples was carried out by modified lytic 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 vortex-ed, 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 1ml nuclease free water, centrifuged for 3 minutes and supernatant discarded. The pellet was re-suspended in 30 µl nuclease free water. Boiled for 10 minutes ,then centrifuged for 3 minutes. Supernatant was used as template for PCR.

For DNA extraction from urine, 1ml urine sample was centrifuged at 1000 rpm for 5 minutes. Supernatant was collected and centrifuged again at 5000 rpm for 30 minutes. Then lysis buffer (900 micro litre) was added to the sediment.

b) Amplification of flagellin gene specific sequence by using nested PCR: Nested PCR was described bySong et al.[14] and was modified by Frankel et al.[15]The following primers was 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=)

For nested PCR, oligonucleotides was 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.

Statistical Analysis: Statistical analysis was performed by SPSS version 22.0. Data were expressed as frequency and percent.

RESULTS

A total 80 clinically suspected typhoid patients were enrolled in this study. Majority (27, 33.75%) of the patients were in 1-5 years age group (Table I).

Table I: Age distribution of study population (n-80)

Age in years	Frequency	Percent
1-5	27	33.75%
6-10	21	26.25%
10 - 18	19	23.75%
>18	13	16.25%
Total	**80**	**100%**

Regarding gender distribution, males are predominant 47(58.75%) and the male:female is 1.4:1(Figure 1).

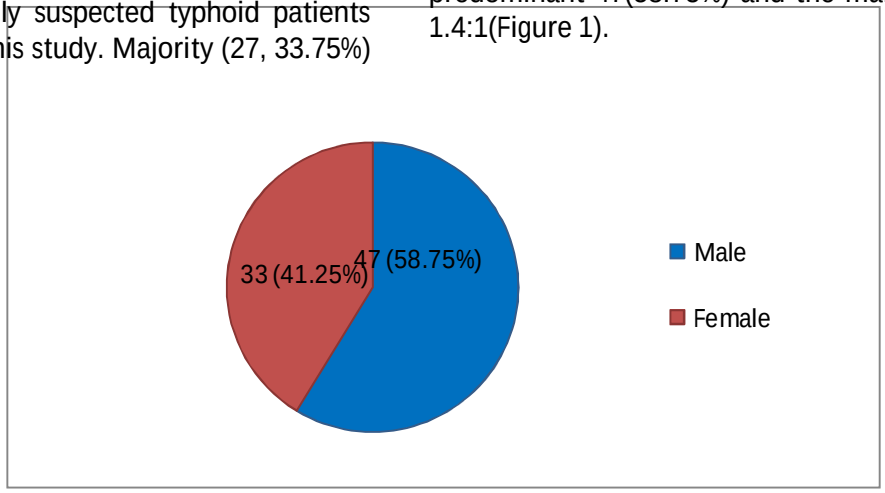

Figure 1: Gender distribution of study population (n-80)

Within 80 suspects, nPCR of blood and urine detected67 (83.75%) and 62 (77.5%) patients respectively as positive for s.typhi (figure 2).

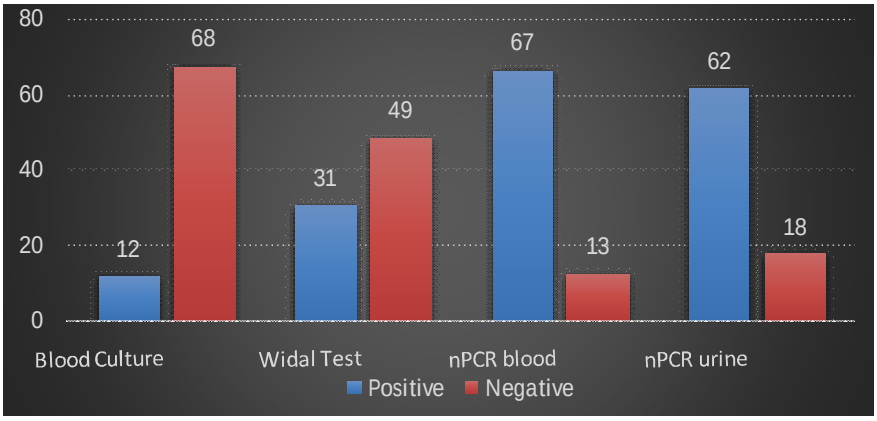

Figure 2: Detection of salmonella by blood culture, widely test and nPCR of blood and urine (n-80)

Within six days of illness, nPCR of blood and urine samples were positive in 31 (91.18%) and 25 (73.53%) cases respectively, but Widal test was negative in all cases. After nine days of illness, most of the cases were Widal test positive (25, 86.21%). Nested PCR of blood and urine samples were more positive in early and late duration of illness of typhoid fever (Table II).

