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Molecular diagnosis of *Salmonella typhi* and its virulence in suspected typhoid blood samples through nested multiplex PCR

Solai Ramatchandirane Prabagaran[a,*], Vellingiri Kalaiselvi[a], Naganathan Chandramouleeswaran[a], Krishnan Nair Geetha Deepthi[a], Kootallur Narayanan Brahmadathan[b], Mariappa Mani[b]

[a] Department of Biotechnology, Bharathiar University, Coimbatore, Tamilnadu 641 046, India
[b] Microbiological Laboratory, Cowley Brown Road (East), R. S. Puram, Coimbatore- 641 002, India

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ABSTRACT

A nested multiplex polymerase chain reaction (PCR) based diagnosis was developed for the detection of virulent *Salmonella typhi* in the blood specimens from patients suspected for typhoid fever. After the Widal test, two pairs of primers were used for the detection of flagellin gene (*fliC*) of *S. typhi*. Among them, those positive for *fli*C alone were subjected to identification of genes in Via B operon of *Salmonella* Pathogenesity Island (SPI-7) where four primer pairs were used to detect *tvi*A and *tvi*B genes. Among 250 blood samples tested, 115 were positive by *fliC* PCR; 22 of these were negative for *tviA* and *tviB*. Hence, the method described here can be used to diagnose the incidence of Vi-negative serovar *typhi* especially in endemic regions where the Vi vaccine is administered.

1. Introduction

Typhoid fever is an acute multisystemic illness caused by *Salmonella enterica* subspecies *enterica serovar Typhi* (*S. typhi*), transmitted through intake of food and fluid contaminated by infected human wastes. According to recent estimates published in 2014, approximately 21 million cases and 222,000 typhoid-related deaths occur annually worldwide (WHO, 2015). Among them Asia was reported as highly affected, especially in south-central and southern Asia (Crump et al., 2004). This sustained maintenance of typhoid is due to dissemination of disease via asymptomatic typhoid carrier (Gunn et al., 2014). Hence there is a critical need to increase the chance of detecting carrier so as to reduce the risk that they pose to the communities. An ideal diagnostic tool for identification of typhoid patients and carrier should be sensitive, specific as well as rapid.

S. typhi is strictly human-adapted pathogen confined to immunocompetent human host causing typhoid fever (Tsolis et al., 2011). The classical and commonly used serological method such as the Widal test is mainly unreliable with single titers in prevalent areas (Mashouf et al., 2007). Confirmation of typhoid fever requires identification of *S. typhi* in clinical specimens. However, blood culture can detect only 45 to 70% of patients as it depends on the amount of blood sample, the bacteremic level of *S. typhi*, type of culture medium and incubation period (Wain et al., 2001, 2008). DNA probe specific to Vi antigen of *S. typhi* had been tried to detect organism in the blood of patients with typhoid fever. However, such hybridization method requires high concentration of bacteria which is seldom possible (Rubin et al., 1988).

It is also recognized that agglutination tests have serious shortcomings viz. false positives and false negatives. Thus, molecular detection methods are regarded suitable to identify pathogens from body fluids and excretions of infected human or mammals, as these methods are found highly sensitive and specific over Widal and blood culture methods. Molecular methods, particularly the polymerase chain reaction (PCR) based assays have been developed over the last few decades to overcome these disadvantages.

In this method, *Salmonella typhi* was precisely identified by amplifying the flagellin (*fliC*) gene locus. In diagnostic laboratories the use of PCR is limited by cost and sometimes the availability of adequate test sample volume. This PCR based tool for the diagnosis of typhoid fever (Nagarajan et al., 2009) and Vi (Wain et al., 2005) are only specific and sensitive for *Salmonella enterica* serovar Typhi. To address this problem, a multiplex PCR targeting flagellin (*fliC*) and virulence genes (*tviA* and *tviB*) was developed. *Salmonella* Pathogenesity Island (SPI-7) carries genes responsible for several pathogenic traits including production of Vi polysaccharide capsule. ViaB operon is found only in organisms which is specific in *S. typhi* that produce Vi capsule or Vi polysaccharide. Vi polysaccharide prevent the organism from phagocytosis when it is outside the cell but inside the host. In the host defense

* Corresponding author.
E-mail address: prabagaran@buc.edu.in (S.R. Prabagaran).

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Table 1
Primers used in this study.

