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ORIGINAL ARTICLE

Rapid Molecular Approach for Simultaneous Detection of *Salmonella* spp., *Shigella* spp., and *Vibrio cholera*

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Abstract
Objectives: Gastrointestinal tract infection is still one of the serious public health problems in many geographic areas and is endemic in most countries including Iran. Early detection of the gastrointestinal tract pathogens can be extremely important. The aim of the current study was to apply a shortened time-multiplex polymerase chain reaction (PCR) for rapid and simultaneous detection of *Salmonella* spp., *Shigella* spp., and *Vibrio cholera*.
Methods: The standard and clinical strains of *Salmonella* spp., *Shigella* spp., and *V. cholerae* were used in the assay. Multiplex PCR was performed and optimized based on amplification of *invA*, putative integrase, and *ompW* genes for detecting *Salmonella* spp., *Shigella* spp., and *V. cholerae*, respectively. The specificity of the assay was evaluated by testing 12 different bacterial species.
Results: Only *Salmonella* spp., *Shigella* spp., and *V. cholerae* strains had positive results when subjected to the assay using multiplex PCR. The assay showed a high sensitivity, and no amplification products were observed in multiplex PCR with any of the other microorganisms.
Conclusion: Our study indicated that the *invA*, putative integrase, and *ompW*-based multiplex PCR assay appears to be an efficient method for rapid and simultaneous detection of *Salmonella* spp., *Shigella* spp., and *V. cholerae*.

1. Introduction

Worldwide, gastrointestinal tract infections are the second most important cause of death; about 25 million enteric infections occur each year. These infections cause significant morbidity and death in children younger than 5 years in particular and in elderly people. It has been estimated that 4–6 million children die each year because

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of diarrheal diseases, particularly in the developing countries [1]. Numerous outbreaks of diarrheal illness caused by various microorganisms have been reported. Microorganisms such as *Shigella*, *Salmonella*, *Vibrio*, *Escherichia coli*, *Campylobacter jejuni*, *Giardia lamblia*, *Cryptosporidium*, and *Rotaviruses* have been reported to be the most important causes of diarrheal outbreaks. *Salmonella* spp., *Shigella* spp., and *V. cholerae* are the most important bacterial causes of diarrhea in Iran [2–5].

The diseases caused by all of these microorganisms could be serious, resulting in death. *V. cholerae* causes cholera, a disease with endemic or pandemic potential characterized by watery diarrhea and vomiting, leading to severe and rapidly progressing dehydration and shock [6]. The symptoms are caused by cholera toxin, which is produced by pathogenic strains of *V. cholerae*. Many efforts have been made to introduce a more effective vaccine, but many researches have shown that the vaccination has no role for cholera; however, new oral vaccines are displaying egregious promise [7].

Shigellosis and salmonellosis are caused by *Shigella* spp. and *Salmonella* spp., respectively. These organisms are likely to be the common cause of diarrhea worldwide. *Shigella* spp. are the causative agents of inflammatory diarrhea and dysentery, thus presenting a serious challenge to public health authorities worldwide [5]. Although shigellosis has no known animal reservoirs, we are still lacking an effective vaccine owing to poor immune responses to oral vaccines and existence of multiple serotypes [8].

Unlike *Shigella*, *Salmonella* spp. (except *Salmonella enterica* subspecies Typhi) are found in many animals. Thus, salmonellosis is well recognized as zoonosis disease [9]. The prevalence of *Salmonella* infection varies depending on the waste disposal, water supply, food preparation practices, and climate. Gastroenteritis is the most common disease among children caused by *Salmonella* [5].

The traditional methods for detection of bacterial infections are still primarily based on culture and serological methods that may take several days to be completed. There has been a general move toward molecular methods for microbial detection, which are based less on phenotypic features and more on stable genotypic characteristics. In recent years, polymerase chain reaction (PCR) and similar nucleotide-based methods have become potentially powerful alternative approaches in microbiological diagnostics because of their higher user-friendliness, rapidity, reproducibility, accuracy, and affordability. These methods have also gained momentum in terms of use for rapid, specific, and sensitive detection of foodborne pathogens [10–15].

Multiplex polymerase chain reaction is a variant of PCR in which two or more loci are simultaneously amplified in the same reaction [16]. This technique is a powerful molecular method in microbiological diagnostics that allows the simultaneous amplification of more than one target sequence in a single PCR reaction, saving considerable time and effort, and decreasing the number of reactions to be performed in order to assess the possible presence of foodborne pathogens [16–18].

