Direct detection of *Salmonella typhi* in blood by polymerase chain reaction

S3-5

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Abstrak

Sepasang primer nested-PCR telah dirancang untuk mendeteksi secara spesifik S. typhi, *agen penyebab demam tifoid. DNA target untuk pasangan primer PCR tersebut adalah daerah* spacer *antara gen 23 S rRNA dan 5 S rRNA dari* S. typhi *(ATCC 167). Pasangan primer ini telah dicoba pada 5 spesimen darah pasien tifoid yang telah dikonfirmasi dengan kultur. Produk* nested PCR *positif yang berupa 140 bp ditemukan pada semua 5 spesimen. Pemeriksaan lebih dari 100 spesimen feses yang kemudian dikonfirmasi dengan kultur* S. typhi *negatif tidak satupun memberikan produk PCR positif. Tingkat deteksi dalam darah yang diinokulasi dengan* S. typhi *dalam jumlah yang diketahui adalah 10 cfu per ml darah. Pasangan primer ini mempunyai potensi untuk pengembangan suatu uji untuk diagnosis cepat demam tifoid.*

Abstrak

A pair of nested PCR primers has been designed for the specific detection of S. typhi, *the causative agent of typhoid fever. The target DNA for this pair of PCR primers is the spacer region between the 23S rRNA and 5S rRNA genes from* S. typhi *(ATCC 167). This pair of primers has been tested against 5 blood specimens from patients. These patients are typhoid cases, confirmed by culture. The positive 140 bp nested PCR product was obtained from all 5 specimens. From more than 100 stool specimens, which were later confirmed to be* S. typhi *negative by culture, none gave the positive PCR product. The detection level in blood inoculated with known numbers of* S. typhi, *is 10 CFU per ml blood. This pair of primers, therefore, has potential for development into a diagnostic tool for rapid diagnosis of typhoid fever.*

MATERIALS AND METHODS

Bacterial strains and specimens

S. typhi (ATCC 167) obtained from the Singapore General Hospital was used as the type strain. Blood and stool specimens were also from SGH. Other *S. typhi* and non-typhoid salmonellae were clinical isolates from various hospitals in Singapore.

Extraction of DNA

Direct extraction of genomic DNA from blood was carried out as according to manufacturer's (Boehringer Mannheim mammalian blood DNA extraction kit) instructions. The DNA precipitate obtained from 1 ml blood was re-dissolved in 100 µl TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5). Five µl of this was used in each PCR. Extraction of genomic DNA from bacteria was carried out as described in Zhu *et al*[1].

Extraction of genomic DNA from stool specimens was carried out as described by Kongmuang *et al*[2].

PCR primers

For specific detection of *S. typhi*, two pairs of primers (F1/R4 & M1/M3) were used. The positions of these two pairs of primers are shown in Figure 1. For PCR ribotyping, four pairs of primers were used. Primers E1/E2 are based on *Escherichia coli* rRNA sequences (Kostman *et al*[3]). Primers K1, K2, K3 (see Figure 1) were used with M3. All primers were synthesized by Bio-Synthesis Inc. (TX, USA).

PCR conditions

The optimum conditions for both primary & nested primers are given in Table 1. All PCR were carried out using Taq polymerase (HT Biotechnology Ltd, U. K.) in a DNA thermal cycler (Hybaid Omnigene). The primary PCR mixture contained 75 mmol l^{-1} KCl, 10 mmol l^{-1} Tris-HCl (pH 9.2), 3.5 mmol l^{-1} $MgCl_2.6H_2O$, 2 mmol l^{-1} of each dNTP, 0.004 µg μl^{-1} of each primer, 0.4 U μl^{-1} Taq polymerase and 0.002 ng μl^{-1} to 0.02 ng μl^{-1} of template DNA. The primary PCR products were diluted 500 fold before being used for the nested PCR. The buffer used for the

