Original Article

Targeting of putative fimbrial gene for detection of *S.* Typhi in typhoid fever and chronic typhoid carriers by nested PCR

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Abstract

Introduction: It is important to identify *Salmonella* Typhi infection quickly to treat acute fever patients and to prevent transmission by chronic typhoid carriers; therefore, a very specific and sensitive diagnostic technique is highly desirable, especially in endemic areas. The objective of this study was to develop a PCR protocol targeting the putative fimbrial *staA* gene of *S.* Typhi. This is a preferred target gene that is specifically amplified in the *S.* Typhi serotype compared to the commonly targeted *fliC* gene which may also be amplified from the non-typhoidal *Salmonella* Munchen serotype.

Methodology: A new nested PCR primer methodology was designed to target the *staA* gene, which is a member of the fimbrial gene family specific to *Salmonella* Typhi only.

Results: The primers were found to be very specific as the desired amplicon (377 bp) could be generated exclusively from *S.* Typhi strains including the reference strain (MTCC 3216) and 78 clinical isolates . Restriction digestion with *Hinf*I confirmed the identity of the amplified DNA fragment in clinical specimens of *S.* Typhi origin. Furthermore, these primers were able to detect a minimum of three colony forming units per ml (1fg) in spiked blood samples. The detection sensitivity of the described primers is comparable to that of previously published primers targeting *fliC* gene sequences.

Conclusions: This study indicates that the primers targeting the putative fimbrial *staA* gene are very specific to the Typhi serotype and may be a better alternative to *fli*C targeted amplification based diagnosis.

Key words: *Salmonella* enterica serovar Typhi; typhoid fever; nested-PCR; putative fimbrial gene

J Infect Dev Ctries 2013; 7(7):520-527. doi:10.3855/jidc.2561

(Received 24 January 2012 – Accepted 03 July 2012)

Introduction

Salmonella enterica subspecies *enterica* serovar Typhi (*S.* Typhi) is the causative bacterial agent of typhoid fever. Typhoid is a public health concern in many areas around the globe, particularly in developing countries where sanitary conditions may be poor [1]. Annually, this pathogen is responsible for more than 25 million enteric fever cases, resulting in approximately 200,000 deaths worldwide [2]. It is estimated that 3-5% of enteric fever cases become chronic typhoid carriers although the prevalence has been reported to be more than 10% by nested PCR and Vi serology in North India [3]. These carriers often harbour *S.* Typhi throughout their lifetime [4, 5, and 6] and chronic carriage has been observed to be associated with many gastrointestinal diseases including malignancies, especially of the gallbladder [5]. The genus *Salmonella* contains many species, subspecies and serotypes and, although they are genetically quite similar, they differ greatly in their host range [7]. Serotype Typhi is a typical human-restricted serotype which affects higher primates only.

Classical diagnosis methods for presence of *S.* Typhi, such as blood culture and Widal test, usually require four or five days to acquire test results, thus delaying the definitive diagnosis. Most of the earlier studies using blood culture have reported sensitivity values ranging from 38% to 42% but these use clinically defined enteric fever as the base line; enteric fever has been emphasized as the gold standard in the recent past [8,9]. An isolation rate for *S.* Typhi of almost 100% has been reported from bone marrow aspirates of typhoid fever patients; however, bone marrow aspiration is a very painful procedure and demands technical expertise to perform the test [9]. The Widal test is difficult to interpret when the single titers are

used in endemic areas [10,11]. A rapid, alternative method is needed for the diagnosis of *S.* Typhi infection. Polymerase chain reaction (PCR) diagnostic tests have been observed to be highly efficient in the diagnosis of typhoid and paratyphoid fever [12,13]. Some researchers have already reported serovar Typhi PCR detection methods that target the *fliC-d* gene [14], the Vi capsular antigen gene [15], and the 16S rRNA gene [16] but strains of serovars other than Typhi were also detected in some cases. Parry *et al*. reviewed various *S.* Typhi specific nucleic acid amplification tests targeting genes including the H1*d* flagellar gene *(fliC-d),* the H*a* flagellar gene (*fliC-a)*, the Vi capsular gene (*viaB),* the tyvelose epimerase gene (*tyv*; previously *rfbE*), the paratose synthase gene (*prt*; previously *rfbS*), and the heat shock *groEL* gene, reporting variable levels of sensitivity and specificity; the most efficient PCR-based detection was reported as targeting the *fliC-d* gene using a nested protocol [17]. However, strains of *Salmonella* serotype Munchen also share the same sequences with the *fliC* gene of serotype Typhi.

