Original article

Detection of *Salmonella* strain by rapid-cycle multiplex PCR

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Abstract

Introduction and objective: Salmonellosis is responsible for large numbers of infections in both humans and animals. Conventional methods of isolation of *Salmonella* strains take 4-7 days to complete and are therefore laborious and require substantial manpower. Our main objective was to develop a rapid detection method using shortened PCR cycles in a conventional thermal cyclers and fast electrophoresis for *Salmonella*.

Materials and methods: The PCR primers for *tyv* (rfbE), *prt* (rfbS) and invA, genes were used for the rapid identification of *S. enterica* serovars *typhi* and *paratyphi* A, with rapid and short cycles of multiplex PCR. By using very fast and simple DNA extraction method in 10mins, rapid PCR cycles with total times of 35mins and rapid electrophoresis procedure with simple and very cheap buffer in 15mins we were able to separate the PCR products.

Results: All references and clinical isolates of *Salmonella* serovars *typhi* and *paratyphi* were accurately identified. Specificity analysis revealed no cross reaction with other enterobacterial strains. The sensitivity of the assay was 1-10 cells. The total time of multiplex PCR from sample preparation to final result is 45 to 50mins.

Conclusion: These data indicate that the specificity and sensitivity of optimized rapid cycle multiplex PCR is a potentially valuable tool for rapid diagnosis of *S. typhi* using a conventional thermal cycler. This method cut the time of a PCR reaction from 3.5h to less than 60mins. These findings could also be applied to other PCR programs detecting various genes allowing researchers to significantly shorten their PCR reaction times.

Keywords: Rapid cycle; Multiplex PCR; *Salmonella typhi*; Conventional thermal cyclers; Rapid electrophoresis

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Introduction

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* serotype *typhi* is a member of the family *Enterobacteriaciae*. 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* CT18, *S. enterica* serotype *typhimurium* LT2, and *Escherichia coli* are essentially collinear, despite the fact that *E. coli* and *S. enterica* diverged about 100 million years ago [3]. In the present study we describe interserovar typing methods for human salmonellosis-associated *Salmonella* and their applicability in detection and in tracing the origin of an outbreak. Gene clusters unique to particular bacteria are likely to represent adaptations to particular environments or may contribute to pathogenicity.

During the progressive development of the PCR over the last two decades, this method became one of the central techniques for nucleic acid analyses and is the most widely used method for pathogen detection. Most of the researchers who have already reported using PCR and real time PCR for detection of serovar *typhi* have employed only one gene of serovar *typhi* 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 studies strains of *Salmonella* serovars other than *typhi* were detected in some cases [4].

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, and the 16S rRNA gene [5].

In this study, we have studied conventional PCR for rapid detection of both typhoid fever and paratyphoid fever based on Vi antigen gene (viaB), H and O antigen synthesis genes (tyv and prt). Applying rapid multiplex PCR technique, we have developed very fast PCR by using conventional thermocyclers and rapid electrophoresis that can be applied in any diagnostic laboratory for detection and identification of *S. typhi*.

Materials and methods

One *Salmonella* strain and five non-*Salmonella* strains were included in this study. The bacterial strains were collected from the clinical laboratory of Baqiyatallah University of Medical Sciences and reference laboratory of Iran, included one strain of *Klebsiella pneumonia, E. coli*, *S. typhi*, *S. infants*, *S. havana*, two strains of *S. typhimurium*, *S. paratyphi* A, *S. paratyphi* B and *S. paratyphi* C. All isolates were identified by biochemical and serological tests. A suspension of bacteria was heated at 100°C for 10mins. The samples were then used for the PCR [6].

PCR primers, DNA amplification and detection

We used the primers tyvF and tyvR for detection of the tyvelose epimerize 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 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 259bp 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 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 frame shift mutation and converts codon four of Tyv to a stop codon [7].

The PCR was carried out with a 50µl mixture containing 10mM Tris-HCl (pH 8.3), 50mM KCl, 1.5mM $MgCl_2$, 1U of Taq DNA polymerase (Sina Gene, Tehran, Iran), 0.2mM dNTP, a 0.1µM concentration (each) of primers (tyvF, tyvR, and 0.2µM concentration (each) of primers prt F, R and 5µl of the DNA sample. The PCR was carried out under the following conditions: 30 cycles with denaturation at 95°C for 30S, annealing at 55°C for 60S, and DNA extension at 72°C for 90S on conventional Eppendorf gradient master cycler. The amplified DNA was separated by 1% agarose gel electrophoresis, stained with ethidium bromide, and visualized by UV Trans illumination.

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 (Difco, Germany) plate, boiled in 50µl distilled water for 10mins and immediately cooled on ice for 5mins. After a short spin, 4µl of this solution was used in PCR.

Sensitivity of PCR

In order to determine the detection sensitivity of PCR, we used dilutions of a genomic DNA extracted from *Salmonella* serovars *typhi*. Briefly, genomic DNA extracted described above was measured with spectrophotometer in 260/280. Ten folds serial dilution was prepared. PCR was performed by using 1µl of each sample as described above.

