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Diagnostic Validity of Flagellin Gene (*fliC*) in Urine for the Detection of Typhoid Fever

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Abstract

Background: Accurate diagnosis of typhoid fever is important issue for the treatment of the patients. **Objective:** The purpose of the present study was to evaluate the nested PCR for the diagnosis of suspected cases of typhoid fever targeting the flagellin gene (*fliC*) of Salmonella Typhi from urine. **Methodology:** This cross sectional study was conducted in the Department of Microbiology of Dhaka Medical College (DMC), Dhaka. Specimens were collected from OPD of DMCH, Dhaka Shishu Hospital (DSH), Dhaka and National Institute of Neurosciences and Hospital (NINSH), Dhaka from July 2013 to June 2014 for a period of one year. All suspected patients of typhoid fever irrespective of age, sex and history of antibiotic intake who attended the OPD of DMCH, DSH and NINSH were selected for this study. Diagnosed cases of febrile illness other than typhoid fever were excluded from the study. Blood and urine were collected and all isolates were tested for antimicrobial susceptibility by Kirby-Bauer modified disc-diffusion technique. Widal test and CRP were performed. Nested PCR was performed in the urine and blood to detect the DNA of Salmonella Typhi. **Results:** Among 72 typhoid fever patients, 33(45.8%) were Widal test positive and 42(55.5%) were blood culture positive. Out of 30 blood culture negative patients nested PCR in urine was positive in 5(16.7%) cases. The sensitivity, specificity, PPV, NPV and accuracy of nested PCR in urine were 97.6%, 83.3%, 89.1%, 96.1% and 91.7% respectively. **Conclusion:** In conclusion the nested PCR for the diagnosis of typhoid fever targeting the flagellin gene (fliC) of Salmonella Typhi from urine may be very useful diagnostic tool.

Keywords: Diagnostic validity; flagellin gene; fliC; urine; typhoid fever

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Introduction

Bangladesh is highly endemic in enteric fever[1]. Currently, various alternative diagnostic tests for typhoid fever have been developed and evaluated.

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Previous studies have shown that various prototypes and commercially available diagnostic tests may have advantages over the Widal test[2-3]. However, there remains no single test that has proven to be sufficiently sensitive, specific, and practical for use in low-income countries, and as such, these diagnostic kits are seldom used on a large scale. Rapid diagnostic test (RDT) evaluations to date have used blood culture primarily as the reference standard for typhoid fever diagnosis. Culture provides definitive evidence of infection, but it fails to detect all cases, due to low numbers of the pathogen in the bloodstream and/or prior exposure to

antibiotics[4]. There is no evidence to date to suggest that automated systems overcome this shortcoming; indeed, in one study manual and automated blood cultures were equally sensitive for the detection of *Salmonella Typhi*[5].

The development of molecular methods for diagnosis of infectious diseases has improved the sensitivity, specificity, quality and availability of diagnosis and treatment[4]. PCR is a sensitive and specific method used for the diagnosis of a number of infectious diseases. In typhoid fever it can be an effective tool because it can be used even in cases where antibiotic therapy has been started or the pathogen load is very low. A nested PCR makes the detection more sensitive and is able to detect the presence of even 3 to 5 bacilli[5].

Few studies have been done to diagnose typhoid fever by detecting DNA of Salmonella in blood by nested PCR[6]. But nested PCR in urine has not yet been done in Bangladesh for diagnosis of typhoid fever. This study was carried out to detect DNA of Salmonella in urine by nested PCR and compared with blood culture and Widal test. The purpose of the present study was to evaluate the nested PCR for the diagnosis of suspected cases of typhoid fever targeting the flagellin gene (*fliC*) of Salmonella Typhi from urine. The purpose of the present study was to evaluate the nested PCR for the diagnosis of suspected cases of typhoid fever targeting the flagellin gene (fliC) of *Salmonella Typhi* from urine.

