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MOLECULAR CHARACTERIZATION OF FLIC GENE BY SEMI NESTED PCR FOR THE DETECTION OF SALMONELLA ENTERICA SEROVAR TYPHI IN THE PERIPHERAL BLOOD OF PATIENTS FROM CHENNAI, INDIA

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ABSTRACT

Typhoid fever caused by *Salmonella enterica serovar typhi (S. enterica serovar typhi*) is one of the important public health problems in many developing countries. Conventional blood culture is currently the gold standard for diagnosis of typhoid fever by this bacterium, but it is time-consuming requiring several days for isolation of bacterium resulting in delay to initiate proper antibiotic therapy and often they are not cultivable. Early diagnosis of the disease is required since 2 to 5% of all deaths in India are due to typhoid fever. Semi-nested polymerase chain reaction (Sn-PCR) specific for the *S. enterica serovar typhi* targeting flagellinC (*fliC*) gene was standardized and applied onto 100 blood specimens. Sn-PCR was specific to *S. enterica serovar typhi* and sensitivity is 10fg/10µl of peripheral blood DNA. Positivity rate was 62% in culture negative specimens and 100% in culture positives (p=0.001). Sn-PCR was highly reproducible and could detect *S. enterica serovar typhi* in less than 12-24 hours after the receipt of the specimen in the laboratory and thus helping in rapid initiation of antibiotic treatment.

KEY WORDS:*Salmonella typhi*, **Typhoid fever, Semi-nested PCR, Flagellin gene**

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INTRODUCTION

Typhoid fever caused by *Salmonella typhi* remains as an important public health problem in India and in many developing countries. It is a major cause of morbidity and mortality worldwide [1, 2, 3]. According to a press release from the Press Information Bureau, Government of India, dated 22 February 2006, the morbidity due to typhoid fever varies from 102 to 2,219 per 100,000 populations in different parts of India, and in some areas, typhoid fever is responsible for 2 to 5% of all deaths[3]. *Salmonella enterica serovar typhi*, is human-specific, causative agent of typhoid fever. It is generally thought that this bacterium is an enterically acquired invasive pathogen, penetrates the ileal epithelium and is transported via underlying macrophages to spleen, liver, and other target tissues during the normal disease course [4]. Almost half of the treated patients continue to excrete the pathogen one month after the symptoms have disappeared, and approximately 5% still do so five months later. Approximately 3% are reported carriers and continue to excrete the organism, often lifelong. The carrier stage may also develop after an asymptomatic infection [5, 6] resulting in dissemination of bacterium among the humanity. Rapid and sensitive laboratory methods to detect the bacterium in typhoid fever are essential for rapid initiation of effective therapy. Although several serological assays for detecting *S. enterica serovar typhi* antigens or antibodies have been used for their rapidity and simplicity, they are not sensitive, ~~and~~ specific and therefore an alterative technique equivalent to culture for typhoid fever are required with high sensitivity and specificity [1]. The classical and the most commonly used serological method, the Widal test, is quiet unreliable with the single titers in endemic areas [2, 7, 8, 9]. Diagnoses of typhoid fever currently relies mainly on blood culture test, but are negative in 30-65% of cases with typhoid fever because of either prior administration of antibiotics or a low number of bacteria which do not adapt to the new culture conditions. Therefore, negative blood culture reports in patients with typhoid fever underestimates the actual incidence of disease. No non-cultural test for typhoid fever has been consistently shown to be sufficiently sensitive and specific [10]. Molecular biological techniques, as is the case in blood culture, target the pathogen itself to enable the early detection of the bacterium. Hybridization by using DNA probes was the first molecular biology technique used for the diagnosis of typhoid fever. The test lacks clinical sensitivity. The advent of PCR technology has provided unparalleled sensitivity and specificity for the diagnosis of typhoid fever [2]. The most predominant target gene in detection of *S. enterica serovar typhi* is the “Flagellin”. This flagellar protein or flagellin constitutes the subunit of the helical filament that forms the flagellar organelle. *Salmonella* flagellin consists of extremely conserved terminal regions and a variable central region. This central region of the molecule carries the antigenic specificity. For the phase-1 flagellin, 63 antigens have been distinguished. For the phase-2 flagellin, 37 antigens have been described. In most isolates of *Salmonella*, two genes encode flagellar antigens; *fliC* encodes the phase 1 antigens, and *fljB* encodes the phase 2 antigens. These genes are coordinately expressed by a phase-variation mechanism [11, 12]. Present study was undertaken to develop and evaluate a specific and sensitive Semi-nested polymerase chain reaction (Sn-PCR) to detect *S. enterica serovar typhi* in peripheral blood of patients clinically suspected to be of enteric fever and thereby helping in the early diagnosis of typhoid fever. This study also envisages the molecular characterization of *fliC* gene of the bacterium to determine the phylogeny of *S. enterica serovar typhi* in our region.