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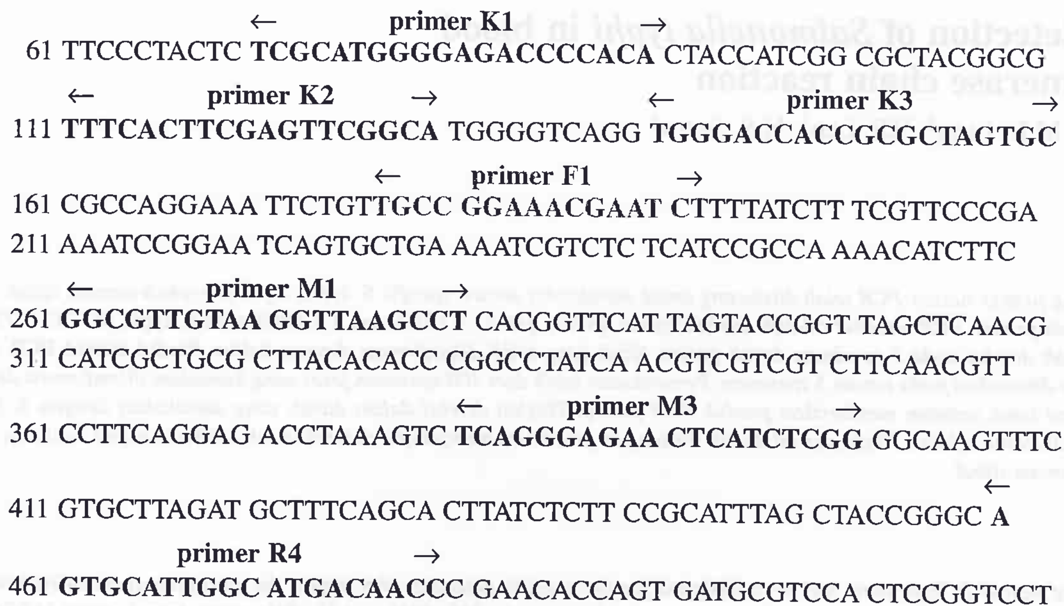

Figure 1. Sequence of the PCR primers and their corresponding regions in the S.typhi (ATCC 16) 5S and 23S rRNA genes (EMBL GenBank accession No. U04734).

nested PCR contained 1.5 mmol l^{-1} $MgCl_2.6H_2O$ and 25 mmol l^{-1} KCl; the other conditions are as for the primary PCR. PCR conditions for ribotyping are the same as for primary PCR except that template concentration was 0.01 $\mu g\ \mu l^{-1}$ and the primer concentration was 0.6mM for E1/E2 and 0.2mM for K/M4. The PCR mixtures (20 µl) were analysed by electrophoresis in 1-1.2 % agarose gel (Sigma Type 1-A). PCR patterns were compared after ethidium bromide staining.

Nucleotide sequence analysis

The PCR amplicons (300 bp & 140 bp) were excised from agarose gels and purified by phenol (Ultrapure,Gibco-BRL) extraction and ethanol precipitation. The purified PCR amplicons were then sequenced using a cycle sequencing Kit (ABI prism d-Rhodamine terminator cycle sequencing reaction kit) with amplitaq polymerase (Perkin-Elmer).

RESULTS

Specificity of the PCR primers

The primary PCR primers were designated F1 and R4 (Zhu *et al*[1]). F1 (5' - TGCCGGAAACGAATCT - 3') is complementary to a segment in the 5S to 23S rRNA spacer region, which is highly variable and specific for *S. typhi*. R4 (5' - GGTTGTCATGCCAA TGCACT - 3') is complementary to a highly conserved region of the 23S rRNA. This pair of primary PCR primers will amplify a 300 bp DNA fragment (nucleotides 177-480 in Figure 1).

The nested primers M1 (5'- GGCGTTGTAAGGTT AAGCCT - 3') and M3 (5' - AGGCTTAACCTTA-CAACGCC - 3') will amplify a 140 bp DNA fragment (nucleotides 261-400 in Figure 1). The identity of both the 300 bp and 140 bp PCR amplicons were confirmed by sequencing. The optimum conditions for both pairs of primers were determined and are given in Table 1.

Table 1. PCR conditions

Process	Primary		Nested		PCR-Typing	
	Temp.	Time taken	Temp.	Time taken	Temp.	Time taken
Pre-denaturation	95°C	5 min	95°C	2 min	94°C	5 min
Denaturation	95°C	50 s	95°C	30 s	94°C	1 min
Annealing	58°C	60 s	52°C	30 s	53°C	1 min
Extension	72°C	50 s	72°C	30 s	72°C	1 min
No. of cycles	35		25		30	
Post-extension	72°C	5 min	72°C	5 min	72°C	5 min
TOTAL		3 h		2 h		3 h

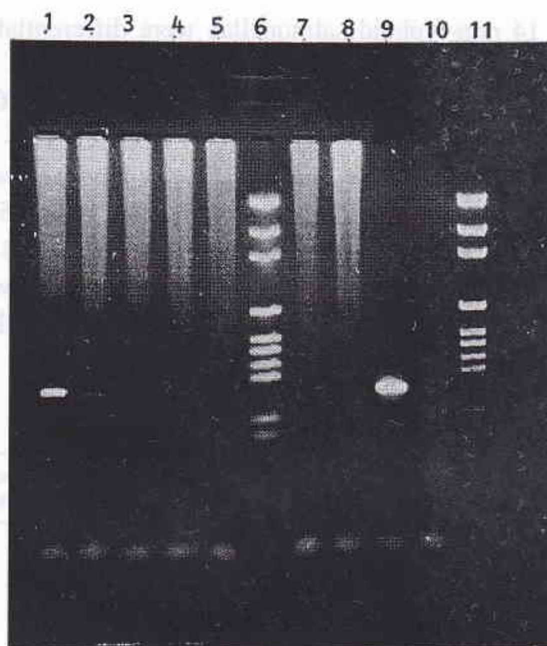

***Figure 2.** Electrophoretogram of primary PCR products using DNA extracted from blood spiked with varying CFU of S. typhi*

lane 1: 10^3, lane 2: 10^2, lane 3: 50, lane 4: 25, lane 5: 10, lane 6 & 11: pGEM, lane 7 & 8: 0, lane 9: S. typhi genome DNA, lane 10: water

