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Original Articles

Rapid Detection of Different Serovares of *Salmonella entrica* by Multiplex PCR

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Abstract

Background: Typhoid fever is still one of the serious public health problems in many geographic areas and is endemic in most countries. Aim of current study was to evaluate a shortened time –Multiplex PCR for rapid detection of different *Salmonella enterica* serovars.

Methods: The PCR primers for three target genes *tyv*, *prt* and *invA* were subjected for amplification by PCR. By using simple DNA extraction method, rapid PCR cycles and rapid electrophoresis procedure with simple and very cheap buffer were utilized in 200 to 300 volts for 15 minutes to separate the PCR products.

Results: The results showed that all reference and clinical isolates of *S. enterica* were accurately identified by this assay with no cross reaction with other enterobacterial strains tested. Detection limit of the reaction was to be fewer than 10^{-1} colony forming unit.

Conclusion: These data indicate that the optimized rapid cycle multiplex PCR is a potentially valuable tool for rapid diagnosis of *S. enterica* using a conventional thermal cycler. This method reduced the reaction time of PCR from 3.5 h to less than 1 h.

Key words: *PCR, Salmonella enterica, Typhoid fever*

Introduction

Typhoid fever is still a serious public health problem in many parts of the world particularly the developing countries (1). The incidence of typhoid fever has been estimated to be between 17 and 33 million cases annually with 600,000 associated deaths (2).

Salmonella strains are not detectable in certain clinical samples that contain small numbers of organisms (3). Methods that allow the detection of low levels of *S. enterica* in food samples start with pre enrichment steps to increase both the total numbers of bacteria and the ratio of *S. enterica* to competing organisms (4). The organisms are then isolated on selective agar plates and are subsequently characterized by serological and biochemical tests. With these conventional techniques, the identification of *S. enterica* takes between 72 and 96 h. Alternate methods, such as immunoassays, have been developed to reduce the time needed for detection. These techniques depend on bacterial numbers up to 10^5 to 10^7 cells/ml to guarantee reliable detection of *S. enterica* (5). Molecular biology-based techniques including PCR assays have been reported for the rapid, specific, and sensitive detection of microorganisms in different clinical samples (6).

In this study, we have evaluated the conventional PCR to detect the causative agents of typhoid and paratyphoid fever based on *tyv* (rfbE), *prt* (rfbS) and *invA*, target genes.

Materials and Methods

Bacterial strains The bacterial strains including different *S. enterica* serovars (Typhi, *Infantis, Havana, Typhimurium, Paratyphi A, Paratyphi B* and *Paratyphi C*), non-*Salmonella* strains including *Klebsiella pneumonia*, and *Es-*

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cherichia coli were obtained from reference laboratory of Iran. All isolates were identified and confirmed by biochemical and serological tests.

DNA extraction DNA extraction was carried out by a modified method of Saito & Miura (7). About 5 ml of an overnight culture in LB broth was harvested by centrifugation at 6000 rpm for 5min. The pellet was resuspended in lysozyme solution (1mg lysozyme ml^{-1} in 0.15M NaCl, 0.1M EDTA, pH 8.0). Bacterial cells were lysed using lysis buffer (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 2 volumes of 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 (10 mM Tris/HCl, pH 7.5, 0.1 mM EDTA) and then serially diluted with deionizer water to concentrations ranging from 100 ng to 1 fg and subjected to PCR amplification (8).

Alternatively, the boiling method was used for direct extraction of bacterial DNA in the strains tested. Boiling method was performed according to the previous study (9). A single bacterial colony was picked up from the Luria-Bertani (LB) agar plate, then was boiled in 50µl of distilled water for 10 min and immediately was cooled on ice for 5 min. After a short spin, 4µl of this solution was used for PCR assay.

Primers and conventional PCR conditions The *Tyv* primers were used for detection of the tyvelose epimerize target 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, prt (rfbS) primer 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*) (10) and invF (gta ttg ttg att aat gag atc cg) and invR (ata tta cgc acg gaa aca cgt t) primers with product size of 373bp.

The *tyv* gene is present in both serovars *Typhi* and *Paratyphi A*, but cannot produces active CDP tyvelose epimerase in serovar *Paratyphi A* due to the 1-bp deletion, which causes the frame shift mutation and converts codon 4 of Tyv to a stop codon (11).

The PCR was carried out with a 50 µl mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5mM MgCl2, 1U of Taq DNA polymerase (Promega, Madison, Wis.), 0.2mM deoxynucleoside triphosphate, a 0.1 µM concentration of tyvF and tyvR_primers , a 0.2 µM concentration of prtF and prtR primers and 5 µl of the DNA sample.

The conventional PCR was performed under the following conditions: 30 cycles with heat denaturation at 95 °C for 30s, primer annealing at 55 °C for 60s, and DNA extension at 72 °C for 90s in conventional Ependorf gradient master cycler (Roche, Mannheim, Germany). The amplified DNA was separated by 1% agarose gel electrophoresis, stained with ethidium bromide, and visualized by UV transilluminator.