Methodology

Study Design and Population: This study was designed as cross sectional study. This study was conducted from July 2013 to June 2014 for a period of one year. This present study was carried out in the Department of Microbiology at Dhaka Medical College (DMC), Dhaka. Specimens were collected from OPD of Dhaka Medical College, Dhaka, Bangladesh, Dhaka Shishu Hospital (DSH), Dhaka, Bangladesh and National Institute of Neurosciences and Hospital, Dhaka, Bangladesh. All suspected cases of typhoid fever patients who attended the OPD of these hospitals of any age with both sexes irrespective of antibiotic intake were selected for this study. The sample was collected by purposive sampling method. This purposive sampling method was used as per inclusion and exclusion criteria. Suspected cases of typhoid fever patients attended the OPD of DMCH, NINSH and DSH irrespective of age, sex and history of antibiotic intake were included for this study. Diagnosed cases of febrile illness other than typhoid fever or severely ill patients were excluded from this study.

Study Procedure: All information regarding clinical features, socio-demographic information and history of antibiotic intake were recorded in a data collection sheet. Mid-stream clean catch urine was collected in a sterile container from each patient. Fresh venous blood was also collected from each patient as well.

Collection, Processing and Storage of Urine: About 50 ml urine was collected in a sterile container and was centrifuged at 3000g for 10 minutes in falcon tubes. The supernatant was discarded and the sediment was collected in the microcentrifuge tube which was further centrifuged at 3000g for 10 minutes and supernatant was discarded and the deposit was kept at -200 C freezer. Blood was collected from the cubital fossa after tightly bind with tourniquet. After the venipuncture site is identified, the rubber septum on the blood culture bottle was disinfected with 70% isopropyl alcohol and was allowed to dry. The site of the venipuncture was then cleaned. The blood was drawn into a sterile disposable syringe and was transferred to the blood culture bottle containing Typtic soy broth with sodium polyanetholsulfonate (SPS) after changing the needle of the syringe. If the patient was under antibiotic therapy, then FAN bottle was used for collection of blood. Blood culture bottles were kept upright after blood collection. Blood culture bottles were inverted gently several times to prevent clotting. Approximately 8 to 10 ml of blood was collected with full aseptic precautions by venipuncture from the cubital vein. The clot was subjected to DNA extraction to carry out nested PCR.

Isolation and Identification of Salmonella Typhi from Blood: Tryptic soy broth in a 30-ml bottle containing 0.3% sodium polyanethol sulfonate was inoculated with 5 ml of blood and was incubated at 37° C overnight. The next day, subcultures were made on MacConkey agar and blood agar media. These plates were incubated overnight at 37°C for bacterial growth. The bottles for negative growth were kept in the incubator for 7 days, and subcultures were made every alternate day on the above-mentioned solid plates. Blood culture in FAN method was performed if the patient was under antibiotic therapy. All isolated Salmonella Typhi were identified by their colony morphology, staining characters and relevant biochemical tests.

Control bacteria strain: Escherichia coli ATCC 25922 was used as control strain to assess the

performance of the method.

Nested PCR: This was performed in the urine and blood to detect the DNA of Salmonella Typhi. The clot was subjected to DNA extraction to carry out nested PCR. A 15-ml deposit of centrifuged urine was subjected to DNA extraction.

DNA Extraction from Urine and Blood Sample: One hundred µl lytic buffer (composition: tris-HCL, proteinase-K and tween 20 solution) was added into tubes having pellet and vortexed thoroughly. The tubes were incubated at 60° C for 2 hours. After incubation the tubes were placed in a heat block (DAIHA Scientific, Seoul, Korea) at 100°C for 10 minutes. Then immediately transferred to the ice and kept for 5 minutes. The solutions were then centrifuged at 14000g at 4◦C for 10 minutes. The supernatant were used as template DNA. The DNA were transferred to other micro-centrifuge tubes and stored at -20°C.

