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Rapid Detection of *Salmonella enterica Serovar typhi* from Humans

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Present study was carried out to investigate *Salmonella enterica* serovar Typhi associated with Typhoid fever in humans. A total of six blood samples were collected from six patients (4 males and 2 females) and processed for the isolation and identification of the causative agents and their virulence determinants. Microbial investigation revealed the causative agents *Salmonella enterica* Serovar Typhi in all the six clinical cases. In polymerase chain reaction (PCR) assay, all the six isolates were found positive for the Invasion gene (*inv*A; 244bp fragment), Tyvelose epimerase gene (*tyv*; 615bp fragment), phage-1 flagellin gene for d-antigen (*fli*C-d; 750bp fragment) and Vi antigen genes (*via*B; 439bp fragment). This study confirms the association of virulent strains of *Salmonella enterica* Typhi in occurrence of the Typhoid fever in humans. Present study suggested that PCR can be a useful, high throughput diagnostic tool for the rapid detection of *Salmonella enterica* Serovar Typhi.

Key words: *Salmonella enterica* Serovar Typhi, PCR, Typhoid, Virulence.

Typhoid is a communicable disease caused by *Salmonella enterica* serovar Typhi remains an important public health problem in many parts of the world including India and other developing countries (Kumar *et al.,* 2006). In recent years, it has been seen that multidrug resistant *S. typhi* is spreading over Indian subcontinent (Agarwal, 1962). Every year, between 17 and 33 million people suffer from typhoid fever with approximately estimated 600,000 deaths worldwide (Pang *et al.,* 1998) and in India more than 3,00,000 cases are reported every year and is associated with significant morbidity and mortality (Agarwal, 1962). Sometimes typhoid fever is fatal infections of children and adults causing bacteraemia and inflammatory destruction of the intestine and other organs (Hirose *et al.,* 2002).

The diagnosis of typhoid fever made by conventional culture methods and biochemical tests takes 2 to 5 days and sometimes takes more than 1 week with blood samples (Hashimoto *et al.,* 1995). The problem with conventional detection methods is that they are laborious and time consuming, and cannot distinguish between the *Salmonella* serovars (Kumar *et al.,* 2006). Therefore, a rapid, alternative method which shows high sensitivity and specificity is needed for the diagnosis of typhoid fever. Polymerase

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chain reaction nowadays has been used a rapid diagnostic technique to detect the presence of virulence genes and to identify the different serovars of *S. enterica* from different clinical cases (Song *et al.,* 1993).

There are nearly 2,000 *Salmonella* serovars and for those tested so far, all seem to contain invasion gene (*inv*), which enable the bacteria to invade host cells (Chiu & Ou, 1996). The O antigen gene- *tyv* encodes CDP-tyvelose epimerase, which converts CDP-paratose to CDP-tyvelose. The *tyv* gene is present in both serovars Typhi and Paratyphi A, but the *tyv* gene of serovar Paratyphi A does not produce active CDP tyvelose epimerase due to the 1-bp deletion which causes the frame shift mutation and converts codon 4 of *tyv* to a stop codon (Verma & Reeves, 1989). All virulent strains of *S.* typhi causing typhoid fever possess the Vi capsular antigen gene. Thus, the DNA sequence encoding the Vi antigen (pertaining to the *via*B region) is useful in developing DNA-based diagnostic tests for *S.* Typhi (Hashimoto *et al.,* 1995). The H antigen gene - *fli*C-d, phage-1 flagellin gene for d-antigen [H:d] encodes for flagellin (Hirose *et al.,* 2002). In this study, the rapid identification of *Salmonella enterica* Serovar Typhi from clinical human samples was carried out by using multiplex PCR technique that detected the *tyv*, *fli*C-d and *via*B genes.

MATERIAL AND METHODS

Sample collection

Blood samples were obtained from six patients (4 males and 2 females) with typhoid fever from Sri Abirami Clinic, Salem District and PSG Hospitals, Coimbatore District in Tamil Nadu, India during the month of November and December 2009. The epidemiological data sheet from each patient was generated (Table 1). Blood samples were transported in an ice cold container and immediately processed for microbial investigation.