In this study, we describe a multiplex PCR assay for the rapid and simultaneous detection of *Salmonella* spp., *Shigella* spp., and *Vibrio cholera*.

2. Materials and methods

2.1. Bacterial strains

The bacterial strains were obtained from the Pasteur Institute, Tehran, Iran and used in this study (Table 1). Clinical isolates of the three most important foodborne bacterial pathogens including *Salmonella* and *Shigella* were obtained from patients admitted to Children's Medical Center and Baqiyatallah Hospitals in Tehran, Iran, during 2012–2014. Subsequently, identification of

Table 1. Bacterial strains included in this study, and performance of the multiplex PCR assay for detecting *Salmonella*, *Shigella*, and *Vibrio cholera*.

Strains	Reference	Multiplex PCR results
Salmonella serovar Albany	ATCC 51960	+
Salmonella serovar Enteritidis	ATCC 4931	+
Salmonella serovar Hadar	ATCC 51956	+
Salmonella serovar Reading	ATCC 6967	+
Salmonella serovar Typhi	ATCC 19430	+
Salmonella serovar Typhimurium	ATCC 14028	+
Citrobacter freundii	ATCC 8090	–
Escherichia coli	ATCC 25922	–
Shigella flexneri	PTCC 1234	+
Shigella soneii	ATCC 9290	+
Staphylococcus aureus	PTCC 1189	–
Vibrio cholerae	PTCC 1611	+

ATCC = American Type Culture Collection (USA); bp = base pair; PCR = polymerase chain reaction; PTCC = Persian Type Culture Collection (Iran).

the references and clinical strains was confirmed by culture, biochemical testing by the API test system (BioMérieux, Marcy-l'Étoile, France), and slide agglutination with serovar specific antisera (Staten Serum Institute, Copenhagen, Denmark). *V. cholerae* isolates were provided by the Molecular Biology Research Center affiliated to Baqiyatallah Hospital.

All bacterial strains were grown either on Brain Heart Infusion (BHI; Difco Laboratories, Detroit, MI, USA) or Luria−Bertani (LB) broth (Merck, Darmstadt, Germany) at 37°C for 18−24 hours.

2.2. DNA extraction

Genomic DNAs from all microorganisms were extracted using the DNA extraction kit (DNP, DNA Extraction Kit; Cinagene Company, Tehran, Iran) according to the manufacturer's instructions. DNA concentration and purity were spectrophotometrically assessed by reading A_{260} and A_{280} and confirmed by visualization on 1% agarose gel. Then, DNA was diluted to 1 mg/mL in nuclease-free water and stored at −20°C until required for analysis.

2.3. Primers and multiplex PCR conditions

The AlleleID software version 7.01 (Premier Biosoft Int., Palo Alto, CA, USA) was used for all oligonucleotide primers designed in this study. All primers were purchased from Bioneer (Daejeon, South Korea). The *in silico* specificity was analyzed using the Basic Local Alignment Search Tool (BLAST) from the GenBank database. The characteristics of the primers used for multiplex PCR are given in Table 2.

PCR was carried out with a 50-μL mixture containing 10mM Tris−HCl (pH 8.3), 50mM.

In this study, we used KCl, 1.5mM $MgCl_2$, 1 U of Taq DNA polymerase (Promega, Madison, WI, USA), 0.2mM deoxynucleoside triphosphate, a 0.1μM concentration of each primers, and 5 μL of the DNA sample.

Multiplex PCR was performed under the following conditions: 35 cycles with heat denaturation at 95°C for 30 seconds, primer annealing at 60°C for 30 seconds, and DNA extension at 72°C for 60 seconds in Eppendorf 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.

2.4. Sensitivity and specificity

To determine the sensitivity of the multiplex PCR assay, 10-fold serial dilutions were made from extracted genomic DNA (498 ng/μL), and the detection limit of the multiplex PCR was defined as the lowest concentration of DNA that could be amplified. The specificity of multiplex PCR was evaluated using three species including *Staphylococcus aureus* PTCC (Persian Type Culture Collection) 1189, *E. coli* ATCC (American Type Culture Collection) 25922, and *Citrobacter freundii* ATCC 8090 as negative controls.

3. Results

The multiplex PCR using three sets of primer pairs targeted for the *invA*, putative integrase, and *ompW* genes, correctly identified *Salmonella* spp., *Shigella* spp., and *V. cholerae* and differentiated them by the different-size bands products: three positive bands, which consist of *invA* (403 bp), putative integrase (159 bp), and *ompW* (592 bp) PCR products (Figure 1). No amplification products were observed in multiplex PCR with any of the other microorganisms subjected to the assay (Table 1). The sensitivity of the multiplex PCR was assessed to be 5 ng/μL of the pure DNA.

