Development of an ultra rapid and simple multiplex polymerase chain reaction technique for detection of *Salmonella typhi*

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ABSTRACT

Objective: To make a rapid and definite diagnosis of *Salmonella enteritis*, using an ultra rapid multiplex polymerase chain reaction (PCR) detection method for major *Salmonella* serotypes, such as *Salmonella typhi, Salmonella Typhimurium* and *Salmonella Havana*.

Methods: We performed this study at the Research Center of Molecular Biology, Institute of Military Medicine, Bagyatallah University of Medical Sciences, Iran from June 2004 to July 2005. The PCR primers for tyv (rfbE), prt (rfbS) and invA genes were designed and used for the rapid identification of *Salmonella enterica* serovars *Typhi* and *Paratyphi A* with multiplex PCR. By using simple DNA extraction method in 10 minutes, rapid PCR cycles with total cycle times of 35 minutes and rapid electrophoresis procedure with simple and very cheap buffer used in 200 to 300 volts for 15 minutes to separate the PCR products.

Results: The results showed that all reference and clinical isolates of *Salmonella serovars Typhi* and *Paratyphi* were accurately identified by this assay. Specificity analysis revealed no cross-reaction with other *Enterobacterial* strains. The sensitivity of the PCR and the multiplex PCR was 1-10 cells. The total time of Multiplex PCR from sample preparation to final result is 45-50 minutes.

Conclusions: These data indicate that the specificity and sensitivity of the PCR and the multiplex PCR make them potentially valuable tools for diagnosis of *Salmonella typhi* bacteria and that they may be used for the identification of *Salmonella enteritidis* responsible for sporadic enteritis cases.

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Salmonellosis is responsible for large numbers of infections in both humans and animals.[1] Typhoid fever and paratyphoid fever are still serious public health problems in many geographic areas and are endemic in most countries, *Salmonella enterica* (*S. enterica*) *serotype Typhi* is a member of the family *Enterobacteriaceae*. Conventional methods of isolation of *Salmonella* strains take 4-7 days to complete and are therefore laborious and require substantial manpower.[2] The genomes of *S. enterica serotype typhi CT 18, S. enterica serotype typhimurium* LT 2, 18 and *Escherichia coli* (*E. coli*) 19 are essentially collinear, despite the fact that *E. coli* and *S. enterica* diverged approximately 100 million years ago. Similar environmental requirements for these enteric bacteria presumably explain this conservation of gene order. Gene clusters unique to particular bacteria are likely to represent adaptations to particular environments or may contribute to pathogenicity. Most of the researchers, who have already reported using PCR for detection of serovar *Typhi*, have employed only one gene of serovar *Typhi*

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in their studies (fliC-d gene, Vi capsular antigen gene or 16S rRNA gene). Since one gene was targeted for the identification of serovar *Typhi* in these methods, strains of *Salmonella* serovars other than serovar *Typhi* were detected in some cases.[3] A rapid, alternative method is needed for the diagnosis of typhoid or paratyphoid fever agent. Some researchers have already reported serovar Typhi detection methods with PCR that use the fliC-d gene,[4] the Vi capsular antigen gene,[5] and the 16S rRNA gene.[6] In this study, we developed a more specific diagnosis method for both typhoid fever and paratyphoid fever based on a multiplex PCR technique that detected the Vi antigen gene (viaB), H and O antigen synthesis genes [tyv and paratose synthase gene (prt)]. Applying multiplex PCR technique, we developed a more specific diagnosis method for both typhoid fever and paratyphoid fever. In this regard, we targeted the tyvelose epimerase, and paratose synthase genes in our study. This system enabled us to identify and distinguish serovars *Typhi* and *Paratyphi A*, which are clinically more important human pathogens, by only a single PCR, when we isolated the bacteria from blood or stool cultures from clinical patients.

Methods. This study was carried out from June to July 2005 at the Research Center of Molecular Biology, Institute of Military Medicine, Bagyatallah University of Medical Sciences, Iran. *Salmonella* strains*, Salmonella typhi, Salmonella Typhimurium Salmonella paratyphi A, Salmonella Paratyphi B Salmonella Paratyphi C, Salmonella infantis and Salmonella havana* and non-*Salmonella strains Klebsiella pneumoniae, Escherichia coli* were included in this study. The bacterial strains were collected from the clinical laboratory of Bagyatallah University of Medical Sciences and Reference Laboratory of Iran. All isolates were identified by biochemical and serological tests. A suspension of bacteria was heated at 100°C for 10 minute. We used the samples for the PCRs.

