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Use of Urine with Nested PCR Targeting the Flagellin Gene (*fliC*) for Diagnosis of Typhoid Fever

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This study was planned to evaluate the efficacy of the use of nested PCR with a large volume of easily available urine as an effort to devise a test that can meet the levels necessary to be considered a gold standard for the diagnosis of typhoid fever. A total of 60 subjects with suspected cases of typhoid fever and 20 apparently healthy control subjects were included in the study. The study period extended from March 2010 to June 2011. Blood, urine, and stool specimens were collected from the participating individuals. Nested PCR was done targeting the flagellin gene (*fliC*) of *Salmonella enterica* subspecies *enterica* serotype Typhi. Specimens in all three categories could be collected from 22 of the subjects with suspected cases of typhoid fever; 21 of the 22 urine samples (95.4%) yielded a desired amplicon of 343 bp, whereas none of the urine samples collected from the 20 control subjects (0%) yielded the amplicon. The analyses of blood and stool samples were found to be inferior to urine sample analysis in sensitivity, with detection rates of 90.9% and 68.1%, respectively. Culture isolation was observed to display very poor sensitivity (31.8%). A large volume of urine may be the ideal specimen for PCR-based detection of typhoid fever.

Typhoid fever is a systemic infection caused by *Salmonella enterica* subspecies *enterica* serotype Typhi. This bacterium is feco-orally transmitted; thus, most infections occur in an environment with overcrowding, poor sanitation, and untreated water (10). Searches for the ideal typhoid diagnostic test are being carried out by several research groups worldwide, with elusive results (2). In regions where typhoid is endemic, clinical diagnosis becomes very difficult due to other prevalent overlapping infectious diseases, e.g., malaria, dengue fever, rickettsioses, leptospirosis, and many unknown vector-borne viral infections. The diagnosis of typhoid fever, as with any other infectious disease, can be made by detecting the microorganism itself or immune responses of the host to the microorganism. The invader may be detected by direct observation (microscopy), culture isolation, detection of its components, *viz*, antigen(s) and nucleic acids, etc. Cultivation of microorganisms and amplification of nucleic acid are the two methods by which copy numbers of the microorganism or target can be increased exponentially; thus, the sensitivity of detection improves significantly compared to microscopy or antigen detection. Although the numbers of specific antibody molecules against the pathogen are also amplified due to proliferation of responder lymphocytes in the natural course of infection, the immune responses elicited in individuals always differ. Sometimes they may be minimal or even absent in immunocompromised hosts. All 3 modalities have been tried in the diagnosis of typhoid fever, which is primarily a bacteremic disease. Rates of isolation from easily available body fluids, *viz*, peripheral blood, urine, and stool, hardly exceed 40% for the routine procedures used in most diagnostic laboratories (1, 11). Bone marrow culture is known to be quite sensitive for the isolation of *S.* Typhi in acute typhoid cases but is not feasible in routine practice, because it is very painful and technically demanding (5). Antibody detection can easily be done using serum samples from patients suspected of harboring the pathogen, but the results of available tests based on agglutination (Widal), enzyme-linked immunosorbent assay (ELISA) (Typhi Dot), chromogenic lateral flow techniques, etc., have been observed to be far away from the recommended standards (8). Per WHO recommendations, the gold standard method for detection of any disease should approach 100% with regard to its sensitivity and specificity as well as positive and negative prediction values (2). There are several published reports showing the efficacy of nucleic acid amplification, specially nested PCR, with results approaching quite closely to the WHO recommendations. The second round of PCR counters the effect of PCR inhibitors, apart from making it more specific, as primers of about 80 to 90 bp hybridize with the targeted bacterial gene sequences (6). Of the 3 specimen types, *viz*, blood, urine, and stool, commonly used for *S.* Typhi isolation and specific DNA amplification, urine might have the most minimal level of PCR inhibitors, as well as being most easily collected in large volumes. PCR-based amplifications for detection of *S.* Typhi have been carried out using urine specimens by few of the previous workers (4, 8). While Hatta et al. (7) previously reported detection sensitivities of 61.8%, 84.5%, 69.3%, 46.9%, and 39.0% by blood culture, nested PCRs using blood, urine, and feces, and the Widal test, respectively, Ambati et al. (1) reported almost the same sensitivity (82.7%) of detection of *S.* Typhi by nested PCR using both blood and urine samples. Although the above-mentioned studies used only 1 ml of urine for DNA extraction, the sensitivity of *S.* Typhi detection seems to have been quite satisfactory. This is why it was speculated that collection of a larger volume of this easily available specimen from typhoid patients might yield better detection efficacy. The present study, therefore, was planned to evaluate the efficacy of nested PCR in suspected cases of typhoid fever by subjecting a larger volume (15 ml) of urine to analysis for diagnosis.