4. Discussion

Salmonella spp., *Shigella* spp., and *V. cholerae* are responsible for large numbers of intestinal infections in humans worldwide. Molecular techniques, such as multiplex PCR, are proving useful in detection of pathogens in a wide spectrum of matrices [10,19]. This technique enables us to identify these three pathogens at one experiment, obviating the need for three separate experiments. The use of multiplex PCR substantially reduces the time and manpower required when compared with conventional methodologies. Here,

Table 2. Primers sequences used for amplification by multiplex PCR.

Primer name	Sequence (5′→3′)	Product (bp)	Target	Reference
Vibrio-F	ATAATGGCTCACCAAGAAGG	592	*ompW*	This study
Vibrio-R	TTAGAACTTATAACCACC			
Shigella-F	TCCGTCATGCTGGATGAACGATGT	159	*Putative integrase*	Ranjbar et al [2]
Shigella -R	ACAGTTCAGGATTGCCCGAGACACA			
Salmonella-F	GTATTGTTGATTAATGACATCCG	403	*invA*	This study
Salmonella-R	ATATTACGCTACGGAAACACGTT			

bp = base pair; PCR = polymerase chain reaction.

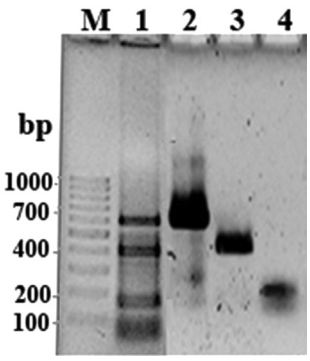

Figure 1. The multiplex PCR results. Lanes 2−4, uniplex PCR of some representative strains of *Vibrio cholera*, *Salmonella* spp., and *Shigella* spp., respectively. Lane 1, multiplex PCR for the same three bacterial strains in a single PCR tube. M = molecular weight (100 bp DNA ladder); PCR = polymerase chain reaction.

we report a multiplex PCR assay for detection of *Salmonella*, *Shigella*, and *V. cholerae* based on *invA*, *ompW*, and putative integrase genes, respectively. Previous studies indicated that these genes are conserved in each species. Many studies noted that *invA* is a specific and sensitive target for detection of *Salmonella* spp. [20,21]. Also, the *ompW* gene has been previously used for identification of *V. cholerae*, owing to its specificity [22]. Furthermore, restriction fragment length polymorphism analysis and nucleotide sequence data have shown that the *ompW* gene is highly conserved among all *V. cholerae* biotypes, suggesting the *ompW* gene can be considered a good target for the specific identification of *V. cholerae* strains [23].

Unlike the above-mentioned species, *Shigella* genomes have a high level of similarity with the *E. coli* genome; hence, the whole sequences of *Shigella dysenteriae, Shigella boydii*, *Shigella flexneri*, and *Shigella sonnei* have ∼3 Mb of genomic DNA in common with all sequenced *E. coli* genomes [24]. However, based on the comparative genomic analysis, a specific target known as putative integrase locus, conserved in all *Shigella* species, was subjected to identification of *Shigella* species. Hence, *Shigella*-specific primers were designed based on putative integrase locus. The results also showed that this locus is a suitable target for specific identification of *Shigella* species.

In many research studies, multiplex PCR has been applied for rapid identification of diarrheal agents [25,26]. All of these studies noted that multiplex PCR is a reliable, useful, and cost-effective assay, which is consistent with our results. Jin et al [27] studied foodborne pathogenic bacteria including *C. jejuni*, *Shigella*, *Salmonella*, *Vibrio parahaemolyticus*, *S. aureus*, *E. coli* O157:H7, and several other bacterial species and showed that multiplex PCR is time-saving assay in comparison with conventional PCR. Furthermore, Paniagua et al [28], who described the detection of different foodborne pathogens by multiplex PCR, noted that this method could be useful for quick detection of foodborne pathogens.

There are inconsistent reports about the sensitivity of multiplex PCR. According to Tsai et al [29], the sensitivity of multiplex PCR is considerably lower than that of monoplex PCR because of the primers' interference, so that it can be decreased several times compared with conventional PCR. However, Al-Talib et al [30] showed that multiplex PCR has a high level of sensitivity, and it might be useful as an alternative diagnostic tool for diarrheal diseases. In our study, a high level of sensitivity (5 ng/μL) was also observed. It appears that the sensitivity of multiplex PCR is related to primer length and can be enhanced by shortening the primers' length. However, this modification leads to low specificity.