Amplification of flagellin gene (flic)-specific sequence by using nested PCR: Nested PCR was described by Song et al. (1993) and was modified by Frankel (1994). The following primers were 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 were used to amplify a 343-bp fragment using the following primers. ST3 (5=-AGA TGG TAC TGG CGT TGC TC-3=) and ST4 (5=-TGG AGA CTT CGG TCG CGT AG-3=)

Mixing of Master Mix and Primer for First Round PCR with DNA Template: Primers were mixed with Tris-EDTA (TE) buffer according to manufacturer's instruction. PCR was performed in a final reaction volume of 25µl in a PCR tube, containing 10 µl of master mix (mixture of dNTP, taq polymerase, MgCl2 and PCR buffer), 1 µl forward primer and 1 µl reverse primer (Promega corporation, USA), 3 µl extracted DNA and 14 µl nuclease free water for monoplex PCR. After a brief vortex, the PCR tubes were centrifuged in a micro centrifuge machine for few seconds.

Amplification in Thermal Cycler (Eppendorf AG, Master cycler gradient, Hamburg, Germany): The amplification reaction was carried out in a thermal cycler (Eppendorf AG, Master cycler gradient, Hamburg, Germany) with the following temperature and duration profile: initial denaturation at 94°C for 5 min followed by 35 cycles each of 1min of denaturation at 940 C, 1min of annealing at 570 C, and 1min of elongation at 72 C, with a final elongation step extended to 7 min.

Mixing of master mix and primer for nested PCR: The nested PCR master mix was the same as that of the first-round PCR, except it was contained 1 µl forward primer and 1 µl reverse primer (Promega corporation, USA) and 1 microliter of DNA template (5times diluted product of the primary cycle). Thermal cycling was carried out as described for first-round PCR, except that the annealing temperature was set at 63° C. The procedure was repeated 2 to 3 times to ensure that the amplification obtained with the primers was reproducible and consistent.

Agarose gel electrophoresis: The DNA fragments of the flagellin (fliC) gene of S. Typhi amplified by PCR were identified by agarose gel electrophoresis. The amplified product (10 microliter) of the second-round (nested) PCR was subjected to electrophoresis using a 1.5% agarose gel (RM 273; Himedia Laboratories, Mumbai, India). Gel was prepared with 1xTBE buffer (Tris Borate EDTA). For 1.5% agarose gel preparation, 0.18 gram of agarose powder (LE, Analytical grade, Promega, Madison, USA) was mixed with 12.5 ml TBE buffer. After mixing thoroughly it was boiled using microwave woven for few minutes to dissolve the agarose completely. Gel was cooled to 60-70◦C before pouring it into the casting tray and was allowed to stand for 30 minutes for solidification before removing the comb. 6 µl amplicon was mixed with 1 µl loading dye on a piece of para film and then the mixture was loaded into the well of agarose gel. 2 µl of DNA ladder was mixed with one µl of loading dye and was loaded into one well of agarose gel. Positive control and negative control was also loaded in separate well. 1x TBE buffer was poured in the electrophoresis tank and the gel with the stand was placed in it. Gel amplicons and DNA ladder was subjected to electrophoresis for 35 minutes at 100 volts. Molecular markers (100-bpDNA Ladder; MBI-Fermentas, Germany) were processed concurrently.

Staining and de-staining of the gel: The gels stained with ethidium bromide was visualized under UV illumination, and images were processed to reveal the presence of 343-bp bands by using a Multi-Image light cabinet (Alpha Innotech Corporation). After electrophoresis, the gel was stained with ethidium bromide (10 µl ethidium bromide in 100 ml distilled water) for 30 minutes. It was then destained with sterile distilled water for 15 minutes.

Visualization and interpretation of results: The gel was then removed from the tray and observed under

UV Transilluminater (Gel Doc, Major science, Taiwan) for DNA bands. The DNA bands were identified according to their molecular size by comparing with the molecular size marker (100 bp DNA ladder) loaded in a separate lane. Samples showing the presence of corresponding bp band were considered positive for the presence of that organism. Exact band size was calculated from a log graph.