Polymerase chain reaction primers DNA amplification and detection. We designed the primers tyvF and tyvR for detection of the tyvelose epimerase gene (tyv, previously called rfbE), forward primer GAG GAA GGG AAA TGA AGC TTT T and reverse primer TAG CAA ACT GTC TCC CAC CAT AC this with PCR product size of 615 bp, primers paratF CTT GCT ATG GAA GAC ATA ACG AAC C and paratR CGT CTC CAT CAA AAG CTC CAT AGA with product size of 259 bp were designed for detection of a paratose synthase gene (prt, previously called rfbS) and primers invF GTA TTG TTG ATT AAT GAG ATC CG and invR ATA TTA CGC ACG GAA ACA CGT T with product size of 373bp. The gene prt encodes CDP-paratose synthase, which converts CDP-4-keto-3, 6-dideoxyglucose to CDP-paratose. The gene prt is present in both serovars *Typhi* and *Paratyphi A*. The gene tyv encodes CDP-tyvelose epimerase, which converts CDP-paratose to CDP-tyvelose. The tyv gene is present in both serovars *Typhi* and *Paratyphi A,* but the tyv gene of serovar *Paratyphi A* does not produce active CDP tyvelose epimerase due to the 1-bp deletion, which causes the frameshift mutation and converts codon 4 of Tyv to a stop codon.[7]

The PCR was carried out with a 50 μl mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl**,** 1.5 mM MgCl2, 1 U of Taq DNA polymerase (Promega, Madison, Wis.), 0.2 mM deoxynucleoside triphosphate, a 0.1 μM concentration (each) of primers (tyvF, tyvR, and 0.2 μM concentration (each) of primers prt F, R and 5 μl of the DNA sample. The PCR was carried out under the following conditions: 30 cycles with heat denaturation at 95°C for 30 seconds, primer annealing at 55°C for 60 s, and DNA extension at 72°C for 90 seconds by a DNA thermal cycler (Eppendorf gradient master cycler). The amplified DNA was separated by 1% agarose gel electrophoresis, stained with ethidium bromide, and visualized by UV transillumination.

Specificity of the PCR. Bacterial strains were used to assess the specificity of the PCR. The boiling method was used to prepare the DNA template. A single bacterial colony was picked from the Luria–Bertani (LB) agar plate, boiled in 50 μl distilled water for 10 min and immediately cooled on ice for 5 minutes. After a short spin, 4 μl of this solution was used in PCR.

Sensitivity of PCR. To determine the detection sensitivity of PCR, we used Serial dilution of a genomic DNA extracted from *Salmonella serovars Typhi*, briefly. Genomic DNA extracted described above was measured spectrophotometer in 260/280. A 10-fold serial dilution was prepared. Polymerase chain reaction was performed by using 1 μl of each sample as described above.

Genomic DNA. Genomic DNA from *Salmonella Typhimurium* was prepared by a modified method of Saito and Miura.[8] Briefly, 5 ml of an overnight culture grown in luna bertina broth was harvested by centrifugation. The pellet was resuspended in lysozyme solution (1 mg lysozyme ml^{-1} in 0.15 M NaCl, 0.1 M ethylenediaminetetraacetic acid, pH 8.0), followed by lysis using 1% sodium dodecyl sulfate, 0.1 M NaCl, 0.1 M Tris/HCl (pH 8.0) at 60°C. Deoxyribonucleic acid was purified by extraction with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) in the presence of 5 M sodium perchlorate. A 1/10 volume

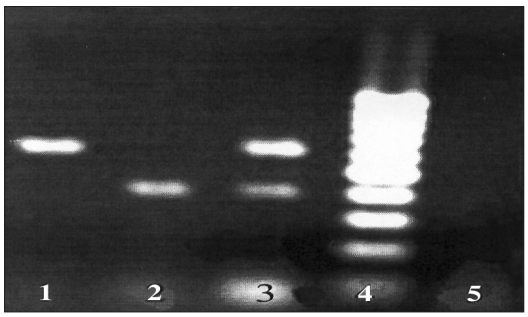

Figure 1 - The results of Uniplex and Multiplex PCR with the short time(32 min). Lane 1: The results of PCR by S1-S2 primers. Lane 2: The results of PCR by S12-S13 primers. Lane 3: The results of Multiplex PCR by S1-S2, S12-S13 primers. Lane 4: Molecular marker (100, 200, 300, 400, 500, 600, 700, 800, 900, 1030 bp). Lane 5: Negative control.

