Short Report

Selective Amplification of \( \text{prt}, \text{tyv and invA} \) Genes by Multiplex PCR for Rapid Detection of \( \text{Salmonella typhi} \)

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ABSTRACT

A multiplex PCR-based assay was developed for detection of \( \text{Salmonella typhi} \) and identification of other salmonella serotypes. Three primer-sets were selected from different genomic sequences, \( \text{malo2-F/malo2-Ra} \) primers from invasion gene, \( \text{Parat-s/Parat-as} \) as well as \( \text{tyv-s/tyv-as} \) primers from O-antigen gene cluster of the genus \( \text{Salmonella} \). This method differentiated \( \text{Salmonella} \) spp., based on size and number of amplified fragments. The \( \text{Salmonella para typhi A and B} \) yielded two bands of 373 bp and 285 bp, respectively, and the other species including \( \text{S. paratyphi C, S. infantis and S. havana} \) yielded only one 373 bp band. The PCR products of \( \text{S. typhi} \) and \( \text{S. enteritidis} \) were 373, 285 and 615 bp. In testing the specificity of the assay, no amplification was observed in non-\( \text{Salmonella} \) species such as \( \text{Shigella, Klebsiella, E. coli, Proteus, Staphylococcus} \) and \( \text{Streptococcus} \). The sensitivity of the method was evaluated about \( 2.5 \times 10^2 \) CFU/ml, that could be detected by the PCR assay. \( \text{Iran. Biomed. J.} \) 9 (3): 135-138, 2005

Keywords: Multiplex PCR, \( \text{Salmonella typhi} \), Rapid detection

INTRODUCTION

Typhoid fever, a septicemic disease caused by \( \text{Salmonella typhi} \), is a serious health problem in developing countries [1]. Different diagnostic methods are used for the diagnosis of typhoid, including blood culture, bone marrow culture, urine culture, rose spot culture, duodenal string culture, Widal test and ELISA. Widal test and blood culture remain the only universally practiced diagnostic procedures, because other methods are either invasive, have failed to prove their utility, or are expensive [2].

Blood cultures are negative in 30-65% of cases with typhoid fever because of prior administration of antibiotics or a low number of organisms. Widal test has been found to be non-specific and difficult to interpret in areas where typhoid fever is endemic [1].

Molecular techniques can be used for early detection of the disease. Hybridization using DNA probes was the first molecular biology technique used for the diagnosis of typhoid fever. This technique is specific, but its sensitivity is poor. The advent of PCR technology has provided sensitivity and specificity for the diagnosis of typhoid [2].

In this work, we developed and evaluated a multiplex PCR-based assay in rapid detecting of \( \text{S. typhi} \) using three primer-sets. We used purified colonies for our multiplex PCR. We plan to examine whether our system is usable for direct detection from clinical samples.

MATERIALS AND METHODS

Bacterial strains. Seven pure cultures of \( \text{Salmonella} \) including \( \text{S. typhi} \), \( \text{S. parai A, B and C} \).
S. havana, S. infantis and S. enteritidis were provided by Reference Laboratory of the Pasteur Institute of Iran (Tehran).

**DNA extraction.** To recover DNA for PCR amplification, the procedure carried out using DNG\textsuperscript{TM}-plus Kit (CinnaGen, Cat no. DN8117C, Iran). Briefly, 100 μl of overnight LB broth bacteria culture (OD\textsubscript{600} = 2) was mixed with 400 μl of DNG\textsuperscript{TM}-plus solution and vortexed for 20 s. Then, 300 μl of isopropanol was added to the sample and gently mixed by inversion (10 times) and put in -20°C for 20 min. The mixture, centrifugated at 12,000 ×g for 10 min, and after 2 times washing with sterile distilled water and incubated at 95°C for 5-10 min. The sample was spinned at 12,000 ×g for 30 s, and supernatant containing purified DNA used for PCR.

**Amplification.** The multiplex PCR was performed using three primer-sets, which were targeted for invasion A (Malo2-F, Malo2-Ra), parataos synthetase (Parat-s, Parat-as) and tyvelose epimerase (tyv-s, tyv-as) genes [3-5]. The O antigen synthesis genes’ primer-sets Parat-s/Parat-as and tyv-s/tyv-as are specific for S. typhi, S. paratyphi A, B and S. enteritidis, and also S. typhi and S. enteritidis, respectively [4, 5]. Uniplex PCR of each primer was optimized individually, then multiplex PCR carried out in a total volume of 25 μl, containing 1 unit of Taq DNA polymerase (CinnaGen, Iran), 0.2 μM of each primer, 0.2 mM of dNTP (Fermentas, Lithuania), 2.5 μl 10 × PCR buffer, 1.5 mM MgCl\textsubscript{2} and 5 μl DNA template. The reactions were performed as follows: predenaturation at 95°C for 3 min, 30 cycles of denaturation at 95°C for 1 min, annealing at 59°C for 1 min, elongation at 72°C for 30 s, followed by a final extension at 72°C for 5 min. PCR products were visualized by electrophoresis in a 1.5% agarose gel stained with ethidium bromide.

In order to evaluate the sensitivity of the method, the overnight culture of a single colony of S. typhi was diluted many times in LB broth and titrated. The DNA extraction from S. typhi was done using the DNG\textsuperscript{TM}-plus kit and applied to multiplex PCR. To validate the specificity of multiplex PCR, 6 non-Salmonella strains including Klebsiella sp., Proteus sp., Shigella sp., E. coli, Staphylococcus spp. and Streptococcus spp. were tested as already described.