Table II: Status of nPCR, blood culture and widal test with mean duration of illness in clinically suspected cases of typhoid fever (n-80)

Duration of illness (days)	Frequency (%)	Blood culture (%)	Widal test (%)	nPCR blood(%)	nPCR urine(%)
<6	34(42.5%)	7 (20.59%)	0	31 (91.18%)	25(73.53%)
6 - 9	17(21.25%)	3 (17.65%)	6 (35.29%)	14 (82.35%)	14(82.35%)
>9	29(36.25%)	2 (6.90%)	25 (86.21%)	22 (75.86%)	23(79.31%)
Total	**80(100%)**	**12(15%)**	**31(38.75%)**	**67(83.75%)**	**62(77.50%)**

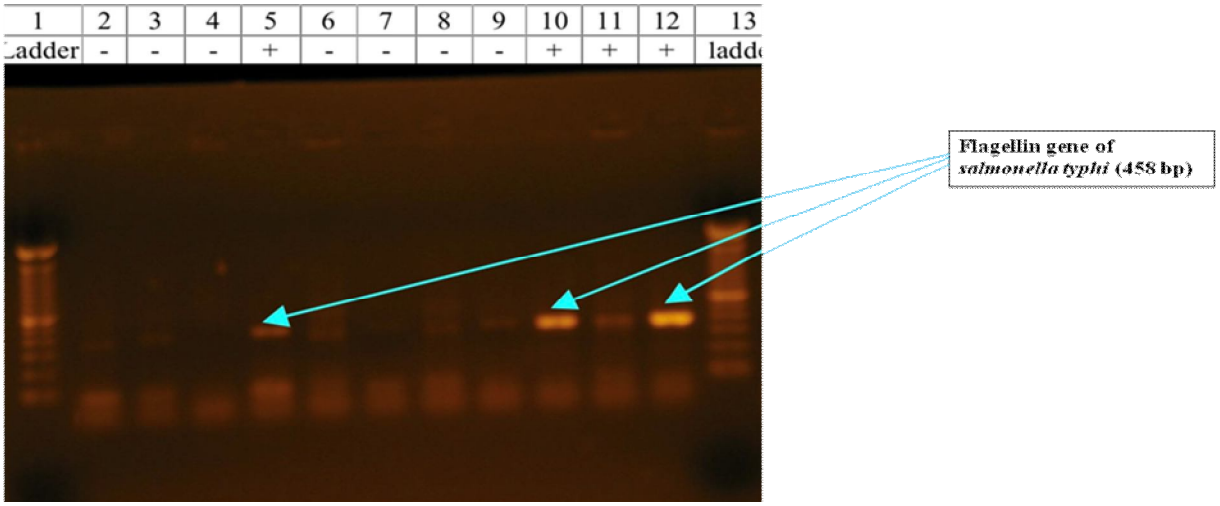

Figure 3: Flagellin gene of Salmonella typhi after first round of nPCR of blood sample

