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Polymerase chain reaction method for the rapid detection of virulent *Shigella* spp.

Majid Alipour [1*], Maryam Talebjannat[2], Mohammad Nabiuni[3]

[1*] *Department of microbiology, Islamic Azad University (IAU) - Babol Branch, Babol, Iran*
[2] *Science and research branch, Islamic Azad University,Tehran, Iran*
[3] *Department of Biological science, Tarbiat moallem university,Tehran, Iran*

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ABSTRACT

Bacillary dysentery, or shigellosis, is a disease of humans in which the colonic epithelium is invaded by bacteria and subjected to inflammatory destruction. The aim of this study was to develop a polymerase chain reaction(PCR) test for detection of virulent *Shigella* spp.. For this purpose, the primers were designed to amplify a 526-bp internal region of the *Shigella* spp. icsA gene, which encodes IcsA (intracellular spread)/VirG protein, a 116-kDa surface exposed outer membrane protein that mediates actin polymerization to aid bacterial movement inside the cell. The use of PCR to amplify a specific icsA gene fragment serves as a highly specific and sensitive method to detect virulent bacteria of the genus *Shigella*. Specific DNA band was obtained by using isolated plasmid DNA of *Shigella* and a bacterial suspension. Amplification of extracted DNA from all other genera of the family Enterobacteriaceae and various other gram-positive bacteria yielded negative results. Therefore this PCR method, can serve as a routine protocol for detecting and identifying virulent *Shigella* spp. from clinical samples.

1. Introduction

There are four sub-groups of *Shigella* (S). These are *S. boydii, S. dysenteriae, S. flexneri,* and *S. sonnei* (Niyogi, 2005). All four sub-groups can cause Shigellosis, otherwise known as bacillary dysentery, which is characterized by the presence of blood and mucus in the stools (Robert, 2009). The infective dose of *Shigella* is as low as 10 cells (Kothary and Babu, 2001). *Shigella* can be transmitted through a variety of salads, raw vegetables, milk meat and other dairy products. Fecally contaminated water and unsanitary practices of food handlers are the most common causes of contamination (Warren et al., 2006). The *Shigella* spp. contains a large, non-conjugative ~220 kb virulence plasmid that encodes critical factors necessary for epithelial cell invasion and spread of the bacterium within the host tissue (Gaynor et al., 2008). It has been shown that the loss of the virulence plasmid results in a virulent strains (Sansonetti et al., 1982). Intra- and intercellular spreading is controlled by the icsA (virG) gene located on the virulence plasmid. The icsA gene encodes the protein IcsA, which enables actin based motility and intercellular spread. IcsA is a surface-exposed outer membrane protein consisting of three distinctive domains: a 52 amino acid N-terminal

[*]Corresponding author: Dr. Majid Alipour
Department of Microbiology, IAU-Babol Branch,
E-mail: alipourma@yahoo.com

signal sequence, a 706 amino acid α-domain, and a 344 amino acid C-terminal β-core (Kerrie and Morona, 2008). The aim of this study was to develop a technique for the identification of the genus *Shigella*. For this purpose, *Shigella* genus-specific primers based on the portion of icsA gene have been designed.

2. Materials and methods

2.1. Bacterial strains, media, and culture conditions:

A collection of bacterial isolates including 2 S. aureus, 2 E. coli, 3 S. dysentriae, 3 S. flexneri, 2 S. sonnei, 2 S. boydii, Bacillus cereus, Salmonella typhi, Proteus spp, Bacillus cereus, Pseudomonas aeruginosa, Clostridium perferingenes and reference strains of S. flexeneri (PTCC 1234), S. dysentriae (PTCC 1188), E. coli O157: H7 (ATCC 43889), Staphylococcus aureus (ATCC 25923), Salmonella typhi (PTCC 1609), Klebsiella pneumonia (ATCC 13883) were used in this study. All Shigella spp. isolates were collected from the Hospitals in Mazandaran province and confirmed in our laboratory. After overnight incubation at 37°C, the salmonella shigella and MacConkey agar plates were checked for non-lactose-fermenting colonies. Suspected Shigella colonies were inoculated into Kligler iron agar, mannitol, citrate, urea, and lysine biochemical test media. After incubation for 18 to 24 hours at 37°C, the test media were read for characteristic Shigella reactions. Slide agglutination with commercially available Shigella antisera was performed for suspicious colonies (Islam et al., 1993). To determine the sensitivity of the PCR protocol used in this study, PCR amplifications were performed using the known number of bacterial CFU as the DNA template in each PCR reaction. The DNA template derived from serially diluted Shigella spp. suspensions. All of the strains were routinely cultured at their optimum temperatures on brain heart infusion (BHI; Difco) and Luria–Bertani agar (LB; Difco). The strains were stored in BHI and LB with 20% glycerol at -70°C.