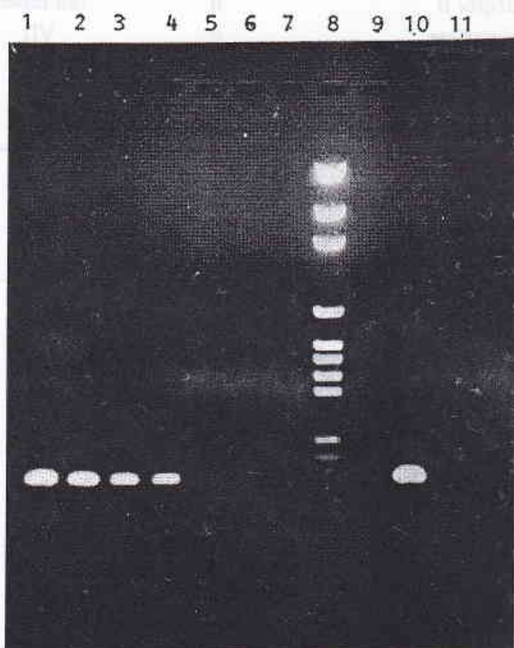

***Figure 3.** Electrophoretogram of nested PCR products using diluted primary PCR from Figure 2 as template*

lane 1: 10^3 CFU, lane 2: 10^2 CFU, lane 3: 50 CFU, lane 4: 25 CFU, lane 5: 10 CFU, lane 6 & 7: 0 CFU, lane 8: pGEM, lane 9: water, lane 10: S. typhi genome DNA,

Sensitivity of the PCR

The primary and nested amplification products are shown in Figures 2 & 3. In Figure 2, only lane 5 did not show the expected 300 bp PCR product, i.e. the detection level was 25 CFU. In Figure 3, only lane 5 showed the 140 bp nested PCR product, indicating that the detection level is 10 CFU. Therefore the use of nested primers has increased the sensitivity level (see Table 2).

Table 2. Detection of *S. typhi* in whole blood by PCR

Concentrations of S. typhi in artificially inoculated blood used for PCR CFU/ml	[1]PCR result (300 bp product)	[2]Nested PCR result (140 bp product)
1 x 10^5	+	+
1 x 10^3	+	+
1 x 10^2	+	+
50	+	+
25	+ (faint)	+
10	+	+ (faint)
0 (uninoculated control)	–	–
Positive control for PCR	+	+
H_20 control for PCR	–	–

[1] *1 ml of the artificially inoculated blood was processed using Boehringer Blood Kit. The extracted DNA was dissolved in 100 µl TE buffer, and (i.e., 5 µl) of which was used for PCRs.*

[2] *A 300-fold dilution of the primary PCR products was carried out, and 5 µl of which was used for the nested PCRs.*

Direct detection of *S. typhi* in blood from typhoid patients

Of five blood specimens from typhoid patients which were tested, all five showed the *S. typhi*-specific 300 bp amplicon (see Figure 4, results from 3 specimens are shown). The use of undiluted DNA extract from typhoid patients' blood as template, yielded the *S. typhi*-specific 300 bp amplicon (see lanes 1, 2 & 3, Figure 4). However there were several non-specific amplicons. Dilution of the template ten-fold removes most of the non-specific amplicons while yielding a fairly distinct *S. typhi*-specific 300 bp amplicon (see lanes 5, 7 & 10, Figure 4).

Direct detection of *S. typhi* in stool specimens

One hundred stool specimens were tested with the primary primers but no *S. typhi* specific PCR amplicon were obtained. These specimens were later shown to culture negative for *S. typhi*. Another ten stool specimens, from typhoid patients who had al-

