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Short Report

Selective Amplification of *prt, tyv and invA* Genes by Multiplex PCR for Rapid Detection of *Salmonella typhi*

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ABSTRACT

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

Keywords: Multiplex PCR, *Salmonella typhi*, Rapid detection

INTRODUCTION

Typhoid fever, a septicemic disease caused by *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 *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 *Salmonella* including *S. typhi*, *S. parai A*, *B* and *C*,

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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^{TM}-plus Kit (CinnaGen, Cat no. DN8117C, Iran). Breifly, 100 µl of overnight LB broth bacteria culture (OD_{600} = 2) was mixed with 400 µl of DNG^{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 -20ºC for 20 min. The mixture, centrifugated at 12,000 ×g for 10 min, and after 2 times washing and drying, the pellet was dissolved in 50 µl of sterile distilled water and incubated at 95ºC 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 $MgCl_2$ and 5 µl DNA template. The reactions were performed as follows: pre-denaturation at 95ºC for 3 min, 30 cycles of denaturation at 95ºC for 1 min, annealing at 59ºC for 1 min, elongation at 72ºC for 30 s, followed by a final extension at 72ºC 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^{TM}-plus kit and applied to multiplex PCR. To validate the specificity of multiplex PCR, 6 non-*Salmonella* strains including *Kelebsiella 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 *flO* 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. entritidis*. 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 1. Selected primer-sets.

Gene and primers	Length (bp)	Amplified fragment size (bp)	Ref.
invA			
Malo2-F: 5'-TATTGTTGATTAATGAGATCCG-3'	23	373	3
Malo2-Ra: 5'-ATATTACGCACGGAAACACGTT-3'	22		
Prt			
Parat-s: 5'-CTTGCTATGGAAGACATACACGAACC-3'	25	258	4
Parat-as: 5'-CGTCTCCATCAAAAGCTCCATAGA-3'	24		
tyv			
tyv-s: 5'-GACGAAGGGAAATGAAGAAGCTTTT-3'	22	615	4
tyv-as: 5'-TAGCAAACTGTCTCCCACCATAC-3'	23		

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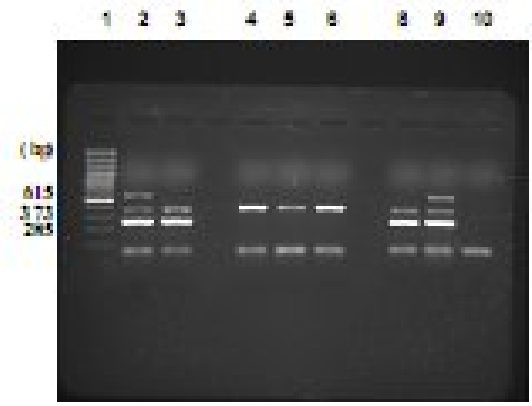

Fig. 1. Agarose gel (1.5%) electrophoresis of multiplex PCR products of reference samples. Lane 1, 100 bp DNA ladder; Lane 2, *S. typhi*; Lane 3, *S. paratyphi A*; Lane 4, *S. infantis*; Lane 5, *S. havana*; Lane 6, *S. paratyphi C*; Lane 7, *S. paratyphi B*; Lane 8, *S. enteritidis*; Lane 9, negative 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 sings, the differentiation of these serotypes is easily accessible (Fig.1).

Six strains of non-*Salmonella* Gram-negative bacteria, including *Shigella, Kelebsiella, 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 obtaind in this experiment was 100% in consistency with the works of other investigators [1, 6, 8, 9,11,14,15].

Fig. 2. Agarose gel (1.5%) electrophoresis of multiplex PCR products of specificity test. Lane1, 100 bp DNA ladder; Lanes 2 and 7, *S. typhi* and *S. paratyphi C,* respectively as positive controls; Lane 3, *Kelebsiella*; Lane 4, *Shigella*; Lane 5, *E. coli*; Lane 6, *Proteus*; Lane 8, *Staphilococcus*; Lane 9, *Streptococcus* and Lane 10, negative control (no DNA template).

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 2.5×10^2 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.

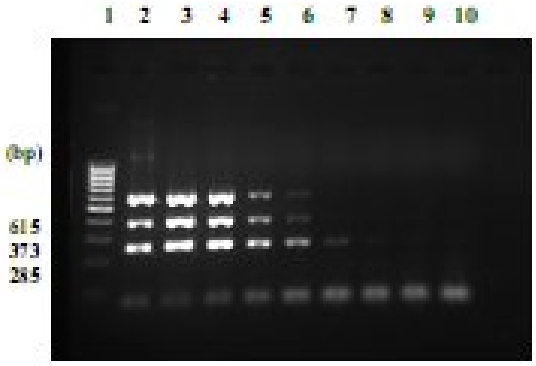

Fig. 3. Agarose gel (1.5%) electrophrosis of multiplex PCR products in sensitivity test. Lane 1, 100 bp DNA ladder; Lane 2, *S. typhi* as positive control; Lane 3, 25×10^4 cells; Lane 4, 25×10^3 cells; Lane 5, 25×10^2 cells; Lane 6, 250 cells; Lane 7, 25 cells; Lane 8, 10 cells; Lane 9, 1 cell; Lane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

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