The present study was planned to design a new PCR methodology targeting the *S.* Typhi specific putative fimbrial *staA* gene, which is not shared with any of the *Salmonella* serotypes. Fimbriae are small hair-like proteinaceous structures present on the cell surface that play a critical role in virulence by allowing bacteria to interact with specific host cell receptors [18]. The involvement of fimbriae in intracellular infection indicates that they may be required for systemic infection at stages beyond the initial colonization of host epithelial surfaces in the intestine [19]. Genes encoding a wide variety of putative fimbriae are present in *Salmonella* serovars, but so far only a few *Salmonella* fimbriae have been characterized. There are a total of 13 known putative operons for fimbriae, *bcf*, *csg (agf), fim*, *saf*, *sef*, *sta*, *stb*, *stc*, *std*, *ste*, *stg*, *sth*, and *tcf*, as well as *pil* coding for the type IV pili in the genome sequence of serovar Typhi [20]. Of the fimbrial genes, one of the virulence factors recently implicated in the adaptation of serotype Typhi to the human host is a fimbrial operon known as *tcf* (Typhi colonization factor) [21]. According to Folkesson *et al*., *tcf* may play a role in the strict human specificity for this serotype [22]. Putative fimbrial gene *staA,* found within the *sta* operon, has been reported to be specific to *S.* Typhi [21].Therefore the present study designed new PCR primers were against the sequence of *staA* utilizing the data from GenBank (accession numbers AE014613, Gene ID: 1067781; t0190).

Methodology

Bacterial strains

Strains cultured for testing the specificity of the novel PCR protocol included 54 *Salmonella* strains comprising 36 typhoid (1 reference and 35 clinical strains), 11 paratyphoid (3 reference and 8 clinical strains) and 7 non-typhoid (2 reference and 5 clinical strains) as well as 26 non-*Salmonella* Gram-negative organisms. The 35 clinical *S.* Typhi strains were isolated from patients with typhoid fever admitted to the Sir Sunder Lal Hospital, Institute of Medical Sciences, Banaras Hindu University, Varanasi, India, a tertiary-care hospital in northern India. All other organisms were blood-borne pathogens isolated from the above mentioned hospital. The reference strain *S.* Typhi (MTCC3216) was procured from Microbial Type Culture Collection Center, Chandigarh, India. This strain has similar phenotypic and genotypic characters as Salmonella ATCC strains.

Collection of clinical specimens

Fifty-five blood (3 ml each), 37 stool (5 g each) and 29 urine (15 ml each) samples were collected from patients with typhoid fever before antibiotic therapy. Additionally, 46 gallbladder stone, 43 gallbladder wall, and 23 carcinoma gallbladder tissue specimens were collected from patients suffering from gallbladder diseases. A of total 30 bone marrow specimens were collected from patients suffering from aplastic anemia. Blood, urine, and stool samples were also collected from nine febrile individuals with no history of typhoid fever.

To determine minimum detection levels in clinical samples, a serial dilution of the reference strain of *S.* Typhi (MTCC 3216) was spiked into each type of sample, *i.e.* blood, stool and urine, before DNA extraction.

Extraction of genomic DNA from bacteria and clinical specimens

DNA was extracted from cultured strains, blood, urine and gallbladder samples following the standard phenol chloroform method [23]. In the case of stool samples, DNA isolation was performed using the modified method described by Van Zwet *et al.*, to minimize PCR inhibitors [24]. About 1-2 g stool sample was added to 10 ml 10% formal saline (formaldehyde 40% W/V and 0.85% W/V NaCl) and mixed well; then 3 ml ether was added, mixed well again, and centrifuged immediately at 4000 rpm for 5 minutes. The supernatant was discarded and the pellet was collected. The pellet was mixed with 567 µl Tris-

EDTA buffer (1 M Tris-Cl [pH = 7.5], 0.5 M EDTA [pH = 8.0]), 50 µl 10% sodium dodecyl sulfate (SDS), and 5µl proteinase-K (20 mg/ml). After incubation at 37°C overnight, 100 µl 5M-NaCl and 150 µl 10% CTAB (cetyltrimethylammonium bromide) were added. Next an equal volume of Phenol: Chloroform: Isoamyl alcohol (IAA) (25:24:1) was added and mixed by vortexing for 15 seconds. After centrifugation at 10,000 rpm for 10 minutes, the aqueous phase was collected. Chloroform: IAA (24:1) in an equal volume was added and mixed by vortexing for 15 seconds. After centrifugation at 10,000 rpm for 10 minutes the aqueous phase was collected. Then an equal volume of isopropanol was added and the solution was kept at room temperature for 5 minutes. After centrifugation at 10,000 rpm for 10 minutes the supernatant was discarded. The pellets were washed in 500 µl 70% ethanol and centrifuged at 10,000 rpm for 10 minutes. The DNA pellets were dried at 37°C for 30 minutes and resuspended in 50 µl TE-buffer (pH 8).