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MATERIALS AND METHODS

In the present study, a total of 80 individuals belonging to age group 3 to 45 years were included. There were 60 patients suspected of suffering from typhoid fever and 20 afebrile and apparently healthy persons. Of the total of 60 patients with suspected cases of typhoid fever, 27 (45%) belonged to age group 3 to 15 years, 29 (48.3%) to age group 15 to 30 years, and 4 (6.7%) to age group >30 years. There were 41 (68.3%) males and 19 (31.7%) females among the patients. Of the 20 control subjects, 8 (40%) belonged to age group 3 to 15 years, 10 (50%) to age group 15 to 30 years, and 2 (10%) to age group >30 years. The male-to-female ratio in the control group was 7:3. Among the 3 specimen categories, *viz*, blood, urine, and stool, samples could be collected only from 22 subjects with clinically suspected cases of typhoid fever and 20 apparently healthy individuals. Of the 22 cases suspected of typhoid fever, 10 (45.5%) belonged to age group 3 to 15 years whereas 11 (50%) belonged to age group 15 to 30 years and 1 (4.5%) belonged to age group >30 years. There were 16 males and 6 females among the 22 cases suspected of typhoid fever (male-to-female ratio, 8:3). This study was carried out during March 2010 to June 2011 in the Department of Microbiology, Institute of Medical Sciences, Banaras Hindu University, Varanasi, India, in collaboration with the Department of Pediatrics and General Medicine.

The control age- and sex-matched subjects were selected from indoor patients admitted for nonfebrile ailments who had no history of typhoid-like fever for the previous year. Well-informed consent was obtained from each of the participants or his or her guardian. The study design was approved by the Institute Ethics Committee of the university.

Sample collection. (i) Blood. Approximately 8 to 10 ml of blood was collected with full aseptic precautions by venipuncture from the cubital vein. About 5 ml of the blood was inoculated for culture isolation, and 3 to 5 ml of the blood was allowed to clot. The serum was subjected to the Widal test, and the clot was subjected to DNA extraction to carry out nested PCR.

(ii) Urine. About 40 to 50 ml of urine was collected in a sterile container. Ten milliliters of fresh urine was subjected for enrichment in selenite-F broth.

(iii) Stool. About 10 to 12 g of a stool sample was collected in a 50-ml sterile container. About 3 g of the specimen was subjected to enrichment in selenite-F broth and also to inoculation on culture plates.

Isolation of *S.* Typhi from different specimens. (i) Blood. Brain heart infusion broth in a 30-ml bottle containing 0.3% sodium polyanethol sulfonate (SPS) was inoculated with 5 ml of blood and incubated at 37°C overnight. The next day, subcultures were made on MacConkey agar (MA) and blood agar (BA). 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.

(ii) Urine. About 10 ml of the urine sample was inoculated in 10 ml of double-strength selenite-F broth and incubated overnight. The next day, subcultures were made on MA and BA plates for bacterial growth.

(iii) Stool. The fresh stool was inoculated onto Xylose Lysine Deoxycholate (XLD) agar and MA, while the enriched stool in selenite-F broth was inoculated onto XLD agar and MA the next day. The plates were incubated overnight, and bacterial growth, if any, was identified as mentioned below. The colonies were subjected to different phenotypic and biochemical tests for identification per the recommendations presented in reference 9. The suspected *S.* Typhi colonies were further identified by serological agglutination using poly- and monophasic antisera, following the standard methodology (3, 9).

Widal test. Estimation of the antibody titers of somatic *S.* Typhi (TO), flagellar *S.* Typhi (TH), and flagellar *S.* Paratyphi A (AH) antibodies was done by using a commercial antigen kit (Tulip Diagnostic, India), strictly following the recommendation of the manufacturer.

Nucleic acid-based detection. (i) Isolation of DNA. About 3 to 5 ml of blood, a 15-ml deposit of centrifuged urine, and 3 g of stool were subjected to DNA extraction. Extraction of genomic DNA was performed sequentially with sodium dodecyl sulfate, proteinase K, and hexadecyltrimethyl ammonium bromide (CTAB) (12). The crude extract was purified by phenol-chloroform extraction, and PCR inhibitors were removed from stool samples by following the procedure described by van Zwet et al. (14) with slight modifications, i.e., 3 g of the stool sample was added to 10% formal saline (40% [vol/vol] formaldehyde and 0.95% NaCl), which was used in place of saline alone.

(ii) Amplification of flagellin gene (*fliC*)-specific sequence by using nested PCR. Nested PCR was performed as described by Song et al. (13) and later modified by Frankel (4). The following primers were used for first-round PCR to amplify a 458-bp fragment: ST1 (5′-ACT GCT AAA ACC ACT ACT-3′) and ST2 (5′-TTA ACG CAG TAA AGA GAG-3′). For nested PCR, oligonucleotides ST3 (5′-AGA TGG TAC TGG CGT TGC TC-3′) and ST4 (5′-TGG AGA CTT CGG TCG CGT AG-3′) were used to amplify a 343-bp fragment.

