Multiplex PCR for differential diagnosis of emerging typhoidal pathogens directly from blood samples

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SUMMARY

Classically *Salmonella enterica* serovar Typhi (*S.* Typhi) is associated with typhoid, a major health problem in developing countries. However, in recent years *S.* Paratyphi A and Vi-negative variants of *S.* Typhi have emerged rapidly. We have developed a nested multiplex PCR targeting five different genes for differential diagnosis of typhoidal pathogens which has been optimized to be directly applicable on clinical blood samples. Of 42 multiplex PCR-positive blood samples, 26, nine, and two were Vi-positive *S.* Typhi, Vi-negative *S.* Typhi and *S.* Paratyphi A, respectively, and five patients were found to have mixed infection. Seventeen patients grew *Salmonella* from blood culture and the remaining 25 were positive in the *Salmonella*-specific PCR. Tests with several common pathogens confirmed the specificity of the assay. We conclude that the proposed multiplex PCR is rapid, sensitive and specific for the diagnosis of typhoidal pathogens directly from blood samples.

INTRODUCTION

In humans, typhoid and related diseases commonly called enteric fever, continues to be one of the most serious public health problems worldwide. The World Health Organization estimates that the current annual global burden of typhoid is about 22 million new cases, 5% of which are fatal [1]. Developing countries have a higher incidence of typhoid because of many interrelated factors [2]. Typhoid fever in endemic areas is common, serious, and increasingly difficult to treat because of resistance to antibiotics [3].

Different microbiological and serological techniques have been used to diagnose enteric fever. These techniques include culture of blood, bone marrow,

faeces, urine [4], duodenal string culture [5], and measurement of specific antibodies by the Widal test [6], ELISA [7] and immunofluorescence [8]. These techniques have limitations in terms of specificity and sensitivity and are being replaced to a great extent by the use of PCR in routine diagnostic laboratories [9–11].

Recently, serotypes of *Salmonella* other than *S.* Typhi have been emerging as causes of typhoid in the Indian subcontinent and in the wider world [12, 13]. Moreover, the incidence of enteric fever caused by drug-resistant *S.* Paratyphi A has markedly increased and in one study represented up to 24% of all isolates [14]. These reports indicate that PCR assays specific for a single serotype will lack sensitivity for the definitive diagnosis of typhoid in all suspected cases. At the same time Vi-negative variants of *S.* Typhi have been detected in various parts of the world, especially in Pakistan, with increasing frequency [13]. These variants must have alternative

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pathogenic mechanisms (as they lack Vi antigen) and thus pose different challenges for treatment. Therefore a molecular diagnostic test giving information about all possible causative agents of typhoidal diseases has the potential to be a very useful tool.

Existing PCR assays for the diagnosis of typhoid fever detect *fliC* [15], *Vi* [16], *groEL* [17], or 16S rRNA [18] and are specific for *S*. Typhi only. To address this problem, a multiplex PCR targeting tyvelose epimerase (*tyv*) (previously *rfbE*), *fliC-d*, paratose synthase (*prt*) (previously *rfbS*), *viaB* and *fliC-a* (phase-1 flagellin; H:a) genes was developed by Hirose *et al.* [12]. The specificity of this assay was reported to be satisfactory but its use is limited to purified bacterial colonies. It lacks the sensitivity to detect directly very small numbers of bacteria that may be circulating in the blood of patients suffering from enteric fever. Its utility is not only limited by the need for culture and isolation of the bacteria, but it exhibited positivity in only 50% of blood culture-positive cases [19] and this figure is reduced for patients who have already taken an antimicrobial drug.

To overcome these problems and to make this technique directly applicable to patient samples, we designed new internal primers and developed a nested multiplex PCR that proved to be sensitive and specific for the detection of typhoidal pathogens in blood samples.

MATERIALS AND METHODS

Bacterial isolates

Thirty *Salmonella* isolates from a local population in the Faisalabad region, Pakistan, were selected from the culture collection of the National Institute for Biotechnology and Genetic Engineering (NIBGE). Representative isolates of *Escherichia coli*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Klebsiella aerogenes*, *Aerobacter* spp., *Micrococcus*, *Staphylococcus*, and *Streptococcus* were also selected as negative controls. Isolates were stored at $-20\,^{\circ}\mathrm{C}$ in TSB tryptic soy broth (TSB; Merck, Darmstadt, Germany) containing 10% dimethyl sulfoxide, and revived in the same broth as required, and subcultured on MacConkey agar for purity.