MATERIALS AND METHODS

Patients and clinical samples

Two ml EDTA blood was collected from patients attending Sundaram Medical Foundation (SMF),

a multispeciality hospital, Chennai with complaints of fever in whom enteric fever/sepsis/pyrexia of unknown origin was suspected, during a period of six months, January – June 2010. This was taken after blood for blood culture was collected and was stored at 4°C until processed for Sn-PCR. A randomized number of fifty blood samples which were known to be *S. enterica serovar typhi* culture positive and fifty samples which were culture negative by Bac T Alert, Bio-Merieux blood culture system were included in this study. The study was carried out at L& T Microbiology Research Centre, Vision Research Foundation, Sankara Nethralaya, Chennai, India. Institutional ethics sub-committees (IRB) of both the hospitals approved the study protocol

Serologic analysis

The Widal test with O antigen (Span Diagnostics, Mumbai, India) was performed and interpreted according to routine laboratory procedures. A titer of 1:80 was considered significant.

Semi-nested PCR (Sn-PCR) targeting *fliC gene* of *Salmonella typhi*

DNA was extracted from the 200µl blood samples using Qiagen DNA Mini Kit (Cat no. 51304) as per the manufacturer's instructions. The eluted DNA was stored at -20º C until further processing. Sn-PCR targeting *flagellinC (fliC) gene* of *S. enterica serovar typhi* was carried out using the primers designed using Primegens software and confirmed for the *invitro* specificity using NCBI Primer blast with a first round product of 250bp and second round of 110bp. GenBank ~~samples~~ Accession Nos.: DQ838233.1, DQ838221.1 and DQ838220.1 were used for primer designing. Compared to the other primer available in literature for the detection of *fliC* gene, these set of promers showed higher *in silico* sensitivity. Primer sequences applied were as shown in table 1.

Table 1
List primers designed by us and used in this study

PRIMERS	Primer sequences- 5'-3'	Product size
First round – FP	AATGGGCGACGATTTCTAT	250bp
Second round – FP	GTGGCGCAAATGGTAAATCT	110bp
First/Second round – RP	TTAAGCTCACCGCCTGTTCT	

The PCR mixture consisted of 20 picomoles of forward and reverse primers, 200µM of each dNTPs, 10X buffer, 10mM $Mgcl_2$ and 1U/µl unit of Ampli*Taq* gold (Applied Bio-system). All other PCR reagents were obtained from Merck, India. To the cocktail 10µl of the extracted specimen DNA was added to make it to a final volume of 50µl reaction. The PCR thermal profile includes initial denaturation at 94ºC for 5 minutes, followed by denaturation at 94ºC for 60 seconds, annealing at 60ºC for 60 seconds, extension at 72ºC for 60 seconds with 40 cycles followed by final extension at 72ºC for 7 minutes. The thermal profile was the same for both the rounds of PCR.