PCR assay. The reaction mixture for the first-round PCR contained 2.5 μl of 10× PCR buffer (100 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl) (Genei Bangalore, India), 10 pmol each of primers ST1 and ST2 (SBS Genetech Co., Ltd., People's Republic of China), 2 μl (2.5 mM [each]) of a mix of deoxynucleoside triphosphates (dNTPs) (Genei Bangalore, India), 1 U of *Taq* DNA polymerase (Genei Bangalore, India), and 5 μl of DNA template (100 ng), and the final volume of 25 μl was achieved by adjustment with deionized water. The amplification reaction was carried out in a thermal cycler (Biometra, Goettingen, Germany) with the following temperature and duration profile: initial denaturation at 94°C for 5 min followed by 35 cycles each of 1 min of denaturation at 94°C, 1 min of annealing at 57°C, and 1 min of elongation at 72°C, with a final elongation step extended to 7 min. The nested PCR master mix was the same as that of the first-round PCR, except it contained 10 pmol each of primers ST3 and ST4 and 1 μl of DNA template (1:5-diluted product of the primary cycle). Thermal cycling was carried out as described for first-round PCR, except that the annealing temperature was set to 63°C. The amplification was repeated 2 to 3 times to ensure that the amplification obtained with the primers was reproducible and consistent.

Detection of PCR product. The DNA fragments of the flagellin (H1-*d*) gene of *S.* Typhi amplified by PCR were identified by agarose gel electrophoresis. The amplified product (10 μl) of the second-round (nested) PCR was subjected to electrophoresis using a 1.5% agarose gel (RM 273; Himedia Laboratories, Mumbai, India), initially at 100 V for 5 min and then at a constant 80 V for 60 min with Tris-borate-EDTA (TBE) buffer.

Molecular markers (100-bp DNA Ladder; MBI-Fermentas, Germany) were processed concurrently. The gels stained with ethidium bromide were 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).

RESULTS

Of the 54 blood samples from suspected typhoid fever cases, 50 (92.5%) were identified as giving positive results for *S.* Typhi by the nested PCR whereas only 12 were positive by culture isolation. Of 29 available urine samples, 28 (96.5%) were positive for the *S.* Typhi-specific amplicon. Similarly, of the 27 available stool specimens, 20 (74%) were identified as positive by amplification. However, none of the stool and urine samples yielded *S.* Typhi bacteria by culture. Of the samples from the 22 suspected typhoid fever patients from whom samples in all three categories could be collected, *S.* Typhi was isolated from 7 of the blood samples (7/22) whereas none of the urine or fecal samples yielded growth of the bacterium. The samples from the 20 control patients failed to yield positive results for *S.* Typhi in any of the three specimen categories, i.e., blood, urine, and stool (Table 1).

A Widal test confirmed the findings of the routine, and all of them were positive for either or both (TO or TH and AH) anti-

TABLE 1 Positivity of nested PCR and culture isolation using urine, blood, and stool specimens and of the Widal test for cases ($n = 22$) and controls ($n = 20$)

	Group A				Group B			
	Positive		Negative		Positive		Negative	
Test	No.	%	No.	%	No.	%	No.	%
nPCR[a] urine	21	95.4	1	4.5			20	100
nPCR blood	20	90.9	2	9			20	100
nPCR stool	15	68.1	7	31.8	3	15	17	85
Blood culture	7	31.8	15	68.1			20	100
Urine culture			22	100			20	100
Stool culture			22	100			20	100
Widal titer	22	100			3	15	17	85

[a] nPCR, nested PCR.

bodies at a titer of ≥1:160. All the 22 (100%) sera from suspected cases had an anti-TO titer ≥ 1:160, while 14 (63.3%) had raised titers against TH/AH. The sera from control subjects were also subjected to Widal testing, and 3 of the patients had raised titers for salmonella flagellar antigen. Two of the 3 patients were found to have anti-TH antibodies at a titer of ≥1:160, while one had the same titer against TO (≥1:160).

Among the results of the *fliC*-specific nested PCR, the highest positivity was observed in urine specimens (95.5% [21/22]) followed by blood (90.9% [20/22]) and stool (68.1% [15/22]). None of the blood and urine specimens were positive for *S.* Typhi-specific amplification in the control group. However, 3 (15% [3/20]) stool specimens from the control group were observed to yield the desired amplicon.