Selection of patients

Sixty patients of all ages were included in the study. All cases were admitted to different hospitals in Faisalabad. Diagnostic criteria for typhoid were fever for $\leqslant 3$ days with enlarged spleen, headache, malaise, abdominal discomfort, and/or agitation. Patients with severe diarrhoea and/or profuse vomiting were excluded.

Blood culture

Blood samples (2 ml each) were collected in tubes containing anticoagulant (20 mM potassium EDTA) and stored at $4\,^{\circ}\mathrm{C}$ and processed for PCR within 48 h of collection. A 2-ml blood sample was inoculated into a culture bottle containing 16 ml TSB with 0·02% SPS (sodium polyanethanol sulfonate) and incubated at $37\,^{\circ}\mathrm{C}$ for 72 h. This was subcultured onto MacConkey agar and after incubation overnight, isolated, colourless, smooth colonies of 2–3 mm diameter were selected for further investigation. Species were differentiated by inoculation into triple sugar iron medium (Merck) and results were interpreted according to the manufacturer's guidelines.

Serology

The presence of Vi antigen in *S*. Typhi isolates was detected by slide agglutination with monovalent Vi antiserum (Bio-Rad, Marnes-la-Coquette, France).

DNA extraction

Total genomic DNA from bacterial isolates was extracted from overnight culture in TSB by the phenol–chloroform method [20]. DNA from 200 μl of each blood sample was recovered with the aid of a DNA extraction kit (Fermentas, Hanover, MD, USA) and dissolved in 100 μl distilled water for use as the template in PCR. The integrity and purity of DNA samples were checked by 1% agarose gel electrophoresis and ratio of $A260/A280$.

Primers

Oligonucleotide primers (Sigma, Dorset, UK) targeting five different genes including *tyv*, *prt*, *fliC-d*, *fliC-a* [12] and *viaB* [16] were used simultaneously for the first round of multiplex PCR (Table 1). The primer sets for nested multiplex PCR (Table 2) were designed from the nucleotide sequence database (GenBank) and PCR conditions were optimized to increase the sensitivity to a level directly applicable on blood samples. The primer sequences and the sizes of

Table 1. *Primers for regular multiplex PCR*

Primers	Gene	Sequences (5′–3′)	Amplicon size (bp)	Ref.
tyv-s	*tyv*	GAGGAAGGGAAATGAAGCTTTT	615	[12]
tyv-as		TAGCAAACTGTCTCCCACCATAC		
parat-s	*prt*	CTTGCTATGGAAGACATAACGAACC	258	[12]
parat-as		CGTCTCCATCAAAAGCTCCATAGA		
vi-s	*viaB*	GTTATTTCAGCATAAGGAG	439	[16]
vi-as		CTTCCATACCACTTTCCG		
fliC-s	*fliC-d*	AATCAACAACAACCTGCAGCG	750	[12]
fliCd-as		GCATAGCCACCATCAATAACC		
fliC-s	*fliC-a*	AATCAACAACAACCTGCAGCG	329	[12]
fliCa-as		TAGTGCTTAATGTAGCCGAAGG		

Table 2. *Primers for nested multiplex PCR*

Primers	Gene	Sequences (5′–3′)	Amplicon	Source	Ref.
N-tyv-s	*tyv*	GTGGCTTCCTTGGGAGTAATCT	532	M29682	This study
N-tyv-as		GTATTCAAACCAAAAATCCTTGC			
N-parat-s	*prt*	GACATAACGAAC CTGCAACAGCT	180	M29682	This study
N-parat-as		CTATAATGGCGGCGGCGAGTTC			
N-vi-s	*viaB*	GATATCTATTTCGGGGGTTGGAGCT	320	D14156	This study
N-vi-as		CGATAATCTCTCTGCTCCGTC			
N-fliC-s	*fliC-d*	GCGTGAACTGGCGGTTCAGTCTGC	702	L21912	This study
N-fliCd-as		CCGTTTTTATCCTCAAACGATAG			
N-fliC-s	*fliC-a*	GCAGCGTGTGCGTGAACTGGCGG	289	X03393	This study
N-fliCa-as		GACTTCGCTCTTCACATCATAT			

their respective amplified products are shown in Tables 1 and 2.