Statistical Analysis: The collected data were analyzed by using SPSS version 22.0. statistical significance was determined by using Pearson chi-square test for all categorical data and result were expressed by frequency and percent and mean with standard deviation respectively. P value of less than 0.05 was taken as statistically significant. Sensitivity was probability that a test result was positive when the disease was present (true positive rate). Specificity was probability that a test result was negative when the disease was not present (true negative rate). Positive likelihood ratio was defined as ratio between the probability of a positive test result given the presence of the disease and the probability of a positive test result given the absence of the disease, i.e. True positive rate / False positive rate = Sensitivity / (1-Specificity); Negative likelihood ratio was defined as ratio between the probability of a negative test result given the presence of the disease and the probability of a negative test result given the absence of the disease, i.e. = False negative rate / True negative rate = (1-Sensitivity) / Specificity; Positive predictive value was defined as probability that the disease was present when the test was positive. Negative predictive value was defined as probability that the disease was not present when the test was negative.

Ethical Clearance: Informed written consent was taken from the patients. If the patient was minor, written consent was taken from legal guardian. The protocol was approved by Research Review Committee (RRC) of Department of Microbiology of Dhaka Medical College. Ethical Clearance was taken from the ethical review committee (ERC) of Dhaka Medical College. All procedures of the present study were carried out in accordance with the principles for human investigations (i.e., Helsinki Declaration) and also with the ethical guidelines of the Institutional research ethics. Participants in the study were informed about the procedure and purpose of the study and confidentiality of information provided. All participants consented willingly to be a part of the study during the data collection periods. All data were collected anonymously and analysed using the coding system.

Results

A total number of 72 patients were recruited for this study. Among 72 patients, 31(43.1%) were in the age group of 10 to 30 years, followed by 19(26.4%) were in 30 to 50 years, 11(15.3%) were in less than 10 years, 7(9.7%) were in 50 to 70 years and 4(5.5%) were in more than 80 years of age group. The mean age of study population was 29.75±16.59 years (Table 1).

Table 1: Distribution of Study Population according to Age

Age Group	Frequency	Percent
<10 Years	11	15.3
10 to 30 Years	31	43.1
30 to 50 Years	19	26.4
50 to 70 Years	7	9.7
>80 Years	4	5.5
Total	**72**	**100.0**
Mean±SD	29.75±16.59	

Among 72 patients, 42(58.3%) cases were male and 30(41.7%) cases were female. The male and female ratio was 1.4:1 (Table 2).

Table 2: Distribution of Study Population according to Gender

Age Group	Frequency	Percent
Male	42	58.3
Female	30	41.7
Total	**72**	**100.0**

Among 42 blood culture positive typhoid fever patients, nested PCR in urine was positive in 41(97.6%) cases. Out of 30 blood culture negative patients nested PCR in urine was positive in 5(16.7%) cases (Table 3).

Table 3: Relationship of Nested PCR in Urine with Blood Culture

Nested PCR of Urine	Blood Culture		Total	P value
	Positive	Negative		
Positive	41(97.6%)	5(16.7%)	46(63.9%)	
Negative	1(2.4%)	25(83.3%)	26(36.1%)	0.0001
Total	**42(100.0%)**	**30(100.0%)**	**72(100.0%)**	

The sensitivity, specificity, PPV, NPV and accuracy of nested PCR in urine were 97.6%, 83.3%, 89.1%, 96.1% and 91.7% respectively (Table 4).

Table 4: Validity parameters of nested PCR in urine

Parameters	Value	95.0% Confidence Interval
Sensitivity	97.6%	94.06 to 100.14
Specificity	83.3%	74.68 to 91.92
Positive Predictive Value	89.1%	81.9 to 96.3
Negative Predictive Value	96.1%	91.63 to 100.57
Accuracy	91.7%	85.33 to 98.07

Discussion

PCR has been used for the diagnosis of typhoid fever with varying success. Nested PCR, which involves two rounds of PCR using two primers with different sequences within the H1-d flagellin gene of Salmonella Typhi, offers the best sensitivity and specificity6. Combining assays of blood and urine, this nested PCR technique has achieved a sensitivity of 82.7% and reported specificity of 100.0%[1]. However, no PCR is widely available for the clinical diagnosis of typhoid fever[8].