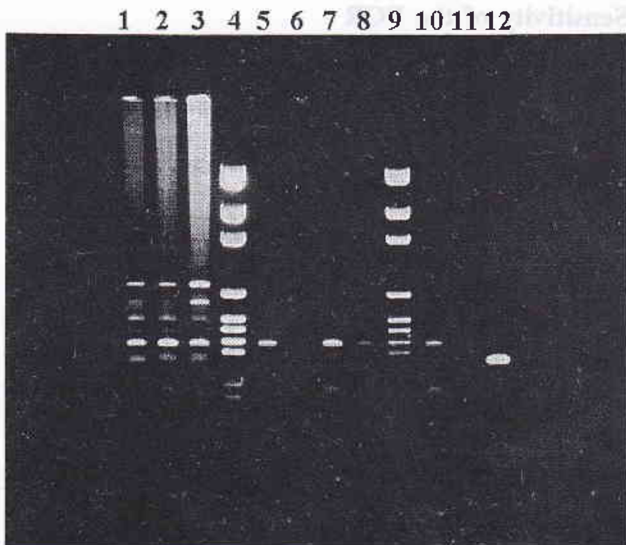

Figure 4. Electrophoretogram of primary PCR products using DNA extracted from blood of typhoid patients

lane 1: B33, lane 2: B34, lane 3: B35, lane 4 & 9: pGEM, lane 5: B33 diluted 1:10, lane 6: B33 diluted 1:30, lane 7: B34 diluted 1:10, lane 8: B34 diluted 1:30, lane 10: B35 diluted 1:10, lane 11: B35 diluted 1:30, lane 12: S. typhi genomic DNA

ready started on antibiotic therapy, were also tested. These also did not yield the *S. typhi* specific PCR amplicon.

PCR-ribotyping

Based on the PCR results, four pairs of primers were designed for use in ribotyping. Using the K3/M3 (K3: 5'-TGGGACCACCGCGCTAGTGC-3') primers, 14 non-typhoid salmonellae were differentiated into 8 ribotypes. Using a mixture of two pairs of primers (E1/E2; E1:5' - TTGTACACACCGCCCGTCA - 3', E2: 5' -GGTACCTTAGATGTTTCAGTTC - 3' & K3/M3), these 14 non-typhoid salmonellae were further differentiated into 9 ribotypes (see Figure 5 & Table 3). The other two pairs of primers (K2/M3 & K3/M3) did not increase the differentiating power when used on its own or together with the other primer pairs.

Table 3. PCR ribotypes

Strains	PCR ribotypes (K3/M3)	PCR ribotypes (E1/E2 & K3/M3)
Salmonella agona	a	I
S. anatum	b	II
S. cervo	b	III
S. choleraesuis	h	X
S. enteritidis	c	IV
S. give	b	V
S. haardt	b	V
S. hader	a	I
S. javiana	e	VI
S. kingston	not tested	I
S. paratyphi A	g	X
S. paratyphi B	h	not tested
S. typhimurium	e	VII
S. weltevreden	f	VIII
S. typhi (ATCC 167)	d	IX
Escherichia coli TG1	i	XI

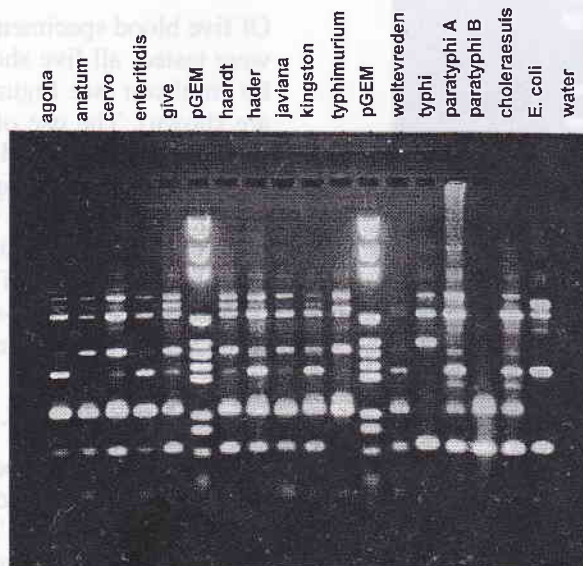

Figure 5. Electrophoretogram of primary PCR products using genome DNA from different Salmonella serotypes as template

DISCUSSION

All the blood specimens from typhoid patients tested positive with the primary primers. The use of nested PCR primers increased the sensitivity of detection to 10 CFUs. Therefore these two pairs of primers have the potential to be developed into a routine diagnostic agent for the rapid diagnosis of typhoid fever.

Conventional ribotyping, using rRNA genes to probe bacterial genomic DNA, has been shown to be useful in strain differentiation in a variety of bacteria (Bingen *et al*[4]). However this method is time-consuming involving electrophoresis, blotting, hybridisation and X-ray film development. PCR ribotyping, on the other hand, is a more rapid method. It involves only PCR and electrophoresis. In this study, both the 16S-23S and 23S-5S rRNA intergenic spacers were used as targets for PCR. The current primer set (E1/E2 & K3/M3) differentiates 14 tested serotypes into only 9 ribotypes. Therefore the differentiation power is as yet inadequate for use in typing salmonellae. Due to the extreme ease and rapidity of PCR-ribotyping, this method has great potential for development into a typing method.

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