Regular multiplex PCR

Each 50 μl of reaction mixture for regular multiplex PCR, in addition to 10 μl of template, contained 1·5 mM MgCl$_2$, 50 nmol of each dNTP, 40 pM of each primer and 5 U of *Taq* polymerase (Fermentas). The thermal cycler (MasterCycler; Eppendorf, Hamburg, Germany) conditions for 30 cycles were: denaturation at 94 °C for 1 min, annealing at 48 °C for 1 min and extension at 72 °C for 1·5 min.

Nested multiplex PCR

A 1/5 dilution (in sterile distilled water) of amplification product of regular multiplex PCR was used as template. Each 50 μl of reaction mixture for nested multiplex PCR, in addition to 10 μl of template, contained 1·5 mM MgCl$_2$, 70 nmol of each dNTP, 50 pM of each primer and 5 U of *Taq* polymerase (Fermentas). Cycling conditions for 30 cycles were: denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1·5 min. The amplification products of both regular and nested multiplex PCRs were separated by electrophoresis in 2% agarose gels, stained, and photographed by a UV transilluminator (Eagle Eye, Stratagene, La Jolla, CA, USA).

Sensitivity of regular and nested PCR

The concentration of DNA was determined spectrophotometrically (μg/μl equal to absorbance at 260 nm multiplied by 10) and extrapolated for the number of bacteria (one *S*. Typhi bacterium contains 4 fg DNA) according to calculations reported by Song *et al.* [15]. Tenfold dilutions of DNA representing viable counts of bacteria ranging from 10^7 to 10 c.f.u./ml were made in sterile distilled water and tested by regular PCR. The amplification products were subjected to nested multiplex PCR after 1/5 dilution in distilled water.

PCR for *Salmonella*

This PCR was used for cross-checking patient blood samples that were positive by multiplex PCR but negative by blood culture. It specifically targets the
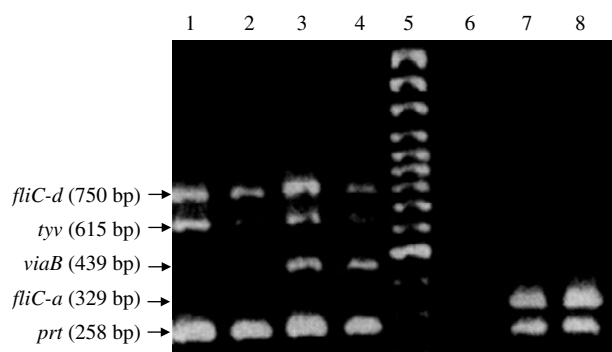
Fig. 1. Regular multiplex PCR for typhoid associated *Salmonella* species. Lanes 1 and 2, Vi-negative *S.* Typhi (*fliC-d, tyv* and *prt* genes); lanes 3 and 4, Vi-positive *S.* Typhi (*fliC-d, tyv, viaB* and *prt* genes); lane 5, molecular weight marker; lane 6, negative DNA template control; lanes 7 and 8, *S.* Paratyphi A (*fliC-a* and *prt* genes).
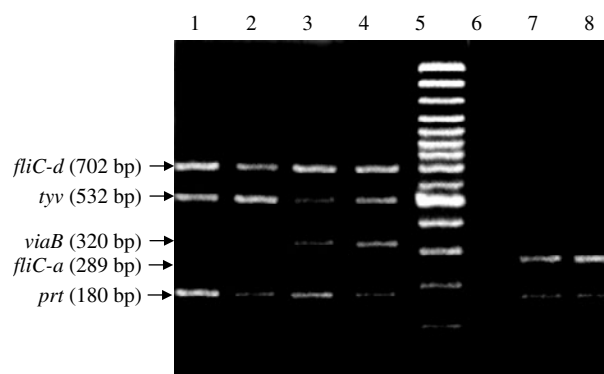