PCR primers

From the sequence of the putative fimbrial staA gene of *S*. Typhi (Accession no. AE014613, Gene ID: 29140506), oligonucleotide primers were designed (Table 1). Oligonucleotides staA F1 and staA R1 were used in the first round of the PCR to amplify a 537-bp fragment which corresponded to nucleotides 33 to 52 and 569 to 550, respectively, of staA. Oligonucleotides staA F2 and staA R2 were used in the nested PCR on the amplified products from the first round of PCR to amplify a 377-bp fragment, corresponded to nucleotides 66 to 85 and 442 to 423, respectively.

PCR assay

PCR was carried out in four different phases. Initially, DNA isolated from *Salmonella* spp. and other organisms was amplified to test the specificity of the PCR products. Later, the minimum detectable level by PCR was established by amplification of the serially diluted DNA from *S.* Typhi (MTCC 3216). To evaluate the influence of DNA from normally present leukocytes in the blood on the sensitivity of PCR, a known amount of DNA (100 ng) from mononuclear cells was spiked to serially diluted DNA from *S.* Typhi. Finally, PCR was performed on DNAs isolated from different clinical specimens.

The reaction mixture for the first-round PCR contained 2.5 µl 10x PCR buffer (100 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl) (Genei, Bangalore, India), 10 pmol each primer *staA* F1 and *staA* R1 **(**SBS Genetech Co, Ltd, China), 2 µl (2.5 mM each) dNTPs (Genei, Bangalore, India), 0.33 µl (1 unit) Taq DNA polymerase (Genei, Bangalore, India), 5 µl DNA template (100 ng used unless indicated otherwise), and the final volume was adjusted to 25 µl with HPLC-grade sterile water. The amplification reaction was performed on a thermal cycler (Biometra, Goettingen, Germany) with the following temperature and duration profile: initial denaturation at 94°C for 5 minutes followed by 35 cycles of denaturation at 94°C for 1 minute; annealing at 57°C for 1 minute, and elongation at 72°C for 1 minute, with a final elongation step at 72°C for 7 minutes. The nested PCR master mix was the same as that of the first round PCR, except it contained 10 pmol of each primer *staA* F2 and *staA* R2 and 2 µl of DNA template (1:5-diluted product of the primary cycle). Thermal cycling was carried out as described for the first-round PCR, with an annealing temperature of 65°C. Each amplification reaction was performed in duplicate or triplicate to ensure that the result is reproducible and consistent.

Table 1. Details of the primers used in to amplify the *staA* gene in this study

Primer sequence	Target gene	*Salmonella* serovar	Amplicon size (bp)	Target position
staA F1 5'-TGG TTA CAT GAC CGG TAG TC-3'	*staA*	*S.* Typhi	537 bp	33-52
staA R1 5'-TAG CTG CCG CAA TGG TTA TG-3'				569-550
staA F2 5'-CAT CGG CAC GAA CGT AAG AC-3'			377 bp	66-85
*sta*A R2 5'-TC AAG CGA CTG ATG GTG ACG -3'				442-423

The amplified DNA fragments were resolved through electrophoresis on 1.5% agarose gel prepared in TBE buffer (54.0 g Tris-base, 27.5 g boric acid, 0.5 M EDTA (pH 8.0) in 1000 ml volume) and visualized using a gel documentation system (Alfa Imager 2200, Alfa Innotech Corporation, California, USA). The 100 bp DNA ladder (MBI Fermentas) was used as the molecular size marker. For the *in silico* restriction analysis study, the sequence of *staA* gene of *S*. Typhi reference strains (MTCC 3216, CT18 and Ty2) were accessed from National Centre for Biotechnology Information (NCBI) Gene Bank. The restriction site for *Hinf*I was identified by using NEB Cutter version 2.0 to analyzed banding pattern of restricted fragment of *staA* gene sequence.