The infections caused by enteric pathogens comprise second commonest medical problems after respiratory infectious disease [31,32]. Salmonella, Shigella, and Vibrio are among the most prevalent and endemic food and water- borne pathogens in Iran [33−36]. Rapid and simultaneous detection of these common bacteria in is extremely important to ensure food and water safety. For this purpose, we developed and successfully applied a multiplex PCR for the rapid identification of *Salmonella* spp., *Shigella* spp., and *V. cholera*. This technique decreases the test time of PCR. This method is simple and rapid, and the results obtained proved to be highly specific and sensitive and can be expanded to additional species. Moreover, multiplex PCR may provide an epidemiological tool to investigate the wide spread of diarrheagenic pathogens in various areas worldwide.

Conflicts of interest

The authors declare that there is no conflict of interests.

References

1. Guerrant RL, Hughes JM, Lima NL, et al. Diarrhea in developed and developing countries: magnitude, special settings, and etiologies. Rev Infect Dis 1990;12:S41−50.
2. Ranjbar R, Afshar D, Mehrabi Tavana A, et al. Development of multiplex PCR for simultaneous detection of three pathogenic *Shigella* species. Iran J Public Health 2014;43:1657−63.
3. Ranjbar R, Rahbar M, Naghoni A, et al. A cholera outbreak associated with drinking contaminated well water. Arch Iran Med 2011;14:339−40.
4. Ranjbar R, Naghoni A, Farshad S, et al. Use of TaqMan® real-time PCR for rapid detection of *Salmonella* serotypes Typhi and Typhimurium. Acta Microbiol Immunol Hung 2014;61:121−30.
5. Mulatu G, Beyene G, Zeynudin A. Prevalence of *Shigella, Salmonella* and *Campylobacter* species and their susceptibility patters among under five children with diarrhea in Hawassa town, south Ethiopia. Ethiop J Health Sci 2014;24:101−8.
6. Akoachere JFT, Mbuntcha CK. Water sources as reservoirs of *Vibrio cholerae* O1 and non-O1 strains in Bepanda, Douala (Cameroon): relationship between isolation and physico-chemical factors. BMC Infect Dis 2014;14:421.