**RESULTS AND DISCUSSION**

There are several target genes and different methods used by researchers to detect Salmonella strains. They used multiplex PCR on rfbE, fliC, invA, virA, spvC, invA, int and f1O as target genes, uniplex PCR on rRNA gene and Nested PCR over fellagelin gene [2, 5-10].

We developed a multiplex PCR method which involves in amplification of invA, prt and tyv genes of Salmonella spp. The primers malo2-F/malo2-Ra from invA [3], parat-s/parat-as from prt, and tyv-s/tyv-as from tyv (the two latter belong to O-antigen synthesis genes) [4] were efficient in this study (Table 1). The invA gene is essential for full virulence in Salmonella and thought to trigger the internalization required for invasion of deeper tissues [5], and is specific for Salmonella spp. [6, 11-13]. The invA primers amplified 373 bp fragment in all seven strains of S. typhi, S. para A, B, and C, S. havana, S. infantis, and S. enteritidis. The parat-s/parat-as primers, amplified 285 bp fragment of prt gene. The prt gene encodes CDP-paratose synthase which converts CDP-4-Keto-3,6-dideoxy to CDP-paratose. This gene is present in S. typhi, S. paratyphi A and B, and S. enteritidis strains. The tyv-s/tyv-as primers amplified the 615-bp fragment of tyv gene only in S. typhi and S. enteritidis strains. The tyv gene encodes CDP-tyvelose epimerase that converts CDP-paratose to CDP-tyvelose [3].

<table>
<thead>
<tr>
<th>Gene and primers</th>
<th>Length (bp)</th>
<th>Amplified fragment size (bp)</th>
<th>Ref.</th>
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<tbody>
<tr>
<td><strong>invA</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Malo2-F: 5'-TATTGTTGATTAATGAGATCCG-3'</td>
<td>23</td>
<td>373</td>
<td>3</td>
</tr>
<tr>
<td>Malo2-Ra: 5'-ATATTACGCAGGAAACTGTT-3'</td>
<td>22</td>
<td></td>
<td></td>
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<tr>
<td><strong>Prt</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parat-s: 5'-CTTGTATGGAAGACATACAGGACC-3'</td>
<td>25</td>
<td>258</td>
<td>4</td>
</tr>
<tr>
<td>Parat-as: 5'-CGTCTCCATACAAAGCTCCATAGA-3'</td>
<td>24</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>tyv</strong></td>
<td></td>
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<tr>
<td>tyv-s: 5'-GACGAAGGGGAATGAGAAAGCTTAT-3'</td>
<td>22</td>
<td>615</td>
<td>4</td>
</tr>
<tr>
<td>tyv-as: 5'-TAGCAAACGTCTCCACCACACAT-3'</td>
<td>23</td>
<td></td>
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</tbody>
</table>
Fig. 1. Agarose gel (1.5%) electrophoresis of multiplex PCR products of reference samples. Lane 1, 100 bp DNA ladder; Lane 2, *S. typhi*; Lane 3, *S. paratyphi* A; Lane 4, *S. infantis*; Lane 5, *S. havana*; Lane 6, *S. paratyphi* C; Lane 7, *S. paratyphi* B; Lane 8, *S. enteritidis*; Lane 9, negative control (no DNA template).

The results indicate that *S. typhi* and *S. enteritidis* have high similarity percentage in O-antigen genes. Based on specific clinical signs, the differentiation of these serotypes is easily accessible (Fig. 1).

Six strains of non-*Salmonella* Gram-negative bacteria, including *Shigella*, *Klebsiella*, *E. coli* and *Proteus*, which have genetic properties similar to *Salmonella* species, along with *Staphylococcus* and *Streptococcus* from Gram-positive bacteria, were tested by primer-sets and no amplified fragments were seen (Fig. 2). The results of the specificity test obtained in this experiment was 100% in consistency with the works of other investigators [1, 6, 8, 9, 11, 14].

The sensitivity of PCR amplification depends on the number of copies of target DNA, and can be evaluated using of extracted pure DNA. Serial dilution of *S. typhi* culture was prepared and tested by multiplex PCR assay. The sensitivity test determined that $2.5 \times 10^2$ CFU/ml target bacteria could be detected by multiplex PCR assay (Fig. 3). The result is in accordance with those of other investigators [1, 2, 7-9, 12, 14]. They performed it with 1, 5, 30, 40, 100 and 300 bacterial cells using different methods. Although, some researchers did not study the sensitivity test [4, 5, 11].

The rapid detection of purified colonies of *S. typhi* by multiplex PCR in our work, could be used for direct detection of clinical samples.

Fig. 3. Agarose gel (1.5%) electrophoresis of multiplex PCR products in sensitivity test. Lane 1, 100 bp DNA ladder; Lane 2, *S. typhi* as positive control; Lane 3, $25 \times 10^4$ cells; Lane 4, $25 \times 10^3$ cells; Lane 5, $25 \times 10^2$ cells; Lane 6, 250 cells; Lane 7, 25 cells; Lane 8, 10 cells; Lane 9, 1 cell; Lane 10, negative control (no DNA template).

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REFERENCES