2.2. Design of specific primers for Shigella spp. Detection

A segment of the icsA gene sequence was used as the PCR target for the specific detection of *Shigella* spp. To design a primer pair, a sequence comparison was made using the known icsA genes of *Shigella* spp., which were retrieved from the Entrez database using the National Center for Biotechnology Information GenBank database and the BLAST search program. The forward primer icsA (5´- ATG CAG GCA TTC TAA AAA TGG - 3´, positions 1621–1641 in the Shigella spp. icsA gene), and the reverse primer icsA (5´-ACA GTG CCC TGT TTC AGG CG -3, positions 2154–2174 in the *Shigella* spp icsA gene) were used.

2.3. Plasmid DNA Extraction

Plasmid DNA was extracted from the *Shigella* spp. by the alkaline lysis method of Birnboim and Doly (Birnboim and Doly, 1979). The extracted plasmid DNA was separated by horizontal electrophoresis in a 1% agarose slab gel in TBE buffer at room temperature and 60 V for 4 hours. After electrophoresis, the gel was stained with ethidium bromide and video images were obtained by a gel documentation system.

2.4. DNA Template Preparation

DNA templates were prepared from overnight cultures in LB broth by boiling bacterial cultures for 15 minutes. Alternatively, 1 to 2 loopful of bacteria (from an agar plate) was resuspended into fresh LB broth followed by boiling. The boiled cell suspensions were used directly as a DNA template for PCR amplification (Houng et al., 1997). For the sensitivity assay, the bacteria were cultured at 37°C and prepared to turbidity equivalent to 0.5 McFarland Standard.

2.5. DNA amplifications (PCR)

Amplifications were made in a 50-µl reaction mixture which contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 200 mM deoxynucleoside triphosphate, 20 pmol of each primer, and 2U of *Taq* polymerase, with either 1 ng isolated plasmid DNA, or from 2 µl of standardized bacterial suspension whose turbidity was adjusted to that of a 0.5 McFarland standard, which corresponds to approximately 1.5×10^8 bacteria per ml. The PCR cycles consisted of an initial denaturation step at 94°C for 3 minutes followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 56°C for 45 seconds with an extension at 72°C for 1 minute followed by a final extension at 72°C for 5 minute.

The products of amplification were electrophoretically resolved on a 1 % agarose gel

and visualized for analysis after being stained with ethidium bromide.

3. Results

The amplified target sequence of the PCR was identified for all species of *Shigella* that were tested. By purification of plasmid DNA of this bacterium, it was determined that the number of its plasmid is 3-4 (Figure 1).

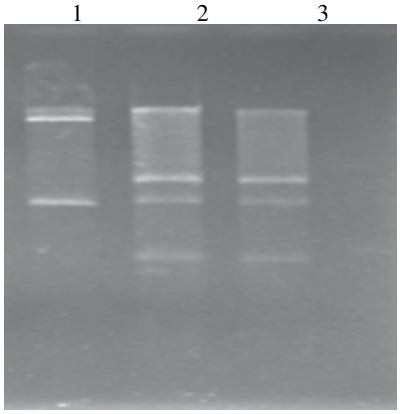

Figure 1. Plasmid profiles of *Shigella* spp in 1% agarose gel. Lane 1, *S. dysentriae* strain (PTCC 1188); lane 2, *S. flexeneri* strain (PTCC 1234); lane 3, *S. sonnei*

The predicted amplification product of 526 bp was seen as a band on 1% agarose gels. This band was not identified for any other genus of bacteria studied, including other members of the family Enterobacteriaceae (Figure 2). Sequencing of the amplified specific product showed identity for *Shigella* spp. Therefore, this pair of primers is specific for the detection of *Shigella* spp.

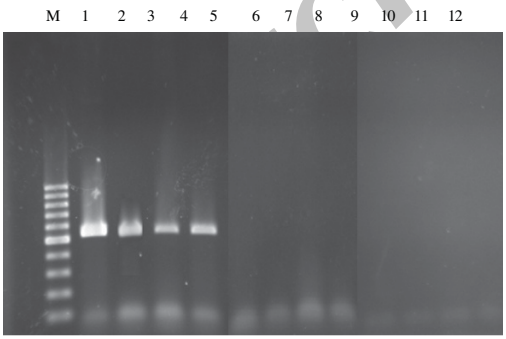

Figure 2. Agarose gel electrophoresis of PCR products amplified with *Shigella* spp. icsA gene primers. Lane 1, *S. dysentriae*; lane 2, *S. flexeneri* strain (PTCC 1234); lane 3, *S. sonnei*; lane 4, *S boydii*; lane 5, *E. coli* (ATCC 43889) O157: H7; lane 6, *Salmonella typhi* strain (PTCC 1609); lane 7, *Klebsiella pneumonia* (ATCC 13883); lane 8, *Proteus spp*; lane 9, *Staphylococcus aureus* (ATCC 25923); lane 10, *Bacillus cereus*; lane 11, *Pseudomonas aeruginosa*; lane 12,*Clostridium perferingenes*; and lane M is DNA molecular weight ladder