Results

Specificity of the PCR primers

Amplification products of the expected sizes were obtained using DNA from a reference strain of *S*. Typhi in both the first round of PCR using primers *staA* F1 and *staA* R1 (537 bp) and in the second round using primers *staA* F2 and *staA* R2 (377 bp). None of the non-*Salmonella* strains or non-typhoidal *Salmonella* strains yielded amplicons of the expected sizes. These results confirmed that there was no cross-reactivity and that the assay is 100% specific to the *staA* gene of *S*. Typhi (Table 2).

Sensitivity of the PCR method

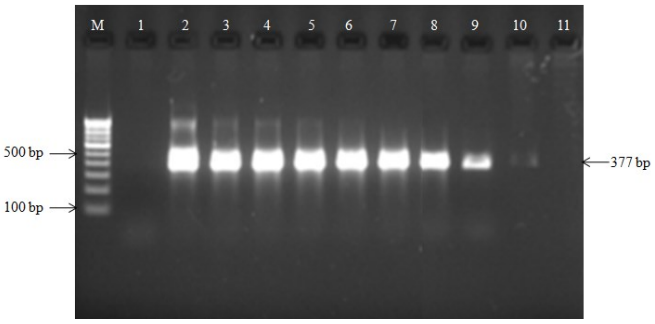
To determine the minimum detectable level of *S*. Typhi DNA, PCR was carried out on a serial dilution of target DNA (Figure 1). The nested PCR amplified 1 fg of target DNA and produced an amplification product of 377 bp.

To determine whether amplification would also be specific and sensitive from clinical samples, a serial dilution of *S*. Typhi [from 3 x 10^8 to one colony forming unit (cfu) per ml] was spiked into blood, stool and urine samples prior to DNA extraction and PCR amplification. Using the single round of PCR using primer *staA* F1 and *staA* R1 in the presence of 2 µg of

Table 2. Evaluation of primer specificity by using *Salmonella* and other Gram-negative bacteria

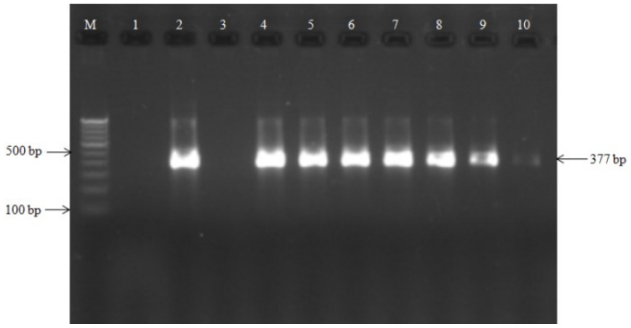
	No. of isolates	Antigen structure			*sta-A* PCR result
***Salmonella enterica* strains**					
Serogroup	**Serovar**	**O**	**H-1**	**H-2**	
A	Paratyphi A (ATCC 9150)	1	1,2,12	a	-
B	Paratyphi B (ATCC 10719)	1	4,5,12	b	1, 2
	Typhimurium (ATCC700720)	1	4,5,12	i	1,2
C	Paratyphi C (ATCC 13428)	1	6,7	c	1,5
D	Typhi (MTCC 3216)	1	9,12,(Vi)	d	-
	Enteritidis (ATCC 13076)	1	1,9,12	g, m	-
Cultured clinical isolates					
	Salmonella Typhi	35			+
	Salmonella Typhimurium	5			-
	Salmonella Paratyphi A	3			-
	Salmonella Paratyphi B	3			-
	Salmonella Paratyphi C	2			-
Non-*Salmonella* strains					
	Pseudomonas aeruginosa	5			-
	Citrobacter freundii	2			-
	Shigella dysenteriae	3			-
	Shigella flexinari	3			-
	Proteus mirabilis	4			-
	Proteus vulgaris	3			-
	Escherichia coli (ATCC 25922)	1			-
	Morganella morganii	2			-
	Klebsiella pneumonae	3			-

Figure 1. Agarose gel image showing the sensitivity of nested PCR amplification with serially diluted *Salmonella enterica* serovar Typhi genomic DNA