As determined according to identification of the true disease status as the gold standard, i.e., the positive cases identified by employing nested PCR in tests of samples from all 22 patients, the sensitivity, specificity, and positive and negative predictive values (PV^+ and PV^-, respectively) observed by each of the methods are shown in Table 1 and Table 2. The positive and negative likelihood ratios (LR^+ and LR^-, respectively) are also shown in Table 2.

DISCUSSION

There are only a few reports available of studies where urine and stool were subjected to PCR-based amplification apart from blood analysis despite the very well known fact that the bacterium is frequently discharged from the body through both of those routes. Further, urine and stool samples can easily be collected in quite large volumes. Ambati et al. (1) subjected 3 ml of blood and 1 ml of urine to testing and reported positivity as determined by nested PCR to be quite satisfactory. On the other hand, Hatta et al. (7) carried out a study using all 3 categories of specimens, i.e., blood, urine, and stool. This group subjected 100 μl of freshly collected blood, 1 ml of urine, and 100 μl of stool samples to DNA extraction for the purpose. In light of the observations made by Wain et al. (15) that the quantity of bacteria per milliliter of blood is generally low, that the majority of blood samples contain <1 organism/ml, and that PCR results are related to the actual numbers of CFU found in the blood, we planned to subject large volumes of urine (15 ml) and stool (3 g) to nested PCR-based amplification apart from blood (3 ml) for DNA extraction. The positivity rates determined by PCR for 22 clinical patients suspected of typhoid fever who had high titers (≥1:160) of antibodies for TO and/or TH were found to be 95.5%, 90.9%, and 68.1% for urine, blood, and stool, respectively. Similar rates of detection could be seen for all available samples, i.e., for urine, 96.5% (28/29); for blood, 92.5% (50/54); and for stool, 74% (20/74). The possibility of *S.* Paratyphi A infection in the only case that could not be detected by targeting *fliC* of serotype *S.* Typhi cannot be denied. Interestingly, a raised antibody titer against somatic antigen of *S.* Paratyphi A was found in this case. If that finding was correct, the sensitivity rate for the urine sample would reach 100%. This observation implies that urine in a large volume should be considered the best specimen for detection of *S.* Typhi in acute typhoid fever cases. The nested protocol used with conventional PCR very efficiently underscored the inhibitory effect of PCR inhibitors during the second round, as a very small volume (2 μl of a sample diluted 1/10) was taken from the first-round amplification tube and thus led to a large dilution of the biological PCR inhibitors present in the primary template. In the real-time PCR system, a single round of PCR is usually done (6). That is why we chose the time-tested conventional nested PCR method in the present study for detection of *S.* Typhi. It is really interesting to see that all (22/22 [100%]) of the serologically positive (TO and/or TH/AH ≥ 1:160) patients included in the present study were positive for the presence of *S.* Typhi in analysis of one or more of the types of specimens collected from them. Moreover, all of them had an anti-TO titer ≥ 1:160, while only 63% had raised titers against TH/AH antigen, indicating that the rise in the antibody titer against somatic antigen is more specific for the diagnosis of enteric fever. The number of cases analyzed in the present study was too low for us to reach definite conclusions regarding this phenomenon, but the strong association of raised anti-TO antibody titers compared to flagellar (TH/AH) titers needs to be explored further. Moreover, there were 3 Widal test-positive cases in the control group: 1 had a raised (≥1:160) antibody titer against TO, while 2 had raised antibody titers against TH. Apart from 3 stool specimens that tested positive for *S.* Typhi-specific amplification, none of the blood, urine, or stool specimens from controls was found positive for *S.* Typhi-specific amplification and/or isolation. Surprisingly, these 3 cases were also found to have raised antibody titers against *S.* Typhi. The possibility of these 3 healthy persons having clinical infection or chronic carriage cannot be ruled out. On the basis of this study, it may be proposed that the option of collection and

TABLE 2 Statistical evaluation of nested PCR using blood, urine, and stool samples and of blood culture in cases ($n = 22$) and controls ($n = 20$)

Tests	Sensitivity (%)	Specificity (%)	PV^+	PV^-	LR^+	LR^-
nPCR[a] blood	90.9	100	100	90.9	∞	0.1
nPCR urine	95.5	100	100	95.2	∞	0.05
nPCR stool	68.1	85	83.3	70.8	4.5	0.37
Blood culture	31.8	100	100	57.1	∞	0.7

[a] nPCR, nested PCR.

extraction of DNA from a large volume of blood may be replaced by collection of a larger volume of urine and/or stool. However, a stool specimen might have a good concentration of other DNA and PCR inhibitors, as is true for the blood. Therefore, nested-PCR-based detection of *S.* Typhi in urine may be considered a rapid, reliable, specific, and sensitive method. Further, asymptomatic patients with raised titers as determined by the Widal test must not be overlooked even in areas of endemicity. It may be suggested that such cases must be investigated for chronic carriage or transient asymptomatic *S.* Typhi infections.

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