Isolation and identification

Three to five millilitres of venous blood was inoculated into 30ml of brain heart infusion broth (Hi-Media, India), incubated at 37°C for 7 days. A minimum blood-to-broth ratio of 1 to 10 was maintained. All tubes were examined daily and if any visible growth was shown were streaked on nutrient agar (Hi-Media, India) supplemented with 10% sheep blood followed by streaking on Xylose Lysine Deoxycholate agar plates (Hi-Media, India) and incubated at 37^0C for 24h. Bacterial colonies were purified based on the size, shape, colour on XLD agar and patterns of haemolysis on blood agar and were subjected to Gram's staining. The isolates were identified based on motility test, citrate utilization, methyl red and Voges Proskauer test, hydrogen sulphide production, fermentation of mannitol, arabinose, sorbitol, dulcitol, lactose, sucrose and glucose (Holt *et al*., 1994).

Detection of virulence genes by polymerase chain reaction

To study the virulence of the organism, isolates were tested for the invasion gene (*inv*A), tyvelose epimerase gene (t*yv*), phage-1 flagellin gene for d-antigen (*fli*C-d) and Vi antigen genes (*via*B) by PCR. The *inv*A gene was detected by single step PCR, whereas, a multiplex PCR was used to detect the *tyv*, *fli*C-d and *via*B genes (Hirose *et al.,* 2002).

The specific forward and reverse primer pairs for *inv*A gene of 244bp were 5/-acagtgctcgttacgacctgaat-3/ and 5/-agacgactggtactgatcgataat-3/ (Chiu and Ou, 1996); *tyv* gene of 615bp were 5/-gaggaagggaaa tgaagctttt-3/ and 5/-tagcaaactgtctcccaccatac-3/ (Hirose *et al.,* 2002); *fli*C-d genes of 750bp were 5/-aatcaacaacaacctgcagcg-3/ and 5/-gcatagccaccatcaataacc-3/ (Hirose *et al.,* 2002) and *via*B gene of 439bp were 5/-gttatttcagcataaggag-3/ and 5/-cttccataccactttccg-3/ (Hirose *et al.,* 2002) were commercially synthesized (Eurofins Genomics India Pvt Ltd., India). *S. enterica* serovar Typhi (MTCC 733) and *Aeromonas hydrophila* (MTCC *646) strain were used as positive and negative controls respectively.

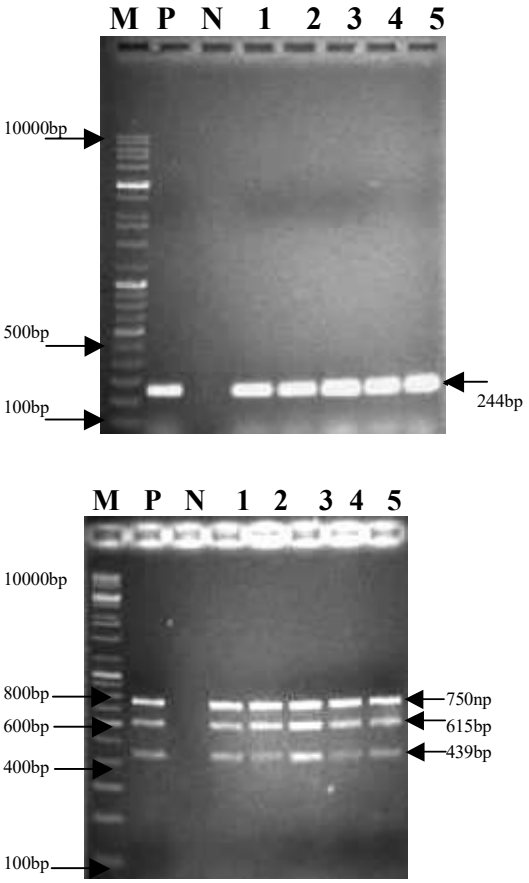
Freshly grown bacterial colonies from solid media plates were suspended in 200µL of sterile distilled water in a micro centrifuge tube, gently vortexed and boiled for 10min in a water bath. Supernatant after centrifugation at 10000*g* for 5min was used as a template DNA. The amplification was carried out in 25µL reaction volume containing 12.5µL of 2× PCR master mix (Promega, USA) containing 4mM magnesium chloride, 0.4mM of deoxynucleotide triphosphates

(dNTPs), 0.5U of *Taq* DNA polymerase, 150mM tris-hydrochlroric acid, pH 8.5 (Promega, USA), 1µM concentration of primers (invA-F and invA-R), 0.1µM concentration of primers (tyv-F, tyv-R, fliC-d-F and fliC-d-R) and 0.2µM concentration of primers (viaB-F and viaB-R) and 2.5µL of template DNA. The PCR reactions were performed in thermal Cycler (Eppendorf, Germany). For the *inv*A gene after initial denaturation at 94^0C for 4 min, the amplification cycle had denaturation, annealing and extension at 94^0C, 56^0C and 72^0C for 30s, 30s and 2min respectively. For *tyv, via*B and *fli*C-d genes after initial denaturation at 95^0C for 4 min, the amplification cycle had denaturation, annealing and extension at 95^0C, 55^0C and 72^0C for 30s, 60s and 90s respectively. Final extension was done at 72^0C for 10min. The PCR amplicons (5µl) were electrophoresed in 1.5% agarose gel in TAE (Tris-acetate-EDTA, pH 8) buffer, stained with ethidium bromide and observed in gel doc system (Universal Hood, BIORAD, Italy).