Detection of PCR Products

A reaction mixture of 10µl was fractionated electrophoretically in 3 % agarose gel (SRL, India) containing 1x Tris-acetate-EDTA buffer, 50ng/ml of ethidium bromide per ml (Hi-Media Laboratories Private Limited, Mumbai.) and was photographed under UV light using the gel documentation system (VILBER LOURMAT – FRANCE). A positive control representing 5 bacteria/ml of *S. enterica serovar typhi (16SrRNA* DNA sequencing proved) and a negative control without any DNA were also included in each lot of PCR reaction. Molecular size markers (100bp DNA ladder; (Merck, Darmstadt, Germany) were run in parallel on all

gels. Positive results were indicated by the presence of an 110bp band seen on the gel.

Specificity and sensitivity of Sn-PCR targeting fliC gene

Specificity of the nested PCR were tested with DNA extracted from *Staphylococcus aureus* (ATCC-6538), *Staphylococcus epidermidis* (ATCC-10211), (ATCC-12384), *Streptococcus pneumoniae* (ATCC-6301), *Mycobacterium tuberculosis H37 RV, M. xenopi,* (ATCC-1432), *Haemophilus influenzae* (ATCC-10211), *Pseudomonas aeruginosa* (ATCC 9742), *Propionibacterium acnes* (ATCC-11828), *Acinetobacter calcoaceticus* (ATCC-9956), *Enterobacter aerogenes* (ATCC-13048), *Escherichia coli* (ATCC-4157), *Herpes Simplex Virus- 1* (VR 733), *Candida albicans* (ATCC 90028) and the laboratory isolates of *Bacillus cereus, Nocardia asteroides, Serratia marscens*, *Citrobacter freundii*, *Proteus mirabilis, Klebsiella pneumoniae, S. enterica serovar typhi, S. enterica serovar paratyphi A*, *S. enterica serovar paratyphi B*, *Human DNA.* All laboratory isolates were identified by conventional biochemical test and confirmed by PCR-based DNA sequencing *(16S rRNA).* To know the minimum detectable level, the analytical sensitivity of the semi-nested PCR was carried out using serial ten-fold dilutions of DNA obtained from *S. enterica serovar typhi.*

DNA sequencing of amplified products

All culture negative, PCR positive amplified products (n=31) and 10 out of 50 culture positive were randomly chosen and was subjected for DNA sequencing. The cycle sequencing reaction was carried out with 4µl of big dye terminator, 2µl of amplified product, 2 picomoles/µl of forward or reverse primer and 2µl of deionized water. PCR profile consisted of denaturation at 96°C for 1min, followed by 25 cycles of 96°C for 10 sec, 50°C for 5 sec and 60°C for 4 min and final extension of 4°C. Products were purified according to standard protocol, loaded onto ABI 3100 Genetic Analyzer (Applied Biosystems, USA.) with polymer POP6 and sequenced. The sequences were analyzed using BIOEDIT, (downloaded from *http://www.mbio.ncsu.edu/BioEdit/bioedit.html*), and finally blasted with NCBI Blast website *http://blast.ncbi.nlm.nih.gov/Blast.cgi* to identify species and their corresponding homology.

RESULTS

Determination of specificity and sensitivity of the PCR

The DNA extracts of Gram positive and Gram negative bacteria including, *S. enterica serovar typhi, S. enterica serovar paratyphi A*, and *B* along with the fungal viral and human DNA that were tested for the specificity resulted in the amplification of *S. enterica serovar typhi* DNA only in both the first round and second round. It was visualized in a 3% agarose gel electrophoresis (figure 1). The analytical sensitivity of the nested PCR was 10fg/10µl of peripheral blood DNA (figure 2).