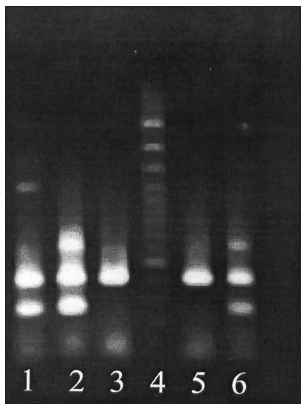

Figure 2 - The multiple PCR results of the clinical templates. Lane 1: The *Salmonella Paratyphi A* (258, 373 bp). Lane 2: The Salmonella *Typhi* (258, 373,615 bp). Lane 3: The *Salmonella* infantis (373 bp). Lane 4: Molecular marker (100, 200, 300, 400, 500, 600, 700, 800, 900, 1030 bp). Lane 5:The *Salmonella havana* (373 bp). Lane 6: The standard *Salmonella Typhi* (258, 373,615 bp).

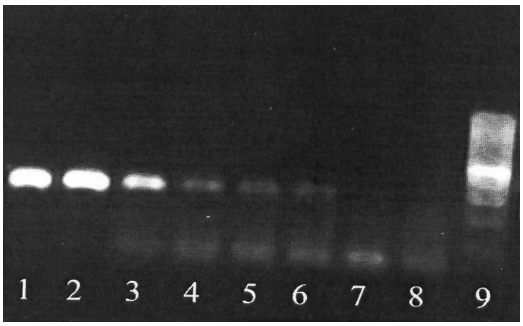

Figure 3 - The results of PCR by different dilutions of genome and S3-S4 primers (615 bp) Lane 1: The template of purify of genome. Lane 2: The 10---1 dilation of genome. Lane 3: The 10---2 dilation of genome. Lane 4: The 10---3 dilation of genome. Lane 5: The 10---4 dilation of genome. Lane 6: The 10---5 dilation of genome. Lane 7: The 1---6 dilation of genome. Lane 8: Negative control. Lane 9: Molecular marker (100, 200, 300, 400, 500, 600, 700, 800, 900, 1030 bp).

of 3 M sodium acetate and 2 volumes absolute ethanol were added and the nucleic acid was then pelleted by centrifugation, washed with 70% ethanol and dried under vacuum. The DNA pellet was resuspended in TE buffer (10 mM Tris/HCl, pH 7.5, 0.1 mM EDTA) and then serially diluted with deionized water to concentrations ranging from 100 ng to 1 fg and subjected to PCR amplification.

Bacterial cell dilutions. An overnight culture of *Salmonella Typhi* was serially diluted 10-fold with LB broth. A 100 μl aliquot of each dilution was boiled for 10 min, snap-cooled and then centrifuged for 1 min at 13000 rpm. A 4 μl aliquot of the supernatant was used as template in the PCR. Viable counts were obtained by plating 100 μl of each dilution of bacterial culture on LB plates and incubating overnight at 37°C.

Rapid PCR. The rapid PCR was carried out under the following conditions: 20 cycles with heat denaturation at 94°C for 30 seconds, primer annealing at 55-57^{0}C for 5 seconds and DNA extension at 72^{0}C for 10 seconds and final extension for 30 seconds (**Figure 1**).

Rapid electrophoresis. We used 10 mM sodium boric acid (Na2B4O7 10 H_2O) as electrophoresis buffer. For this purpose, 3.58 gram of boric acid was dissolved in one liter of double distilled water. Polymerase chain reaction fragments were separated in Mini sub marine apparatus on 200-300 volts for 5-15 minutes. For rapid staining and analysis Ethidium Bromide in concentration of 0.5 ug/ml were added to the buffer.

Results. The multiplex PCR using 6 sets of primer pairs, which were targeted for the invA, prt, tyv, genes, correctly identified *Salmonella serovars Typhi* and *Paratyphi A* and differentiated the 2 serovars by the combinations of the different-size bands produced: 4 positive bands, which consist of InvA, prt, tyv PCR products, in serovar Typhi and 2 positive bands, which consist of prt and PCR products, in serovar Paratyphi A (**Figure 2**). As expected, the prt primers in this study reacted with both serovars *Typhi* and *Paratyphi A*, yielding PCR products of the same size. The presence in both *Serovars Typhi* and *Paratyphi A* of the prt gene was consistent with the findings of a previous report.[7] The primers for tyv specifically detected the tyv gene of *Serovar Typhi*.

