



Development of Multiplex PCR for Simultaneous Detection of Three Pathogenic *Shigella* Species

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Abstract

Background: *Shigella* species are among the common causes of bacterial diarrhoeal diseases. Traditional detection methods are time-consuming resulting in delay in treatment and control of *Shigella* infections thus there is a need to develop molecular methods for rapid and simultaneous detection of *Shigella* spp. In this study a rapid multiplex PCR were developed for simultaneous detection of three pathogenic *Shigella* species.

Methods: For detection of *Shigella* spp., a pair of primers was used to replicate a chromosomal sequence. Three other sets of primers were also designed to amplify the target genes of three most common species of *Shigella* in Iran including *S. sonnei*, *S. flexneri* and *S. boydii*. The multiplex PCR assay was optimized for simultaneous detection and differentiation of three pathogenic *Shigella* species. The assay specificity was investigated by testing different strains of *Shigella* and other additional strains belonging to non *Shigella* species, but responsible for foodborne diseases.

Results: The *Shigella* genus specific PCR yielded the expected DNA band of 159 bp in all tested strains belonging to four *Shigella* species. The standard and multiplex PCR assays also produced the expected fragments of 248 bp, 503 bp, and 314 bp, for *S. boydii*, *S. sonnei* and *S. flexneri*, respectively. Each species-specific primer pair did not show any cross-reactivity.

Conclusion: Both standard and multiplex PCR protocols had a good specificity. They can provide a valuable tool for the rapid and simultaneous detection and differentiation of three most prevalent *Shigella* species in Iran.

Keywords: Multiplex-PCR, *Shigella* spp., Shigellosis

Introduction

Shigella species annually cause an estimated 164.7 million cases of shigellosis worldwide, resulting in 1.1 million deaths (1). Shigellosis as an endemic disease in Iran is one of the major causes of morbidity in children with diarrhea in this country (2-4).

There are many ways for detection of *Shigella* species including conventional culture and molecular methods (5). Conventional methods are usually

problematic process and require several days to give results (6). Moreover, these methods are relying on the viable organisms to multiply in media. Taken as a whole, conventional methods have less sensitive because there is not enough number of organisms in some specimen and the numbers of organisms decrease during specimen transport (5). In many studies, molecular methods for detection of *Shigella* spp. and other intestinal pathogens have

been developed (7-11). Multiplex PCR is one of molecular methods that have been used frequently in many studies because of rapidity and its capability for simultaneous detection of several microorganisms in a single sample (12).

The genus of *Shigella* has four species that are able to cause severe disease in humans. *S. sonnei* is the major cause of shigellosis in industrialized countries. Otherwise *S. flexneri* is the most prevalent *Shigella* serotype in developing countries (13-15). *S. dysenteriae* is usually the cause of epidemics of dysentery is detected mostly in South Asia and sub-Saharan Africa and *S. boydii* has been less frequently reported worldwide compared to other *Shigella* serogroups (16). Of four species, three including *S. sonnei*, *S. flexneri* and *S. boydii* are common species in Iran (17-20).

This study aimed to develop a Multiplex-PCR assay for simultaneous detection of three most common *Shigella* species in Iran.

Materials and Methods

Bacterial species

Clinical *Shigella* isolates were recovered from patients with *Shigella* infections admitted to several hospitals including Children Medical Center, Emam Khomeini and Baqiatallah hospitals in Tehran, Iran, during 2008-2010. Bacterial positive controls were also used to check the specificity of the assay (Table 1). Subsequently, identification and confirmation of the reference and clinical strains were carried out by culture, biochemical and serological testing.