Target gene	PCR	Primer	Primer sequence (5′-3′)	Amplicon
fliC	First PCR	ST1	TATGCCGCTACATATGATGAG	495 bp
		ST2	TTAACGCAGTAAAGAGAG	
	Nested PCR	ST3	ACTGCTAAAACCACTACT	363 bp
		ST4	TGGAGACTTCGGTCGCGTAG	
Vi				
tviA	First PCR	V1	GTTATTTCAGCATAAGGAG	599 bp
		V2	ACTTGTCCGTGTTTTACTC	
	Nested PCR	V3	GTGAACCTAAATCGCTACAG	308 bp
		V4	CTTCCATACCACTTTCCG	
tviB	First PCR	tviB-F	CGAGTGAAACCGTTGGTACA	846 bp
		tviB-R	CAATGATCGCATCGTAGTGG	
	Nested PCR	tviB-in-F	GAATCGGGGAGATATTGTGG	774 bp
		tviB-in-R	TGCCATACTCTCGTCTTACC	

mechanism, though macrophages are produced against both the capsulated and uncapsulated pathogens, the capsulated organisms are more pathogenic in nature due to its inhibition of opsonophagocytosis, a method concerning the detection of underlying structures by complement components and/or antibody leading to engulfment by professional phagocytes (Nelson et al., 2007). To identify this virulence region, *tvi*A and *tvi*B specific primers were used to detect Vi negative *S.typhi* in *fli*C positive blood samples.

Often, where the bacterial load is less than 5 CFU/mL in the blood of patients, it could be difficult for technical advancement in this area. However it can be overcome by the use of nested PCR, which has been reported as more sensitive and specific compared with the conventional PCR (Haque et al., 2001). Multiplex PCR, target more than one sequence which can be amplified by using more than one set of primers in the reaction potential to produce considerable savings of time and effort within the laboratory without compromising test quality (Elnifro et al., 2000).

Hence, in the present study nested multiplex PCR (m-PCR) detection of *fli*C gene, *tvi*A and tviB genes of viaB operon was compared with the Widal test on samples from suspected cases to evaluate their relative utility, specificity and sensitivity.

2. Materials and methods

2.1. Bacterial strains

The virulent strain of *Salmonella typhi* (MTCC 734) was used for standardization of PCR conditions and as reference strain.

2.2. Collection of blood samples

Blood samples were obtained from patients suspected for typhoid fever through hospitals and diagnostic centers in Coimbatore. Around 250 blood samples were collected irrespective of age and duration of illness. A total of 250 (139 males and 111 females) samples with clinical suspicion of typhoid fever was collected from diagnostic lab and hospitals of Coimbatore.

2.3. DNA isolation from bacterial culture

Bacterial DNA was extracted with chloroform: isoamyl alcohol isolation method (Sambrook et al., 1989). Overnight cultures (2 mL) were taken and centrifuged at 6000 rpm for 10 min at room temperature. The pellet was resuspended in 500 μL of 1 × STE by vortexing for 2 min. Then 50 μL of 10% SDS and 150 μL of 5 M NaCl were added. The contents were mixed well and equal volume of chloroform: isoamyl alcohol (24:1) mixture was added, followed by centrifugation at 12000 rpm for 10 min at room temperature. To the aqueous layer, one third volume of isopropanol was added and centrifuged at 12000 rpm for 10 min at room temperature. The resulting DNA pellet was air dried, resuspended in 20 μL of sterile water and incubated in water bath maintained at 37 °C for 3 h or overnight. For long-term storage, DNA was resuspended in 20 μL of TE buffer and stored at − 20 °C until use.

2.4. DNA isolation from blood samples

DNA from blood samples was extracted by the procedure described by Haque et al., 1998. About 500 μL to 1 mL of blood containing 20 mM Potassium EDTA as anticoagulant was centrifuged at 10,000 rpm for 5 min. One milliliter of lysis buffer (0.2% Triton × 100 in Tris HCl, pH 8.0) was added to the pellet. The mixture was gently aspirated multiple times to produce hemolysis. Then the tube was centrifuged at 12,000 rpm for 6 min and the supernatant was discarded. The pellet was washed again with lysis buffer followed by distilled water. Finally the pellet was resuspended in 20 μL of distilled water. The tubes were sealed and subjected to lysis in boiling water for 20 min, and brought back to room temperature before being used as a template for PCR.

2.5. Primers and PCRs

The PCR primers for *S. typhi* were designed according to the flagellin (*fliC*) and Vi-negative (*tviA* and *tviB*) genes were supplied by Sigma (Dorset, United Kingdom) and Imperial Life Sciences (Pvt) limited, India (Table 1). The nested multiplex PCR assay was optimized using a Master Cycler R Eppendorf and the specificity of each primer were confirmed through single PCR. The first round multiplex PCR was performed by the addition of 5 μL of template to 40 μL of PCR mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 1 mM of each primer, 2 mM of each deoxynucleoside triphosphate, 0.5 units of *Taq* polymerase (Fermentas), and distilled water up to 40 μL. The reaction mixture was subjected to initial denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min followed by a final extension at 72 °C for 5 min.

The nested PCR was performed by the addition of 5 μL of the PCR product to a total volume of 40 μL of PCR mix contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatine, 1 mM of each primer, 2 mM of each deoxynucleoside triphosphate, 0.5 units of *Taq* polymerase (Fermentas), and distilled water up to 40 μL. The reaction mixture was subjected to initial denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 50 to 60 °C for 30 s and 72 °C for 1 to 2 min, followed by a final extension at 72 °C for 5 min.

3. Results

3.1. Determination of sensitivity of PCR

DNA was extracted from reference strain *S. typhi* and serial dilutions