To examine the possible cross-reactions of the selected invA, prt and tyv, primers among several genera of the family *Enterobacteriaceae*, some strains were tested by the multiplex PCR assay; none showed positive results. To evaluate the primer specificities for *Salmonella species*, we tested several kinds of *Salmonella serovars*. Detection of both prt and fliC-a

correctly identified serovar Paratyphi A. Detection of the combination of InvA, tyv, and correctly identified serovar Typhi. We plan to examine whether our system is usable for direct detection from clinical samples. Standards and clinical isolate of *Salmonella* were examined and were accurately identified by this assay (**Figure 2**). Specificity of the assay was evaluated by different gram negative and gram positive bacteria. Taken together, the methods described here could make possible detection or identification of clinically important strains of *Salmonella serovars Typhi* and *Paratyphi A* strains within a few working days of the arrival of specimens in the diagnostic microbiology laboratory. The PCR produced an intense band of the expected 784 bp with all the *Salmonella* strains; none of the *non-Salmonella* strains provide any amplification, indicating 100% specificity. Repeat PCR amplifications provide similar reproducible results. A sensitivity of 3 x 104 c.f.u. ml was observed when serial dilutions of bacterial cell culture were used as PCR template. This amount was equivalent to 120 c.f.u. per PCR (3 x 104 c.f.u. ml in 4 μl) (**Figure 3**). As expected, the sensitivity of the PCR decreased to 3 x 105 c.f.u. ml (1200 c.f.u. per PCR) in the presence of normal flora and inhibitors in the stool sample when direct stool samples were used as template in PCR. However, after 4 and 6 hour enrichment periods, the sensitivity increased to 3 x 104 c.f.u. ml (120 c.f.u. per PCR) and 3 x 102 c.f.u. ml (1.2 c.f.u. per PCR). Repeated PCR amplifications to test the sensitivity of the primers provide similar reproducible results. Polymerase chain reaction products were sequences by dideoxy method. Nucleotide sequences were submitted to Genbank, AY771364, AY771362, AY771363.

Discussion. During the process of *Salmonella* infection, invasion genes are required for bacterial entry into host cells. Many of these genes are encoded on *Salmonella* pathogenicity island 1 (SPI 1),[10] which is present in all invasive strains of *Salmonella*[11] and not present from closely related genera such as *E. coli*.[12] The expression of these invasion genes is activated by InvA, a gene also encoded in SPI 1.[13] The hilA gene is an important feature of *Salmonella* pathogenesis, as it is required for bacterial colonization of the extracellular, luminal compartment of the host intestine.[14] Hence, in the present study, the specificity and sensitivity of a pair of primers targeting the hilA gene of *Salmonella* serovars[15] were assessed for the detection of *Salmonella* species in human feces. This method is simple and rapid, and results obtained in less than 60 minutes proved to be highly specific and sensitive. Although multiple bands of nontarget size were occasionally observed in PCR products of *Salmonella* samples at lower dilutions of crude DNA template, this did not obscure the distinct and clear band of the expected size. The use of a hot start-PCR has been proposed to reduce non-specific priming.[16] Although we did not experience any problems with inhibitors in our spiked specimens, the method will have to be evaluated further on more fecal samples to ensure that the pre-enrichment step does eliminate inhibitory substances, since there may be specimen variation in levels and types of inhibitors. Our results confirmed that hilA gene-targeting primers are specific for *Salmonella* and that the PCR assay presented here is a promising technique for diagnosing infections with *Salmonellae* using clinical specimens as well as for detecting carriers of *Salmonella* species. In this study, we used the primers for invA, tyv, prt genes only. However, if specific primers are designed for the amplification of other flagellin genes or other O-antigen synthase genes, it might be possible to identify other human-pathogenic *Salmonella serovars* by the combination of O- and H- antigen-specific gene- targeted PCR primers with multiplex PCR. Multiplex PCR is a variant of PCR which enabling simultaneous amplification of many targets of interest in one reaction by using more than one pair of primers. Since its first description in 1988 by Chamberlain et al, this method has been applied in many areas of DNA testing, including analyses of deletions, mutations, and polymorphisms, or quantitative assays and reverse transcription PCR. Typically, it is used for genotyping applications where simultaneous analysis of multiple markers is required, detection of pathogens or genetically modified organisms (GMOs), or for microsatellite analyses. Multiplex assays can be tedious and time-consuming to establish, requiring lengthy optimal procedures.

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