Table 1: Primers used in this study

Primers	Sequence	Locus	Species	Band size(bp)
GF GR	TCCGTCATGCTGGATGAACGATGT ACAGTTCAGGATTGCCCGAGACACA	NC_004337: 559294-559452 NC_004741: 555187-555345 NC_007384: 759977-760135 NC_007613: 642360-642518 NC_008258: 602392-602550 NC_010658: 652993-653151	<i>Shigella</i> spp.	159
BF BR	TCTGATGTCACCTCTTTGCGAGT GAATCCGGTACCCGTAAGGT	NC_007613: 1360607-1360854 NC_010658: 1782921-1783168	<i>S.boydii</i>	248
SF SR	AATGCCGTAAGGAATGCAAG CTT- GAAGGAGATTTCGCTGCT	NC_007384: 1665725-1666227	<i>S.sonnei</i>	503
FF FR	ACCGGTTATGAACCCTCCAT TGGTGCTTGTGAGCAACTC	NC_004337: 1412593-1412906 NC_004741: 1898025-1898338 NC_008258: 1883992-1884305	<i>S.flexneri</i>	314

Bacterial DNA extraction

Bacterial strains were grown on LB broth and incubated at 37°C for 24 hr. The culture of each *Shigella* isolate was centrifuged at 6000 RPM for 10 min. Genomic DNA of the *Shigella* strains was extracted using a DNA extraction Kit (Cat. No. 11 814 770 001, Roche, Germany) according to the manufacturer's instruction.

Genomic PCR targets and primers

We designed four sets of primers to amplify the target genes of *Shigella* spp. (Putative Integrase

and of three *Shigella* species including *S. sonnei* (Putative Restriction Endonuclease), *S. flexneri* (Putative Bacteriophage Protein) and *S. boydii* (Conserved Hypothetical Protein). The list of the primers and their sequences are presented in Table 2.

To avoid cross-reactivity with *Shigella* related bacteria and within each other *Shigella* species, genus and species-specific regions of the *Shigella* genome were considered to design the primers, respectively.

Table2: *Shigella* species and non- *Shigella* microorganisms included in this stud

Bacterial strains	<i>Shigella</i> spp. PCR results	<i>S. flexneri</i> specific-PCR results	<i>S. boydii</i> specific-PCR results	<i>S. sonnei</i> specific-PCR results	Reference
<i>Shigella</i> spp.					
<i>S. flexneri</i>	+	+	-	-	ATTC9290
<i>S. boydii</i>	+	-	+	-	ATTC 9207
<i>S. sonnei</i>	+	-	-	+	ATTC12022
<i>S. sonnei</i>	+	-	-	+	17clinical isolates
<i>S. dysenteriae</i>	+	-	-	-	3 clinical isolates
<i>S. flexneri</i>	+	+	-	-	6 clinical isolates
<i>S. boydii</i>	+	-	+	-	4 clinical isolates
Non- <i>Shigella</i> organisms					
<i>Salmonella enteritidis</i>	-	-	-	-	ATCC 4931
<i>Salmonella typhimurium</i>	-	-	-	-	ATCC 14028
<i>Campylobacter jejuni</i>	-	-	-	-	ATCC 33560
<i>Escherichia coli</i>	-	-	-	-	ATCC 25922
<i>Vibrio cholerae</i>	-	-	-	-	PTCC 1611
<i>Escherichia coli</i>	-	-	-	-	ATCC 35150

PCR assay

First, a standard PCR assay was performed using standard (*Shigella* and non *Shigella* strains) and 30 clinical strains (17 *S. sonnei*, 6 *S. flexneri*, 4 *S.boydii* and 3 *S. dysenteriae*). The PCR was carried out using a total volume of 25 µL containing 1× PCR buffer, 1 mM MgCl₂, 1 U Taq DNA polymerase, 200 µM dNTP, 0.5 µM of each primers and 2.5 µL of DNA template. The PCR condition consisted of 5 min at 95 °C, followed by 30 cycles of 60 s at 95 °C of denaturing temperature, 60s at 60 °C of annealing temperature, and 1 min at 72 °C of extension temperature. At the end of the 30 cycles, a final extension of 10 minutes at 72 °C was used.