Among 72 patients, 43.1% were in the age group of 10 to 30 years, followed by 26.4% were in 30 to 50 years, 15.3% were in less than 10 years, 12.5% were in 50 to 80 years and 2.7% were in more than 80 years of age group. The mean age of study population was 29.75±16.59. Similar to the present study Kumar et al[7] have reported that younger age is the most commonly affected group. Pervin[9] has reported that young adult group of patients are most commonly affected age group which justify the present findings. In areas of endemicity and in large outbreaks, most cases occur in persons aged between 3 and 19 years7. Uneke[10] has mentioned that young adult is most commonly affected by typhoid fever which is consistent with the present study. The younger populations are mainly infected by Salmonella. The reason behind it might be due to, younger people have less immunity against Salmonella and they are fascinated to outdoor foods.

Among 72 patients, 42(58.3%) were male and 30(41.7%) were female. The male and female ratio was 1.4:1. Similar to this result Kumar et al[6] have reported that gender variation is not significant; however, female are more commonly developed chronicity of typhoid fever. Pervin[10] has reported that male are most commonly affected than female by Salmonella Typhi (M:F=1.2:1) which is consistent with the present study. Khan et al[11] have reported that the frequency of typhoid fever patients among male is higher than female (1.1:1) which is similar to the present study. In Bangladesh males move outside the home for earning and they have to take outdoor foods and have more chance of getting infections[12].

The relationship of nested PCR in urine with blood culture is recorded. Among 42 blood culture positive typhoid fever patients, nested PCR in urine was positive in 97.6% cases. Out of 30 blood culture negative patients nested PCR in urine was positive in 16.7% cases. The validity parameters of nested PCR in urine to detect flagellin gene is recorded. The sensitivity, specificity, PPV, NPV and accuracy of nested PCR in urine were 97.6%, 83.3%, 89.1%, 96.1% and 91.7% respectively.

Similar results have been reported by Patel et al[13] and have mentioned that the sensitivity, specificity, NPV, PPV of nested PCR in urine were 95.1%, 80.2%, 93.1%, 91.2% respectively. McQuiston et al[14] have reported that a DNA-based assay based on alleles of genes encoding the three phases of flagellar H antigen to identify serovar-specific antigens has also been reported to show partial success which is dissimilar to the present study. Usually the nested PCR is more specific test which has given high sensitivity and specificity[15].

Conclusion

It is concluded that the nested PCR for the detection of *fliC* gene from urine of typhoid fever patients is superior with very high sensitivity and specificity. The sensitivity of PCR in detecting *fliC* gene in urine is very high; however, the specificity is also high for diagnosis of typhoid fever. So *fliC* gene detection in urine is more sensitive and alternate test for the diagnosis of typhoid fever patients. Nested PCR for diagnosis of typhoid fever should be carried out to detect *fliC* gene.

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None.

Conflict of Interest
The authors have no conflicts of interest to disclose

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Authors' contributions
Jahan N, Yusuf MA, Shamsuzzaman SM conceived and designed the study, analyzed the data, interpreted the results, and wrote up the draft manuscript. Jahan N, Yusuf MA contributed to the analysis of the data, interpretation of the results and critically reviewing the manuscript. Huq N, Yusu MA, Morshed MS, Chhobi FK involved in the manuscript review and editing. All authors read and approved the final manuscript.

Data Availability
Any inquiries regarding supporting data availability of this study should be directed to the corresponding author and are available from the

corresponding author on reasonable request.

Ethics Approval and Consent to Participate
Ethical approval for the study was obtained from the Institutional Review Board. As this was a retrospective study the written informed consent was not obtained from all study participants. All methods were performed in accordance with the relevant guidelines and regulations.

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