Fig. 2. Nested multiplex PCR for typhoid associated *Salmonella* species. Lanes 1 and 2, Vi-negative *S.* Typhi (*fliC-d, tyv* and *prt* genes); lanes 3 and 4, Vi-positive *S.* Typhi (*fliC-d, tyv, viaB* and *prt* genes; lane 5, molecular weight marker; lane 6, negative DNA template control; lanes 7 and 8, *S.* Paratyphi A (*fliC-a* and *prt* genes).
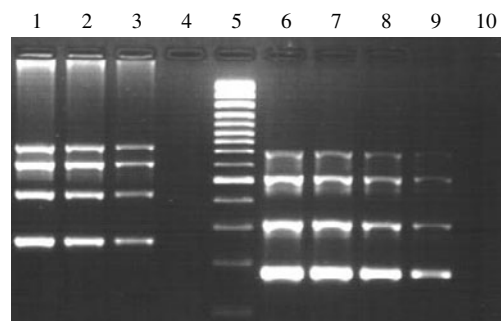

Fig. 3. Sensitivity of regular and nested multiplex PCR. Lanes 1–4, regular PCR on DNA of Vi-positive *S.* Typhi representative of 10^7, 10^6, 10^5 and 10^4 c.f.u./ml respectively; lane 5, molecular weight marker; lanes 6–10, nested PCR on DNA of Vi-positive *S.* Typhi representative of 10^4, 10^3, 10^2, 10 and 5 c.f.u./ml respectively.

aroC gene of *Salmonella*. The primers for regular PCR were 5′-GGCACCAGTATTGGCCTGCT-3′ and 5′-CATATGCGCCACAATGTGTTG-3′ [21]. The nested primers were 5′-CCTATGAGCAGAAA-TACGGC-3′ and 5′-GATTTCATCGCGATTCTG-GC-3′. For PCR, each 50 μl of reaction mixture, contained 1·5 mM MgCl₂, 40 nmol of each dNTP, 30 pM of each primer and 2 U of *Taq* polymerase (Fermentas) in addition to template. Cycling conditions for 20 cycles were: denaturation at 94 °C for 45 s, annealing at 57 °C for 1 min and extension at 72 °C for 1·5 min. Conditions were similar for the nested PCR except for annealing temperature (50 °C) and extension time (1 min). The size of amplification products was 639 bp and 460 bp for the regular and nested PCR respectively.

RESULTS

Regular and nested multiplex PCR

The regular multiplex PCR showed amplified products of expected sizes (Table 1) for *tyv, prt, fliC-d, fliC-a* and *viaB* genes with template DNA from Vi-positive and Vi-negative *S.* Typhi, and *S.* Paratyphi A (Fig. 1). In spiking experiments, the limit of detection for this PCR was 10^5 bacteria/ml.

The second round nested multiplex PCR, successfully amplified the same five genes with predicted product sizes (Table 2) from the panel of isolates (Fig. 2). All control non-*Salmonella* species were negative in the PCR. In the nested PCR of the 30 *Salmonella* isolates, 21 were Vi-positive *S.* Typhi (positive for *tyv, viaB, fliC-d* and *prt* genes), six

Vi-negative *S.* Typhi and three *S.* Paratyphi A (*prt* and *fliC-a* genes only). The nested PCR detected DNA equivalent to 10 c.f.u./ml (Fig. 3).

Diagnosis of suspected typhoid patients

Of the 60 patient cases, 17 were blood culture positive; 16 were *S.* Typhi and the other was *S.* Paratyphi A. Four of the 16 *S.* Typhi isolates were Vi-negative. By nested PCR, 26/60 cases were positive for Vi-positive *S.* Typhi, nine with Vi-negative *S.* Typhi and two with *S.* Paratyphi A. Five formed products consistent with mixed infection of Vi-positive *S.* Typhi and *S.* Paratyphi A (Fig. 4). All blood culture-positive cases were detected by PCR. Blood samples from the 25 culture-negative, PCR-positive patients were
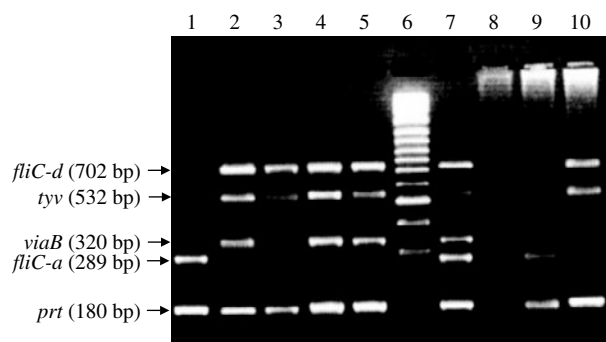
Fig. 4. Nested multiplex PCR for *Salmonella* directly from blood samples. Lanes 1 and 9, *S*. Paratyphi A (*fliC-a* and *prt* genes); lanes 2, 4 and 5, Vi-positive *S*. Typhi (*fliC-d*, *tyv*, *viaB* and *prt* genes); lane 6, molecular weight marker; lanes 3 and 10, Vi-negative *S*. Typhi (*fliC-d*, *tyv* and *prt* genes); lane 7, amplified products from mixed infection by *S*. Typhi and S. Paratyphi A; lane 8, negative case.