Each multiplex PCR mixture in was prepared using a total volume of 25 µL containing 0.5 µM of each primer (four pairs), 2,5 µL PCR buffer 10X, 2 U Taq DNA polymerase , 1 mM MgCl₂, 200 µM dNTPs and 1 µL DNA template. The multiplex PCR was carried out through 30 cycles following a pre-heat step at 95 °C for 5min. Each cycle consisted of denaturation at 95 °C for 60 s, annealing at 60 °C for 1min, and extension at 72 °C for 1min. After the 30 cycles, samples were

maintained at 72°C for 10 min. Sterile distilled water was included in each PCR assay as a negative control. The amplified DNA was separated by 1% agarose gel electrophoresis, stained with ethidium bromide, and visualized by UV transillumination.

Results

The *Shigella* genus specific PCR produced the expected amplified DNA band in all *Shigella* species strains tested. Figure 1 shows the specific band of 159 bp obtained from standard and clinical isolates belonging to four *Shigella* species. Any positive reaction with non *Shigella* strains including *Salmonella* and *Escherichia coli* was detected. Standard PCR assays also produced the expected fragments of 248 bp, 503 bp, and 314 bp, for *S. boydii*, *S. sonnei* and *S. flexneri*, respectively. While *S. dysenteriae* was used as negative control for detection assay of three pathogenic *Shigella* species, any DNA band was not amplified using specific *Shigella* species primers (Fig. 1).

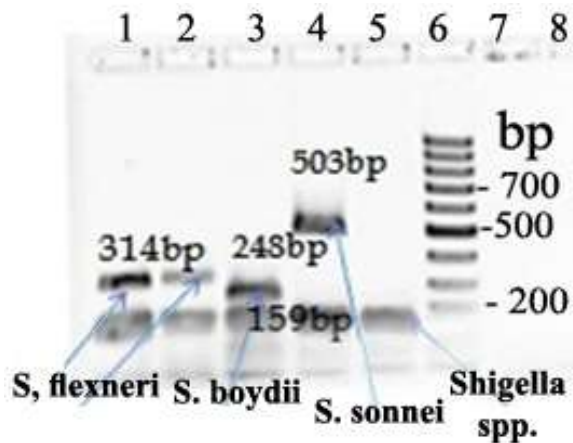


Fig. 1: Detection of specific *Shigella* species genes by PCR : lane 1, *S. flexneri* ATTC9290 (314bp); lane2, clinical isolate of *S. flexneri*(314bp); lane 3, clinical isolate of *S. boydii* (248bp); lane 4, *S. sonnei* ATTC12022 (503bp); lane 5 clinical *Shigella* spp. (*S. dysenteriae*) (159bp); lane 6 100bp ladder; lanes 7 and 8, non *Shigella* isolates including *Salmonella enteritidis* and *E. coli* respectively

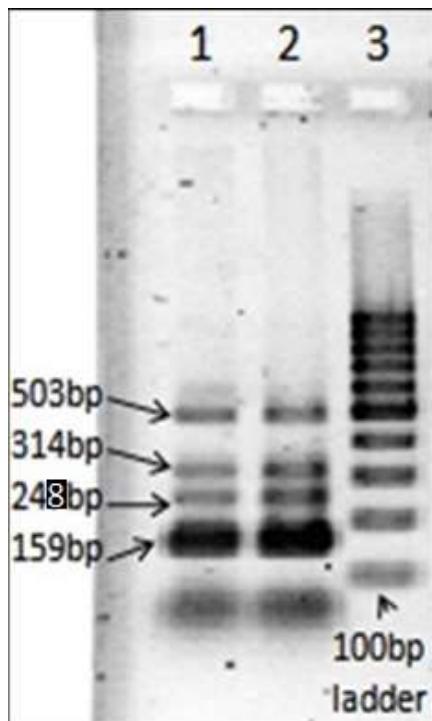


Fig. 2: Multiplex PCR : lane 1 clinical species, lane2 standard species and lane3 100bp ladder. *Shigella* spp ;159bp, *S. sonnei*; 503bp, *S. flexneri*; 314bp, and *S. boydii*; 247bp

As shown in Table 2, the standard PCR showed the same results on 30 clinical *Shigella* isolates. These isolates were recently recovered from pediatric patients in Tehran, Iran.