Figure 1

Agarose gel electrophoretogram showing results of specificity of Semi-nested PCR is shown in Figure 1. The amplified product of 110bp size S. enterica serovar typhi is only present (Lane 17)

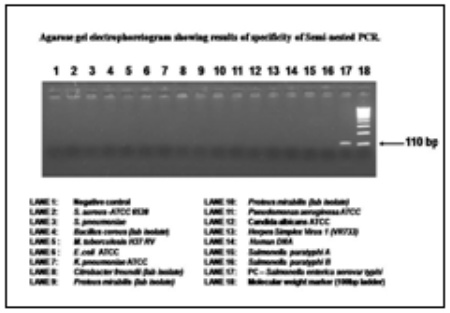
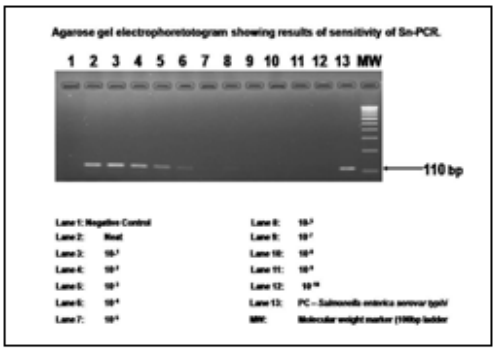


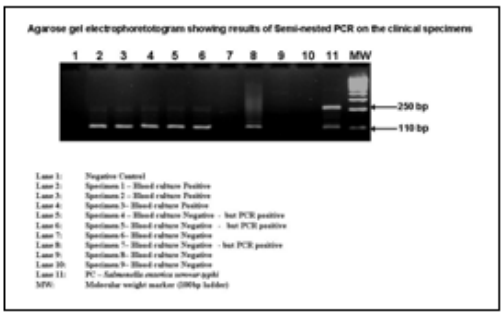
Figure 2
Agarose gel electrophoretogram showing results of sensitivity of Semi-nested PCR is shown in Figure 2. The lowest limit of detectable amount peripheral blood DNA is 10fg/10µl



Amplification of clinical samples

The semi-nested PCR designed specific for the detection of *S. enterica serovar typhi* was tested on randomized 50 known culture positives and negative ones. All the blood specimens positive for the isolation of *S. enterica serovar typhi* had significant titre (1:80) by the widal test and were tested positive for *S. enterica serovar typhi* by Sn-PCR (n=50, 100% positivity) in the second round giving a product size of 110 bp (figure 3).

Figure 3
Agarose gel electrophoretogram showing results of Sn-PCR on the clinical specimens is shown in figure 3



All the 50 *S. enterica serovar typhi* culture negative blood samples were tested negative for *S. enterica serovar typhi* species specific antigen by widal method, whereas 31 (62.0%) samples were tested positive for *S. enterica serovar typhi.* Sn-PCR and identity was confirmed by DNA sequencing. *S. enterica serovar typhi* blood culture positivity were detected within a mean of 2.5 days duration of incubation of blood cultures in suspected cases of typhoid fever where as specimen was reported blood culture negative after 7 days of incubation (table 2). The clinical sensitivity ~~was~~ increased by 62% by Sn-PCR and the statistical significance of the Sn-PCR is calculated by the Fisher's exact test. ~~and~~ The p value is found to be 0.001.

Table 2
Comparison Sn-PCR, blood culture results, mean duration of incubation of blood cultures in suspected cases of typhoid fever

No. of cases	Blood culture results	No. Sn PCR Positive	Mean duration of incubation of blood culture(days)
100	50 - Positive	50 (100%)	2.5 days
	50 - Negative	31 (62%)*	> 7 days

**** The clinical sensitivity of Sn-PCR increased by 62% with a p value of 0.001 (Fisher's exact test) indicating statistical significance of the test.***

Analysis of DNA sequencing results

The DNA sequences of the *fliC* coding region of 110 bp second round amplified products were blasted using NCBI blast and all the isolates showed 98- 99% homology with *fliC* gene of *Salmonella enterica serovar typhi.*