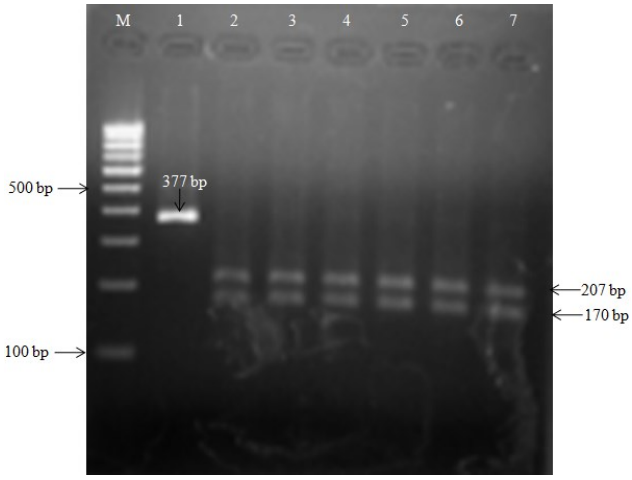
Lane M: 100 bp DNA ladder; lane 1: negative control (199 ng/µl); lanes 2-11 represent amplifications from serially diluted *S.* Typhi DNA from 100 ng/ul to 0.1 fg/ul. where 5□l DNA was used in each reaction

Figure 2. Agarose gel showing the sensitivity of nested PCR amplification after spiking *S.* Typhi (MTCC 3216) in human blood



Lane M: 100 bp DNA ladder; lane 1: positive control unrestricted amplicon of 377 bp *staA* gene of *S.* Typhi (MTCC 3216); lanes 2-7: restriction fragments of *staA*-specific amplicon of clinical samples

Figure 3. Agarose gel showing PCR amplification of *S.* Typhi specific putative fimbrial *staA* gene in clinical samples



Lane M: 100 bp DNA ladder; lane 1: negative control; lane 2: positive control (*S.* Typhi MTCC 3216), lanes 3-14: clinical samples

mononuclear cell DNA, only 0.1 ng of *S.* Typhi DNA (corresponding to 3 x 10^4 organisms) produced an amplification product of 537 bp visible by gel electrophoresis (data not shown). However, additional use of primers *staA* F2 and *staA* R2 in nested PCR produced an amplification product of 377bp (Figure 2). These results suggested that nested PCR is necessary to specifically detect small numbers of organisms such as may be present in clinical blood, stool, urine and benign gallbladder samples, which normally contain human and other bacterial DNA in excess amount.

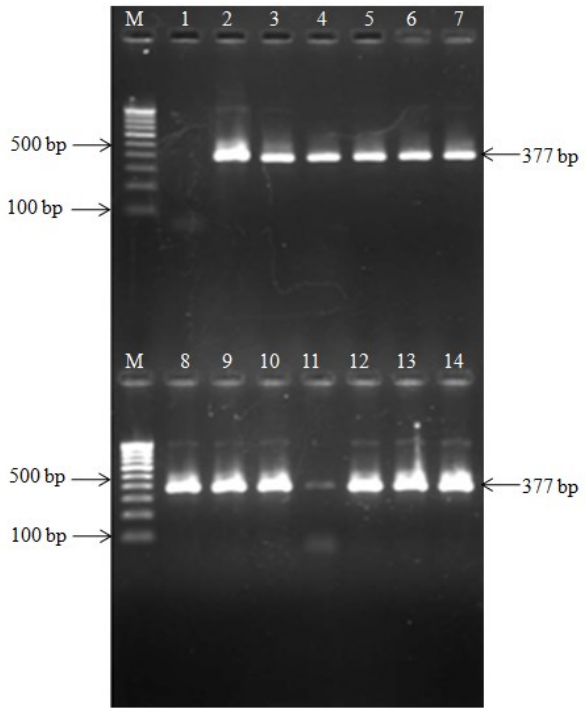
Amplification of staA gene from clinical specimens

Using the nested PCR protocol on clinical specimens from patients suffering from typhoid fever (blood, stool, urine, gallbladder wall, gallbladder stone, carcinoma gallbladder and bone marrow), amplicons of the correct size were obtained (12 representative samples are shown in Figure 3).