positive for the *aroC* gene indicative of *Salmonella* species [21].

DISCUSSION

Typhoid, usually caused by Vi-positive *S*. Typhi, is an important health problem especially in the developing countries with about 22 million new cases each year, 5% of which are fatal [1, 22]. However Vi-negative variants of *S*. Typhi and *S*. Paratyphi A are emerging as a cause of typhoidal disease. These strains lack Vi antigen which is considered to be the main virulence factor and is the major target for vaccine development [23]. Other pathogenicity mechanisms must therefore play a role in infection and if uncontrolled as a result of poor immune protection these strains may emerge as competitors to Vi-positive *S*. Typhi. The foremost step towards their control is an early diagnosis.

Conventionally, blood culture and Widal test are the most commonly used techniques for diagnosis. Blood culture ensures the diagnosis in the first week of infection but has poor sensitivity because as few as 10 bacteria/ml can cause disease [24]. The sensitivity of the blood culture is also affected by the host immune response and the intracellular characteristics of *S*. Typhi [25]. The Widal antibody test is non-specific and prone to false positivity [26], and antibodies may take a week or more to reach detectable levels. The Widal test does not distinguish between Vi-positive and Vi-negative *S*. Typhi.

PCR for the diagnosis of typhoidal diseases is now well established and offers superior sensitivity and

specificity compared with conventional methods [11, 15, 16]. However, most PCR protocols detect single pathogens and are inadequate for differential diagnosis. Multiplex PCR has been successfully applied for differential diagnosis of many diseases caused by viruses, bacteria, fungi, and parasites [27]. Hirose *et al.* [12] reported a multiplex PCR that could differentiate between typhoidal pathogens. They targeted five genes: *prt* (present in both *S*. Typhi and *S*. Paratyphi A) that encodes CDP-paratose synthase, which converts CDP-4-keto-3,6-dideoxyglucose to CDP-paratose; *tyv* (present in both *S*. Typhi and *S*. Paratyphi A) that encodes CDP-tyvelose epimerase, which converts CDP-paratose to CDP-tyvelose but the described primers target a specific region of the *tyv* gene of *S*. Typhi that has been deleted in *S*. Paratyphi A causing a frameshift mutation that converts codon 4 of *tyv* to a stop codon [28]; *viaB* which is only found in Vi-positive organisms and is involved in synthesis of the capsule polysaccharide; the *fliC-d* gene (phase-1 flagellin gene for d antigen [H:d]) which is specific for *S*. Typhi; and *fliC-a* gene (phase-1 flagellin; H:a) which is present only in *S*. Paratyphi A. This PCR proved to be effective for the identification of isolated typhoidal bacteria but it lacked the sensitivity to be directly applicable for blood samples. The need to culture blood samples, isolate the bacteria and perform the PCR incorporates the inherent lower sensitivity of blood culture.

We developed a nested multiplex PCR as an extension of the original PCR by carefully designing highly specific internal primers (Table 2, Fig. 2). PCR conditions were optimized to detect at least 10 c.f.u./ml so that it could be successfully applied to blood samples directly as a diagnostic tool. False-negative results are theoretically highly unlikely owing to simultaneous amplification of *prt* and *tyv* genes which are exclusively and universally present in *S*. Typhi and *S*. Paratyphi A [12]. We verified the specificity of our nested PCR procedure by its negative reactions with control species.

The technique was evaluated directly on blood samples of 60 clinically suspected cases of typhoid. PCR detected typhoidal species in 42 cases, only 17 of which were culture positive. The presence of *Salmonella* in these samples was confirmed with genus-specific primers [21]. These results show that the nested PCR has good potential to be a rapid tool for the definitive, differential diagnosis of typhoid and is superior to conventional methods.

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