7. Sack DA, Sack RB, Nair GB, et al. Cholera. Lancet 2004;17:223–33.
8. Kweon MN. Shigellosis: the current status of vaccine development. Curr Opin Infect Dis 2008;2:313–8.
9. Okoro CK, Kingsley RA, Connor TR, et al. Intra-continental spread of human invasive *Salmonella* Typhimurium pathovariants in sub-Saharan Africa. Nat Genet 2012;44:1215–21.
10. Karami A, Ranjbar R, Ahmadi Z, et al. Rapid detection of different serovares of *Salmonella entrica* by Multiplex PCR. Iran J Publ Health 2007;36:38–42.
11. Malorny B, Hoorfar J, Bunge C, et al. Multicenter validation of the analytical accuracy of *Salmonella* PCR: towards an international standard. Am Soc Microbiol 2003;69:290–6.
12. Popovic T, Bopp C, Olsvik O, et al. Epidemiologic application of a standardized ribotype scheme for *Vibrio cholerae* O1. J Clin Microbiol 1993;31:2474–82.
13. Vargas M, Gascon J, Jimenez De Anta MT, et al. Prevalence of *Shigella* enterotoxins 1 and 2 among *Shigella* strains isolated from patients with traveler's diarrhea. J Clin Microbiol 1999;37:3608–11.
14. Roy S, Thanasekaran K, Dutta Roy AR, et al. Distribution of *Shigella* enterotoxin genes and secreted autotransporter toxin gene among diverse species and serotypes of *Shigella* isolated from Andaman Islands, India. Trop Med Int Health 2006;11:1694–8.
15. Orrett FA. Prevalence of *Shigella* serogroups and their antimicrobial resistance patterns in southern Trinidad. J Health Popul Nutr 2008;26:456–62.
16. Akiba M, Kusumoto M, Iwata T. Rapid identification of *Salmonella enterica* serovars, Typhimurium, Choleraesuis, Infantis, Hadar, Enteritidis, Dublin and Gallinarum, by multiplex PCR. J Microbiol Methods 2011;85:9–15.
17. Kong RY, Lee SK, Law TW, et al. Rapid detection of six types of bacterial pathogens in marine waters by multiplex PCR. Water Res 2002;36:2802–12.
18. Amini K, Zahraei-Salehi T, Nikbakht G, et al. Molecular detection of *invA* and *spv* virulence genes in *Salmonella enteritidis* isolated from human and animals in Iran. Afr J Microbiol Res 2010;4:2202–10.
19. Bohaychuk VM, Gensler GE, McFall ME, et al. A real-time PCR assay for the detection of *Salmonella* in a wide variety of food and food–animal matrices. J Food Prot 2007;70:1080–7.
20. Rahn K, De Grandis SA, Clarke RC, et al. Amplification of an *invA* gene sequence of *Salmonella typhimurium* by polymerase chain reaction as a specific method of detection of *Salmonella*. Mol Cell Probes 1992;6:271–9.
21. Mainar-Jaimea RC, Andrésa S, Vicoa JP, et al. Sensitivity of the ISO 6579:2002/Amd 1:2007 standard method for detection of *Salmonella* spp. on mesenteric lymph nodes from slaughter pigs. J Clin Microbiol 2013;51:89–94.
22. Wei S, Zhao H, Xian Y, et al. Multiplex PCR assays for the detection of *Vibrio alginolyticus, Vibrio parahaemolyticus, Vibrio vulnificus*, and *Vibrio cholerae* with an internal amplification control. Diagn Microbiol Infect Dis 2014;79:115–8.
23. Nandi B, Nandy RK, Mukhopadhyay S, et al. Rapid method for species-specific identification of *Vibrio cholerae* using primers targeted to the gene of outer membrane protein *OmpW*. J Clin Microbiol 2000;38:4145–51.
24. Yang F, Yang J, Zhang X, et al. Genome dynamics and diversity of *Shigella* species, the etiologic agents of bacillary dysentery. Nucleic Acids Res 2005;33:6445–58.
25. Chen J, Tang J, Liu J, et al. Development and evaluation of a multiplex PCR for simultaneous detection of five foodborne pathogens. J Appl Microbiol 2012;112:823–30.
26. Vondrakova L, Pazlarova J, Demnerova K. Detection, identification and quantification of *Campylobacter jejuni, coli* and *lari* in food matrices all at once using multiplex qPCR. Gut Pathog 2014;6:12.
27. Jin SQ, Yin BC, Ye BC. Multiplexed bead-based mesofluidic system for detection of food-borne pathogenic bacteria. Am Soc Microbiol 2009;75:6647–54.
28. Paniagua GL, Monroy E, García-González O, et al. Two or more enteropathogens are associated with diarrhoea in Mexican children. Ann Clin Microbiol Antimicrob 2007;6:17.
29. Tsai YL, Tran B, Sangermano LR, et al. Detection of poliovirus, hepatitis A virus, and rotavirus from sewage and ocean water by triplex reverse transcriptase PCR. Appl Environ Microbiol 1994;60:2400–7.
30. Al-Talib H, Latif B, Mohd-Zain Z. Pentaplex PCR assay for detection of hemorrhagic bacteria from stool samples. J Clin Microbiol 2014;52:3244–9.
31. Ranjbar R, Soltan Dallal MM, Pourshafie MR, et al. Serogroup distribution of *Shigella* spp. in Tehran. Iranian J Publ Health 2004;33:32–5.
32. Ranjbar R, Salimkhani E, Sadeghifard N, et al. An outbreak of gastroenteritis of unknown origin in Tehran. Pak J Biol Sci 2007;10:1138–40.
33. Ranjbar R, Rahbar M, Naghoni A, et al. A cholera outbreak associated with drinking contaminated well water. Arch Iran Med 2011;14:339–40.
34. Pourshafie MR, Bakhshi B, Ranjbar R, et al. Dissemination of a single *Vibrio cholerae* clone in cholera outbreaks during 2005 in Iran. J Med Microbiol 2007;56:1615–9.
35. Ranjbar R, Giammanco GM, Farshad S, et al. Serotypes, antibiotic resistance, and class 1 integrons in *Salmonella* isolates from pediatric cases of enteritis in Tehran, Iran. Foodborne Pathog Dis 2011;8:47–53.
36. Ranjbar R, Hosseini MJ, Kaffashian AR, et al. An outbreak of shigellosis due to *Shigella flexneri* serotype 3a in a prison in Iran. Arch Iran Med 2010;13:413–6.