Restriction analysis of staA *amplicon*

The *staA* gene sequences of *S.* Typhi strains CT18, Ty2 and MTCC 3216 were retrieved from the National Center for Biotechnology Information (NCBI) Gene

Figure 4. Agarose gel showing *Hinf*I digested 377 bp amplicon targeting *staA* gene



Lane M: 100 bp DNA ladder; lane 1: positive control unrestricted amplicon of 377 bp *staA* gene of *S.* Typhi (MTCC 3216); lanes 2-7: restriction fragments of *staA*-specific amplicon of clinical samples

Table 3. Comparison between total percentage positivity by nested PCR targeting *S.* Typhi specific flagellin (H1*d*) gene and putative fimbrial *staA* gene

Sample	Total number of cases	Number culture positive (%)	Number positive by nested PCR (%)	
			Flagellin H1*d* gene (Song *et al.*, 1993)	Putative fimbrial *staA* gene (Present study)
Blood	55	18 (32.7)	51 (92.7)	51 (92.7)
Stool	37	4 (10.8)	22 (59.45)	23 (62.2)
Urine	29	NA	28 (96.5)	28 (96.5)
Gallbladder Wall	43	3 (6.9)	7 (16.2)	9 (20.9)
Gallbladder stone	46	5 (10.9)	32 (69.6)	34 (73.9)
Carcinoma gallbladder tissue	23	NA	18 (78.3)	18 (78.3)
Bone marrow	30	NA	7 (23.3)	7 (23.3)
Blood, stool and urine (negative control)	27 (9 each)	0	0	0

Bank and *Hinf*I restriction analysis sites identified using NEB Cutter version 2.0.

In silico restriction analysis showed identical banding patterns of the 377 bp PCR amplicon after digestion using the *Hinf*I restriction enzyme. Two bands of sizes 207 bp and 170 bp were produced. The bands were found to be identical for *S*. Typhi reference strains MTCC 3216, CT18 and Ty2.

Amplicons of the expected size from the clinical specimens yielded an identical restriction pattern (six samples shown in Figure 4).

Comparative study of alternative nested PCR methods

To compare the sensitivity and specificity of the amplification of the *staA* gene in clinical samples with a PCR targeting the *S.* Typhi specific flagellin (H1*d*) gene, we used the primers designed by Song *et al*. [14] and modified by Frankel [25]. Both methods detected S. Typhi in the majority of samples, but the novel *staA* primer sets detected *S.* Typhi in slightly more stool, gallbladder wall, and gallstone samples (Table 3). The sensitivity of detection by both of the target genes was comparable, and neither method detected *S*. Typhi in negative clinical samples.

Discussion

Fimbriae operons (*tcf* and *sta*) are believed to be important *Salmonella* pathogenicity factors and *S.* Typhi harbours two intact operons which are not encoded by the *S.* Typhimurium genome. Moreover, fimbrial proteins *staA* and *steD*, as well as the gene encoded within the Type IV pilus cluster of SPI-7, play a major role in the pathogenesis of the *S*. Typhi serotype [29]. The (*stgA*) is the first gene of the fimbrial operon gene. Grace *et al*. were the first to use the fimbrial operon *staA* gene as a PCR target, and observed it to be very specific for the detection of serovar Typhi [30]; however they did not use a nested protocol for the amplification, which has been reported to be essential for clinical specimens where PCR inhibitors are abundant [31]. The s*taA* gene sequence is unique to serotype Typhi; therefore, this gene was targeted for amplification using a nested PCR protocol. The observations made in the present study suggest that PCR based on *staA* can be used very efficiently to detect *S*. Typhi, especially in clinical specimens, using a nested protocol. In the present study, nested PCR was found to be very specific and sensitive in detecting *S*. Typhi DNA, down to 3 cfu/ml blood. The specificity of the PCR in clinical practice was confirmed by the results of PCR performed on DNA from clinical specimens of patients with other acute febrile diseases, which were consistently negative for *S.* Typhi DNA. The purpose of using buffy coat instead of whole blood was to separate the bacteria from erythrocyte debris or serum proteins which could interfere with the detection of *S.* Typhi DNA in blood. Low levels of bacteremia in typhoid patients can fail to be detected using *S.* Typhi culture diagnosis, particularly if the patient has been treated with antibiotics before testing, but the present nested PCR was able to detect S. Typhi DNA in typhoid fever patients. The present protocol detected *S*. Typhi DNA in more stool, gallbladder stone and gallbladder wall samples than a PCR targeting *fliC*; moreover, the *fliC* gene sequence is also shared by *Salmonella* Munchen as well as Typhi.

We conclude that the putative fimbrial *sta)* gene could be a potentially useful diagnostic marker using the method presented here, in typhoid fever and other clinical entities associated with chronic typhoid carriers.

Acknowledgements

We gratefully acknowledge the financial support from the Council of Scientific and Industrial Research (CSIR), New Delhi, India, in the form of a Junior Research Fellowship awarded to Mr. Chandra Bhan Pratap.

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Conflict of interests: No conflict of interests is declared.