3.1. Sensitivity of Detecting Shigella spp by PCR Amplification

The lowest *Shigella* spp. cell concentration that could yield a positive PCR product was ~1.5 x 10^3 CFU/ml. Thus, the minimum *shigellae* needed to yield a positive PCR product is approximately 300 per PCR reaction (1.5 x 10^3 CFU/ml × 2µl = 300 CFU) (Figure 3).

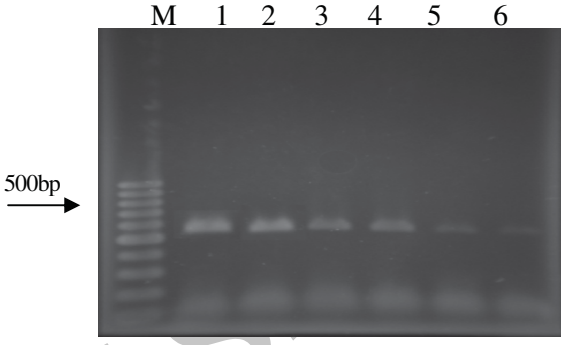

Figure. 3 Agarose gel electrophoresis of *Shigella* PCR products obtained by the analysis of aqueous solutions of different concentrations of *S. flexeneri* strain (PTCC 1234). Lanes 1–6: single PCR of 9600, 4800, 2400, 1200, 600, and 300 cfu per reaction.

4. Discussion

All pathogenic *Shigella* spp. carry the 220-kb virulence plasmid, and the major virulence genes required for bacterial invasion and the protein export system are located in a 30-kb pathogenicity island (Gunnar and Hilbi, 2008). The capacity for Shigella to spread intracellularly and infect adjacent epithelial cells is critical in the infection process. Intra- and intercellular spreading is controlled by the icsA (virG) gene located on the virulence plasmid. The icsA gene encodes the protein IcsA, which enables actin-based motility and intercellular spread (Warren et al., 2006). The icsA gene is distributed at one pole of the outer surface of the membrane. This asymmetrical distribution allows the polar formation of actin tails, and thus directional movement of Shigella within host cell cytoplasm (Steinhauer et al., 1999). The previous studies have indicated that all *Shigella* spp. harbor 1 to 11 plasmids, ranging in size from 1.35 to 230 kb (Hoe et al., 2005). The results of current investigation are consistent with previous reports. To find oligonucleotide sequences specific for *Shigella* spp., a multiple alignment in NCBI has been performed. Conserved sites were identified and a primer pair

was designed. The icsA gene, which encodes the IcsA protein, is highly conserved among four *Shigella* species. The standard procedure for *Shigella* spp. detection is based on isolation of *Shigella* by selective culture media followed by identification by biochemical tests and agglutination assays. This process may take 48 to 72 hours or even longer to obtain results (June et al., 1993). The PCR technique can detect a small number of culturable as well as non-culturable organisms. Such detection is specifically important for *Shigellae,* since they can produce disease by as few as 10-100 organisms (Levine et al., 1973). The time requirement of this technique is lower compared to that of the culture techniques.

The PCR technique can be chosen as an alternative to the culture technique, and can further be used for identifying asymptomatic carriers, serving as potential reservoirs of *Shigellae* silently transmitting the disease within communities. Conventional or nested PCR methods have been developed to detect various species of *Shigella* and the gene targets include *ipaH, virA,* and plasmid DNA (Wen et al., 2010; Deanne and Keith, 2010). In this study, we describe the highly sensitive and specific detection of virulent *Shigella* organisms by icsA gene that exist only *Shigella* spp.. To specific detection of *Shigella* spp., a segment of the gene was selected that was conserved in *Shigella* spp.. We detected *Shigella* spp. successfully by the icsA gene from pure cultured broth, and plasmid DNA. In this study, all the *Shigella* strains were positive for the presence of icsA confirming that icsA gene is highly conserved in *Shigella* spp. This protocol identifies solely invasive Shigella spp. the strains that lose virulence plasmid convert to virulence that do not hybridize to specific primer. Using icsA-specific primers, it was estimated that the minimal *Shigellae* detected through PCR amplification is 300 bacteria for each PCR reaction. These data indicate the possible use of this method for rapid identification of shigella spp. in clinic and food samples.

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