To determine the detection limit of the PCR, we used serial dilution of a genomic DNA extracted from *S. enterica* serovar *Typhi*. Extracted genomic DNA was measured by spectrophotometer in 260/280 nm wavelengths. Ten folds serial dilution was prepared. PCR was performed by using 1ul of each diluted sample.

Bacterial cell dilutions An overnight culture of *S. enterica* serovar *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 4µl aliquot of the supernatant was used as template in the PCR reaction. Viable counts were obtained by plating 100 µl of each dilution of bacterial culture on the LB plates and incubating at 37 °C overnight.

Rapid PCR and electrophoresis The rapid PCR was carried out using a conventional Ependorf gradient master cycler under the fol-

lowing conditions: 20 cycles with heat denaturation at 94°C for 30s; primer annealing at 55-57°C for 5s; DNA extension at 72°C for 10s; and final extension for 30s.

Ten mM sodium boric acid (Na2B4O7 10H2O) was used as electrophoresis buffer. PCR fragments were separated in Mini sub marine apparatus on 200 to 300 volts for 5- 15 mints. For rapid staining and analysis, Ethidium Bromide (0.5 ug/ml) were added to the electrophoresis buffer (12).

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 two serovars by the combinations of the different-size bands products: three positive bands, which consist of InvA, prt, tyv PCR products, in serovar Typhi and two positive bands for 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 primers for tyv specifically detected the *tyv* gene of serovar *Typhi*.

To examine the possible cross-reactions of the selected primers among the several genera of the family *Enterobacteriaceae*, some strains were tested by the multiplex PCR assay; none showed positive results. For further evaluation of the primer specificities for *Salmonella* species, several *Salmonella* serovars were tested. Detection based on the combination of *InvA*, *tyv*, correctly identified serovar *Typhi*. We also evaluated the method for direct detection of organism in the clinical samples. Standards and clinical isolates of *Salmonella* were examined and were accurately identified by this assay. (Fig.1) Specificity of the assay was evaluated by different species of gram negative and positive bacteria.

The PCR produced an intense band of the expected 373 bp with all the *Salmonella* strains; none of the non-Salmonella strains showed the positive reaction, indicating 100% specificity for PCR. Repeated PCR amplifications showed similar positive results. Detection limit of the reaction was to be fewer than 10^{-1} colony forming unit when serial dilutions of bacterial cell culture were used as PCR template.

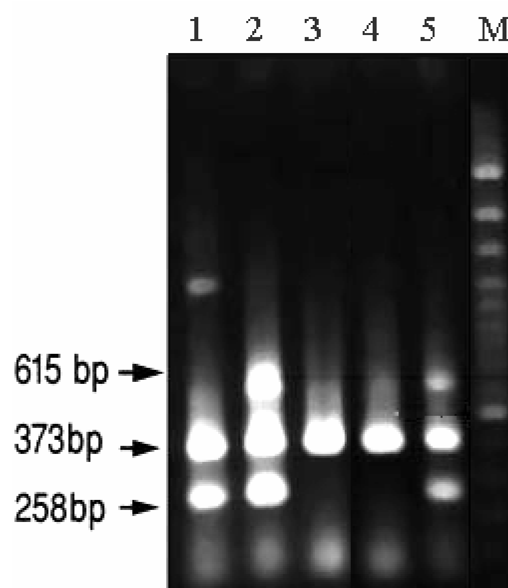

Fig. 1: The Multiple PCR results of the clinical templates. lane1, *S. enterica* serovar *paratyphi* A (258, 373 bp). lane2, *S. enterica* serovar *Typhi* (258, 373, 615bp). lane 3, *S. enterica* serovar *Infantis* (373bp). Lane 4, *S. enterica* serovar *Havana* (373bp). Lane 5: The standard *S. enterica* serovar *Typhi* (258, 373,615bp). MW: (100, 200, 300, 400, 500, 600, 700, 800, 900, 1030 bp).

Discussion

Salmonellosis is responsible for large numbers of infections in both humans and animals (13). Polymerase chain reaction (PCR) provides a rapid means to monitor specific microorganisms in a variety of samples. Several amplification methods such as Uniplex PCR, Nested PCR, and Real time PCR have been used for detection of bacterial pathogens such as *Salmonella* species (14).

Most of the researchers who have already applied conventional and Real time PCR for detection of *S. enterica* have used often only one gene of this organism in their studies. The

serovars other than *Typhi* have been detected in some cases due to cross reaction of single gene directed PCR (15). Others have already used fliC-d 4, the Vi capsular antigen 5, and the 16S rRNA genes for rapid detection by PCR (16).

PCR based methods dramatically reduce the time required to detect *Salmonella* in samples, in comparison with the standard culture methods. The developed methods also offer the potential to provide more information if extended to include multiplex detection with more than one primer set, allowing strain-identification capacities equal to, or better than phage-signature data. The rapid availability of such detailed information is likely to have significant value in epidemiological and outbreak investigations.