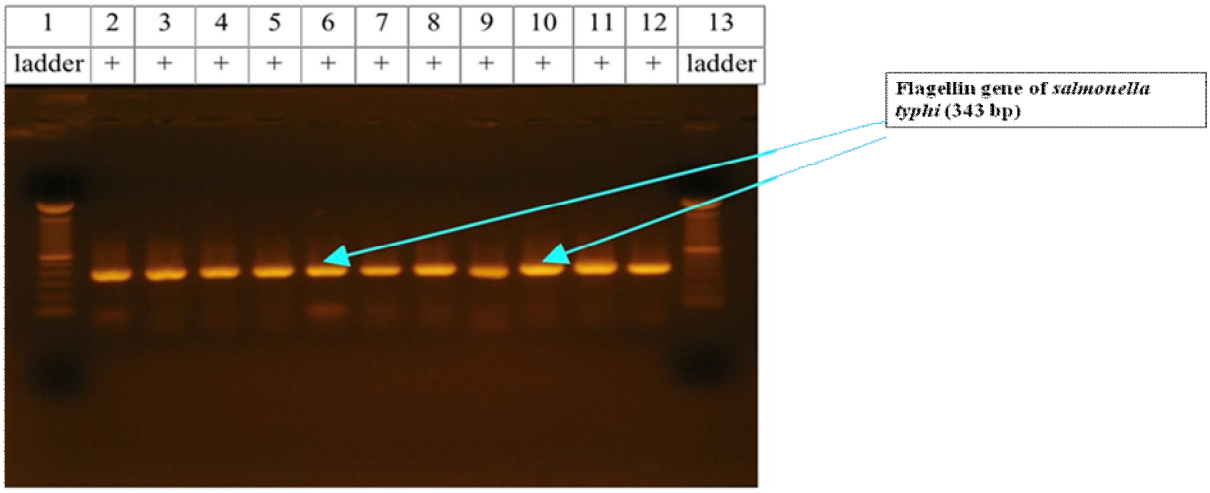

Figure 4: Flagellin gene of Salmonella typhi after second round of nPCR of blood sample

DISCUSSION

Typhoid fever is an endemic disease of Bangladesh. All the signs and symptoms of the disease are nonspecific. Although blood culture is gold standard but it can be detected in few cases and 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. On the other hand, nPCR assay can detect maximum number of typhoid fever cases in spite of having previous history of

antibiotic intake and those with varying clinical manifestations.

In this study, 80 clinically suspected cases of typhoid fever were included. The disease affected all ages, however majority (27, 33.75%) of the patients were in the age group of 1-5 years. This findings correlates with the Saha et al.[16]who found that 35.6% were in 2-3 years age group. Almost similar study done by Brooks et al.[17]showedthat, the prevalence of typhoid fever in under 5 years children were 8.9 times higher than other age group. The age group <5 years were more prone to typhoid fever due to a lack of immunity and poor sanitation practices.

Most of the patients were male (47, 58.75%) than female. Similar findings were observed by Butler et al.[18] They explain this higher incidence of male is due to consumption of contaminated food and water outside the home.

Blood culture yielded growth of S. typhi in 12(15%) cases in this study. Similar finding was also reported by Begum et al.[19] in Bangladesh in 2007, where they isolate 14% S. typhi. Similarly Saha et al.[16] in Kolkata, India reported that an isolation of 21.1% S. typhi . Hossain from Bangladesh reported 16.67%[20] isolation rate of Salmonella typhi. In first week of fever, blood culture is very specific but poorly sensitive due to indiscriminate use of antibiotics, type of culture medium used, length of incubation and variations of bacteria.[21,22]

In this study, the nPCR of blood and urine showed 67 (83.75%) and 62 (77.5%) cases positive respectively. Nested PCR can detect cases as positive where blood culture detects them as negative. Almost similar study was done in Delhi, India where they detect 25 cases as positive by nPCR within which 5 cases were detected as negative by blood culture.[4] Another study by Khan et al.[23] showed that, nPCR and blood culture detect 65% and 40% cases as positive where nPCR detected cases as positive which are negative by blood culture. A study conducted by Jahan et al.[24] showed in 2023 that 63.95% cases were positive by nPCR of urine where only 42% cases were detected as positive by blood culture. Blood culture could not detect rest of the cases as positive which was done by nPCR of urine. Similar studies have been reported by Patel et al.[25] and have mentioned that the sensitivity, specificity of nPCR of urine were 95.1% and 80.2% respectively.

Only one (1) ml of blood is needed for nPCR, whereas up to 10 ml of blood is needed for culture. Nested PCR of urine is a better substitute for establishing diagnosis of typhoid fever as it is noninvasive and rapiddetectionof cases. So, if PCR facilities are available everywhere then nPCR method will be an alternative tool for confirm clinical diagnosis of typhoid fever. Nested PCR of blood and urine showed positive result for typhoid patients who were detected as negative in blood culture and Widal test. So, nPCR can be a very promising, fast and precise technique for diagnosis of typhoid fever.

Limitations: The study subjects were recruited exclusively from a single hospital, which could restrict the applicability of the findings to the broader population of the country.The study was conducted over a short duration, which may influence the comprehensive understanding of long term trends.

The sample size was relatively small due to limitation of budget, manpower and time.

CONCLUSION

Nested PCR of blood and urine can detect maximum number of cases of typhoid fever irrespective of history of taking antibiotic, varying symptoms and duration of illness. While nPCR availability is limited, establishing it in tertiary hospitals is recommended to reduce mortality, morbidity and transmission of typhoid fever, especially in developing countries like Bangladesh.

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Conflict of Interest: The author (s) has no conflicts of interest to disclose.

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