RESULTS AND DISCUSSION

All the isolates were grown in BHI broth and were non haemolytic on sheep blood agar and showed pink colour colonies with black centre on XLD agar. All isolates fermented glucose, mannitol, sorbitol and arabinose. In Triple Sugar Iron slants, the butt and slant turned into yellow and red colour respectively indicating the fermentation of glucose alone and production of acid in the butt. The isolates also showed production of hydrogen sulphide in the TSI agar. Isolates were negative for indole production, urease production and citrate utilization. All the isolates were found to be Gram negative, flagellated and motile. Upon detailed bacteriological investigation, six isolates were identified as *S.* typhi (Wain *et al*., 1998).

In PCR assay, all the six isolates were found positive for the *inv*A, *tyv, fli*C-d and *via*B genes of desired fragments 244bp, 615bp, 750bp and 439bp respectively (Table 1, Fig. 1). Similar detection of *inv*A genes (Chiu and Ou, 1996) and *tyv, fli*C-d and *via*B genes from the isolate of *S.* typhi associated with typhoid fever by PCR and mPCR respectively has been reported (Hirose *et al.,* 2001 & Hirose *et al.,* 2002). In our study, *tyv, fli*C-d and *via*B genes were detected from all the six clinical isolates of *S.* typhi. This is in complete agreement with the previous observation demonstrating that all the isolates of *S.* Typhi causing typhoid fever carry *tyv, fli*C-d and *via*B genes (Hirose *et al*., 2002 & Karami *et al*., 2007), while the genus specific *inv*A gene is also present in all the *Salmonella* serovars irrespective of clinical or healthy cases (Chiu & Ou, 1996). The detection of the combination of Vi antigen (*via*B), H antigen (*fli*C-d) and O antigen (*tyv*) by mPCR



a: Detection of *inv*A (244bp) gene by PCR
b: Detection of *tyv* (615bp), *fli*C-d (750bp) and *via*B (439bp) genes by mPCR
Lane P: Positive control (*S*. Typhi MTCC 733);
Lane N: Negative control (*A. hydrophila* MTCC *646);
Lane 1-5: Field isolates positive for toxin genes;
Lane M: High range DNA ruler.

Fig. 1. Detection of virulence genes from *Salmonella enterica* serovar Typhi by PCR.

Table 1. Epidemiological data and details of virulence genes detected by PCR from *S.* Typhi

S. No	Sample No	Isolate No	Sources	Sex	Age (Yrs)	Health Condition	Sample collected from the day of onset of disease (Days)	Place of Sample Collection	Detection of toxin genes by PCR			
									*inv*A	*tyv*	*fli*C-d	*via*B
1	77321BC	HST1	Human	Male	12	Typhoid fever	7	Abirami	+	+	+	+
2	89323BC	HST2	Human	Female	50	Typhoid fever	8	Sri	+	+	+	+
3	93217BC	HST3	Human	Male	23	Typhoid fever	8	Clinic, Salem	+	+	+	+
4	M3209	HST4	Human	Male	16	Typhoid fever	6	PSG Hospitals,	+	+	+	+
5	F3541	HST5	Human	Female	18	Typhoid fever	7	Coimbatore	+	+	+	+
6	M4367	HST6	Human	Male	15	Typhoid fever	7		+	+	+	+

correctly identified *S.* typhi strains associated with the typhoid fever.

In view of the practical use of PCR, this finding is useful because PCR technique can be applied to reinforce the clinical diagnosis of typhoid fever in patients with suspected clinical symptoms of typhoid fever, such as high fever, leukopenia and hepatosplenomegaly. This study confirmed the association of virulent strains of *Salmonella enterica* serovar Typhi in the occurrence of the typhoid fever in humans in Tamil Nadu. Present study suggested that PCR can be a useful, high throughput diagnostic tool for the rapid detection of *Salmonella enterica* serovar Typhi.

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