Genomic DNA

Genomic DNA from *S. typhimurium* was prepared by a modified method of Saito & Miura [8]. Briefly, 5ml of an overnight culture grown in LB broth was harvested by centrifugation. The pellet was resuspended in lysozyme solution (1mg lysozyme ml-1 in 0.15M NaCl, 0.1M EDTA, pH 8.0), followed by lysis using 1% SDS, 0.1M NaCl, 0.1M Tris/HCl (pH 8.0) at 60°C. DNA was purified by extraction with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) in the presence of 5M sodium perchlorate.

A 1/10 volume of 3M sodium acetate and two voles absolute ethanol were added and the nucleic acid was then polluted by centrifugation, washed with 70% ethanol and dried under vacuum. The DNA pellet was resuspended in TE buffer (10mM Tris/HCl, pH 7.5, 0.1mM EDTA) and then serially diluted with deionizer water to concentrations ranging from 100ng to 1fg and subjected to PCR amplification [9,10].

Bacterial cell dilutions

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

Rapid PCR

The rapid PCR was carried out on conventional Ependorf gradient master cycler under the following conditions: 20 cycles with heat denaturation at 94°C for 30 seconds, primer annealing at 55-57°C for

5S, DNA extension at 72°C for 10S and final extension for 30S (Fig. 1) [8,11] .

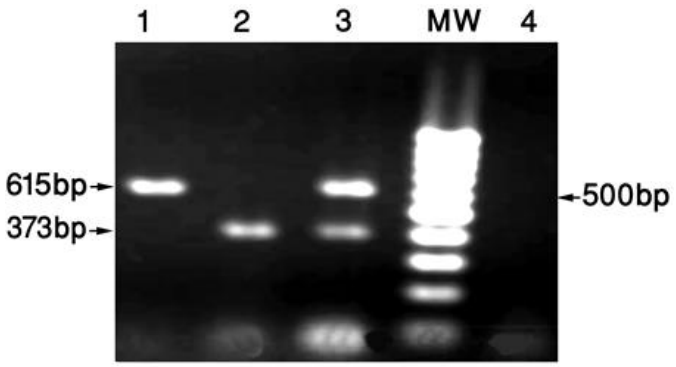

Fig. 1: The results of Uniplex and Multiplex PCR with the short time (32mins)
lane 1: The results of PCR by S1-S2 primers. lane 2: The results of PCR by S12-S13 primers. lane 3: The results of Multiplex PCR by S1-S2, S12-S13 primers. MW: (100, 200, 300, 400, 500, 600, 700, 800, 900, 1030 bp). lane 4: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

Rapid electrophoresis

We used 10mM sodium boric acid ($Na_2B_4O_710H_2O$) as electrophoresis buffer. For this purpose 3.58g of boric acid was dissolved in one liter of DDW. PCR fragments were separated in Mini sub marine apparatus on 200 to 300 volts for 5-15mins. For rapid staining and analysis ethidium bromide in concentration of 0.5µg/ml were added to the buffer [12].

Result

The multiplex PCR using six sets of primer pairs, for the invA, prt, tyv, genes, correctly identified *Salmonella* serovars *typhi* and *paratyphi* A and differentiated the two serovars by the combinations of the different-size bands produced: four positive bands, which consist of InvA, prt, tyv PCR products, in serovar *typhi* and two positive bands, which consist of prt and invA PCR products, in serovar *paratyphi* A (Fig. 1). 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 possible cross-reactions of the invA, prt and tyv, primers among several *Enterobacteriaceae*, some strains were tested by the multiplex PCR assay; none showed positive results. To further evaluate the primer specificities for *Salmonella* species, we tested several *Salmonella* serovars. Both prt and fliC-a were correctly identified serovar *paratyphi* A. In addition, the combination of InvA and tyv, were also correctly identified serovar *typhi*. We plan to examine whether our system is usable for direct detection from clinical samples. Standards and clinical isolates of *Salmonella* were examined and were accurately identified by this assay (Fig. 2).

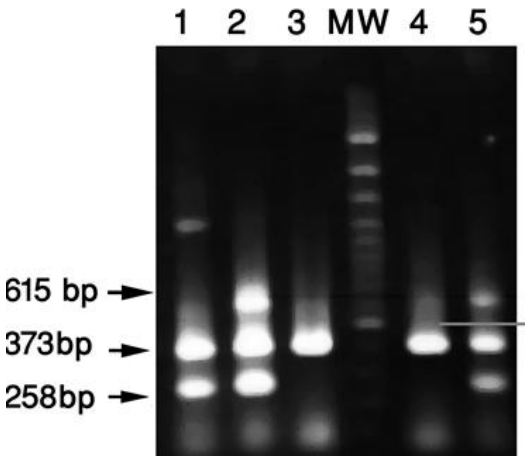

Fig. 2: The Multiple PCR results of the clinical templates
lane 1: The *S. paratyphi* A(258, 373bp). lane2: The *S. typhi* (258, 373,615bp). lane 3:The *S. infantis* (373bp). MW: (100, 200, 300, 400, 500, 600, 700, 800, 900, 1030 bp). Lane 4:The *S. havana*(373bp). Lane 5: The standard *S. typhi* (258, 373, 615bp).