The real-time 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 (17). Other studies have reported the successful detection of *Salmonella* in artificially inoculated and naturally contaminated food products using real-time PCR procedures.

Our data indicate that the Multiplex PCR test using three gene primers was as sensitive as a standard culture method in detecting *Salmonella*.

In conclusion, we 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 (17). The specificity and sensitivity were comparable to the currently used standard culture method. We believe that this is the fastest method based on conventional PCR for detection of different *S. enterica* serovars reported so far. This method is simple and rapid, and results obtained in less than 60 min proved to be highly specific and sensitive.

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References

1. Kumar S, Balakrishna K, Batra HV (2006). Detection of *Salmonella enterica* serovar Typhi (S. Typhi) by selective amplification of invA, viaB, fliC-d and prt genes by polymerase chain reaction in multiplex format. *Lett Appl Microbiol,* 42:149-54.
2. Pang T (1998). Genetic dynamics of *Salmonella typhi* diversity in clonality. *Trends Microbiol*, 6: 339-42.
3. Fricker CR (1987). The isolation of *Salmonellas* and *Campylobacters*. *J Appl Bacteriol,* 63: 99-116.
4. Reissbrodt R (1995). Conventional and alternative methods for isolation and identification of *Salmonella*- an overview. *Biotest Bull*, 5: 143-56.
5. Janyapoon K, Korbsrisate S, Thamapa H, Thongmin S, Kanjanahareutai S, Wongpredee N, Sarasombath S (2000). Rapid Detection of *Salmonella enterica* Serovar *Choleraesuis* in Blood Cultures by a Dot Blot Enzyme-Linked Immunosorbent Assay. *Clin Diagn Lab Immunol,* 7: 977-9.
6. Baumler AJ, Heffron F, Reissbrodt R (1997). Rapid Detection of *Salmonella enterica* with Primers Specific for iro B. *J Clin Microbiol,* 35:1224-30.
7. Saito, H. & Miura, K (1963). Simple and rapid detection of *Salmonella* strains by direct PCR amplification of the *hilA* gene. *J Med Microbiol*, 52: 773-80.
8. Molecular Cloning: A Laboratory Manual (2000). Joseph Sambrook and David W Russell, Cold Spring Harbor Lab Press; 3rd edition.
9. Woo PC, Fung AM, Wong SS, Tsoi HW, Yuen KY (2001). Isolation and characterization of a *Salmonella enterica* serotype Typhi variant and its clinical and public health implications. *J Clin Microbiol,* 3: 1190-94.

10. Hirose K, Itoh K, Nakajima H, Kurazono T, Yamaguchi M, Moriya K, Ezaki T, Kawamura Y, Tamura K, Watanabe H (2002). Selective amplification of *tyv (rfbE), prt (rfbS), viaB, and fliC* genes by multiplex PCR for identification of *Salmonella enterica* serovars Typhi and Paratyphi A. *J Clin Microbiol*, 40: 633-6.
11. Deng W, Liou SR, Plunkett G , Mayhew GF, Rose DJ, Burland V, Kodoyianni V, Schwartz DC, Blattner FR (2003). Comparative Genomics of *Salmonella enterica* Serovar Typhi Strains Ty2 and CT18. *J Bacteriol*, 185:2330-37.
12. Reeves MW, Evins GM, Heiba AA, Plikaytis BD, Farmer JJ (1989). Clonal Nature of *Salmonella typhi* and its genetic relatedness to other salmonellae as shown by multilocus enzyme electrophoresis, and proposal of *Salmonella bongori* comb. Nov. *J Clin Microbiol*, 27: 313-230.
13. Keusch GT (2002). Systemic gastro-intestinal infections: a clinical overview. In: *Molecular Medical Microbiology*. Eds, M Sussman and San Diego. 2nd ed, Academic Press, pp. 1357-63.
14. John J, Farrell JD, Rachel MA, Barth R, Geraldine SH; Gary W (2005) Broad-Range (Pan) Salmonella and Salmonella Serotype Typhi Specific Real-Time PCR Assays Potential Tools for the Clinical Microbiologist Potential Tools for the Clinical Microbiologist. *Am J Clin Pathol*. 123(3): 339-45.
15. Tindall BJ, Grimont PAD, Garrity GM, Euzeby JP (2005). Nomenclature and taxonomy of the genus *Salmonella*. *Int J Syst Evol Microbiol,* 55: 521-24.
16. S. Kumar, K. Balakrishna and H.V. Batra (2006). Detection of Salmonella enterica serovar Typhi (S. Typhi) by selective amplification of invA, viaB, fliC-d and prt genes by polymerase chain reaction in mutiplex format. *Letters in Applied Microbiology*, 42 (2): 149-56.
17. Van Kessel JS, Karns JS, Perdue ML (2003). Using a portable real-time PCR assay to. Detect *Salmonella* in raw milk. *J Food Prot*, 66: 1762-67.