DISCUSSION

Typhoid fever is one of the most common infectious disease in India and in developing countries. Early and definitive detection of the causative agent of the disease is not only important in relieving patient's suffering, but also critical in avoiding fatal complications such as perforation of the intestines. Early detection of the pathogen makes institution of specific treatment possible at an early stage, which leads to the rapid elimination of the same. Otherwise, the patient's excreta especially stool; serve as the predominant source in transmitting of the organism mainly through the fecal-oral route by the consumption of contaminated water and food and thus become a constant source of spread of the disease [2, 4]. The presence of a convalescent patient or a carrier actively shedding the pathogen poses an increased risk for infection. In non-endemic areas, disease outbreaks may occur from a unique source of food or carrier [14]. In disease-endemic areas a recent contact with a patient or carrier has been identified as a major risk factor, but other risk factors include poverty, low education level, poor hygienic conditions, contaminated water supplies, and eating outdoors at food stalls [5]. Although various diagnostic techniques have been used, Widal test and blood culture are the most favored methods. Widal test is not a useful test, since it is not specific and has to be standardized for to each laboratory. Blood culture has the promise of diagnosis in the first week and is very specific, but its sensitivity is poor due to various factors. The most important factor is the very few numbers of bacteria needed to cause severe infection, which can be as low as 10/mL. Hence, positive culture yields are very low and elude definitive diagnosis. Other limiting factors, beside the bacteriostatic effect of antibiotics (already administered before the culture sample is collected), or the nature of culture medium employed, the time period of illness when the blood was collected, the host's immune response system, and the intracellular characteristics of *Salmonella typhi* [15]. The flagellar antigen of *S. enterica serovar typhi* is encoded by a 1,530-bp DNA sequence. Although flagellar antigen is not a structure specific to *Salmonella* species and d antigen is also present in many *Salmonella* species other than *S. enterica serovar typhi* [8], the flagellin gene of *S. enterica serovar typhi* has unique nucleotide sequences in the hyper variable region of the gene. Semi-nested PCR targeting the *fliC* gene for 50 culture negative samples resulted in 62.0% positivity for *S. enterica serovar typhi* PCR and were with 98-99% correlation with the DNA sequencing results. Our evaluation on the specificity of semi-nested PCR was specific only to *S. enterica serovar typhi* and not any other bacteria, non-salmonella strains including the subspecies of *Salmonella*. Sensitivity tests

determined that 10fg of *S. enterica serovar typhi* target DNA could be detected by the PCR assay. No amplification was seen in samples from patients who were culture positive for organisms other than *S. enterica serovar typhi*. Statistical significance of the Sn-PCR is calculated by the Fisher's exact test and the p value is found to be 0.001. Phylogenetic analysis results revealed that almost all the sequences both from culture positive and culture negative were similar and further larger base pair regions should be analyzed before confirming its significance. Song *et al*., 1993 described a polymerase chain reaction (PCR)-based test for the detection of *Salmonella typhi* in blood specimens from patients with typhoid fever oligonucleotide primers were designed to amplify a 343-bp fragment of the flagellin gene of the bacterium, the detectable level after the second round was 10 bacteria in the leucocytes. Frankel *et al*., 1994 has critically analyzed this finding and found that this PCR was not specific only for *S. enterica serovar typhi*. Prakash *et al*., 2005 also developed a nested PCR for the detection of *S. enterica serovar typhi* genome in the peripheral blood, and compared with widal test which is an indirect method and this comparison is not of value for further discussion. Nandagopal *et al.,* 2010 *has* designed a nested PCR targeting flagellin gene (*fliC*) which has a lower limit of detection of 0.01 colony-forming units per ml of blood than Sn-PCR described in this paper. Superiority of Sn-PCR using in-house-designed primers compared to preceding studies is its high degree of sensitivity compared other available primers targeting the *fliC* gene. The turnaround time for the detection of *S. enterica serovar typhi* by PCR was less than 8 hours, compared with five to seven days for conventional blood culture. Therefore, PCR was a rapid, sensitive, and specific test for the diagnosis of typhoid fever, especially where blood culture was negative because of prior antibiotic treatment, low level of bacteremia, and culture done in late stages of disease, thus enabling the clinician to use appropriate treatment and to avoid diagnostic delay. Thus the PCR appeared to be a promising diagnostic test for typhoid fever.

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