Specificity of the assay was evaluated by different Gram-negative and Gram-positive bacteria. Taken together, the methods described here may make possible the detection and/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 a strong band of the expected 784bp size with all the *Salmonella* strains; none of the non-*Salmonella* strains gave any PCR products, indicating 100% specificity. Repeated PCR amplifications gave similar reproducible results. A sensitivity of 3×10^4CFU ml was observed when serial dilutions of bacterial cell culture were used as PCR template. This amount was equivalent to 120CFU per PCR (3×10^4CFU ml in 4µl).

As expected, the sensitivity of the PCR decreased to 3×10^5CFU ml (1200CFU 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 6h enrichment periods, the sensitivity increased to 3×10^4 CFU ml (120CFU per PCR) and$\times10^2$CFU ml (1.2CFU per PCR). Repeated PCR amplifications to test the sensitivity of the primers gave similar reproducible results. PCR products were sequenced by dideoxy method (Fig. 3).

Discussion

Rapid detection and identification of pathogens is crucial for effective disease control. Polymerase chain reaction amplification provides a rapid means to monitor specific microorganisms in a variety of samples. Several amplification methods like Uniplex PCR, Nested PCR, and more rapid method like Rapid and real time PCR have been used for detection of bacterial pathogens like *Salmonella* species [13]. Many studies have used fluorogentice PCR for the rapid detection of *Salmonella* spp. from culture and other samples [9].

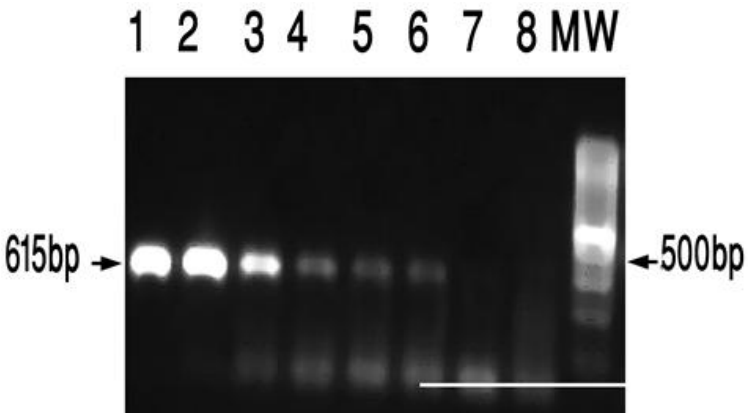

Fig. 3: The results of PCR by different dilutions of genome and S3-S4 primers (615 bp)

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. MW: (100, 200, 300, 400, 500, 600, 700, 800, 900, 1030 bp).

Currently, the detection of *Salmonella* is primarily accomplished through traditional culturing techniques that take up 2-6 days to complete and it is too time-consuming in cases where rapid pathogen identification is critical. In one study, the real-time PCR method using the 16S rRNA primers was performed successfully after a selective enrichment in broth culture [14].

The developed method dramatically reduces the time required to detect *Salmonella* in samples, in comparison with the standard culture method. This method also provides more information if extended to include multiplex detection with more than one primer set. The rapid availability of such detailed information is likely to have significant value in epidemiological and outbreak investigations. The multiplex PCR method could be of benefit when information on the presence of *Salmonella* in food samples is required rapidly such as in an outbreak investigation [15].

Other studies have reported the successful detection of *Salmonella* in artificially inoculated and naturally contaminated food products using real-time PCR. Our data indicate that the Multiplex PCR test developed in this study using three gene primers was as sensitive as a standard culture method in detecting *Salmonella*. In conclusion, this study developed and successfully applied a conventional thermocyclers for rapid multiplex PCR detection of *Salmonella* within a much shorter time than even other PCR methods comparable to real time [15]. The specificity and sensitivity were comparable to the currently used standard culture method. The developed fast PCR method could be used in other areas too.

This study compares a developed PCR method for *Salmonella* with the ISO culture method and shows it to have good promise. Further research would be necessary to fully validate the developed method against the gold standard culture for routine testing [16]. Thus, the whole procedure DNA preparation (10mins), detection of *Salmonella* genes (35mins and electrophoresis (20mins) for detection could be completed in less than one hour.

Conclusion

We believe that this is the fastest *S. typhi* detection method reported so far. Hence, in the present study, the specificity and sensitivity of a pair of primers targeting the hilA gene of *Salmonella* serovar 15 were assessed for the detection of *Salmonella*

species in human faces. This method is simple and rapid, and results obtained in less than 60mins proved to be highly specific and sensitive. Although multiple bands of no target 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 [17].

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