Multiplex PCR was successfully optimized for rapid and simultaneous detection of three pathogenic *Shigella* species.

Multiplex PCR was able produced the expected DNA bands for standard and clinical isolates of *S. boydii*, *S. sonnei* and *S. flexneri* in a single reaction. No non specific amplification products were observed with *S. dysenteriae* (as negative control) and non *Shigella* strains. Figure 2 shows the specific amplified bands obtained by multiplex PCR on the three pathogenic *Shigella* species.

Discussion

A specific PCR using Putative Integrase locus was evaluated for the rapid and specific detection of *Shigella* species. The results showed that this locus is an appropriate target for this purpose. This locus is conserved in all *Shigella* species and has not been studied previously.

A new multiplex PCR was also designed using four sets of primers to identify common *Shigella* species in our country. Accordingly, the designed method was successfully able to detect *S. boydii*, *S. flexneri* and *S. sonnei*. No nonspecific amplification was observed, confirming that this assay is specific for detection of these three most common pathogenic *Shigella* species in Iran.

Several previous studies have used standard PCR for rapid detection of bacterial pathogens such as *Shigella* species. However, multiplex PCR deserves special interest because of the possibility to rapid and simultaneous detection and identification of several target genes in a single reaction (21-26). Optimization of annealing temperature is very important in this technique. The annealing temperature of 60°C proved to be optimal for detection and differentiation of the three *Shigella* species under study. Analysis of quality assessment results of standard strains in combination with clinical samples indicated that the multiplex PCR was reliable and suitable method for the simultaneous detection of different *Shigella* strains. PCR results ob-

tained from the clinical samples were consistent with results from the standard strains.

Previously some researchers have applied the multiplex PCR for rapid detection and differentiation of prevalent *Shigella* species. The primers used in many studies have been designed for detection of plasmid genes encoding virulence factors (27). Vantarakis et al. designed a multiplex PCR using two sets of primers which targeted *invA* and *virA* genes for simultaneous detection of *Salmonella* s and *Shigella* specie, respectively (28). Aranda et al. evaluated two multiplex PCR assays for simultaneous detection of typical and atypical *E. coli* pathovars and *Shigella* species. (29). Their results showed that the multiplex PCR was a potentially valuable tool for rapid diagnosis of *Shigella* species and *E.coli* pathovars.

Thong et al. designed a multiplex PCR assay for simultaneous detection of chromosomal-and plasmid-encoded virulence genes (*set1A*, *set1B*, *ial* and *ipaH*) in *Shigella* species. Unlike our study, the limitation of their Multiplex PCR assay was its inability to differentiate *Shigella* species (30). This problem may be resulted from losing of virulence genes in some strains owing to plasmid-curing or hot spot regions for deletion (31).

The described assay showed to be specific for detection and differentiation of the three *Shigella* species tested. No false positive and negative results occurred during the assay indicating that target loci used in the study were specific for *Shigella* species. One of *Shigella* Multiplex PCR limitations is its inability to discriminate *Shigella* isolates from EIEC (32). However, this problem was resolved by using specific primers so we found any cross reaction with EIEC, bioinformatically.

Our results also showed that the multiplex PCR using four primers sets was able to detect *Shigella* species and to differentiate three species of *Shigella* simultaneously in a single reaction by the combinations of the different-size amplicons without any cross-reactivity.

Conclusion

The method presented here showed a good specificity and proved to be able to offer an important

diagnostic tool for the rapid and simultaneous detection of the three most prevalent species of *Shigella* in Iran.

Ethical Considerations

All ethical issues including plagiarism, Informed Consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc. have been completely observed by the author.

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