Standardization of PCR Assay for the Confirmation of Faecal Isolates of *Shigella* spp. by Targeting *ipaH* Gene

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ABSTRACT

Shigellosis is an infectious disease, a major public-health problem in developing countries which is often associated with significant morbidity and mortality. The present study was undertaken to standardize PCR assay for the confirmation of *Shigella* spp. isolated from faecal samples. All 8 *Shigella* isolates identified to be *Shigella* spp. by cultural and biochemical tests were further confirmed by PCR, targeting *ipaH* gene which yielded specific amplification of 619 bp gene segment.

Keywords: Diarrhoeal disease, *ipaH* gene, PCR, *Shigella flexneri*, shigellosis

Foodborne diseases are a worldwide problem. Recent developments in food production and processing techniques and the subsequent changing trends in food consumption have resulted in the emergence of new hazards. The number of individuals at risk due to these hazards may rise due to increase in life expectancy. Mead et al. (1999) mentioned that although the number of reported cases of shigellosis arising from food is much lower than that of salmonellosis or campylobacteriosis, it is still an important cause of foodborne bacterial infection in developed countries. Shigellosis is caused by *Shigella* species which are gram-negative, non sporulating, rod-shaped bacteria that belong to the family Enterobacteriaceae and have long been known to be closely related to *E. coli*. The genus *Shigella* with four species viz; *S. dysenteriae*, *S. flexneri*, *S. boydii* and *S. sonnei* were formally recognized, and the traditional classification followed the recommendations of Ewing (Ewing, 1949).

Song et al. (2005) reported that genetically, the enteroinvasive *E. coli* (EIEC) are closely related to *Shigella* spp., and the *ipaH* gene encoding the invasive plasmid antigen is carried by both pathogens. It was also reported by Sansonetti et al. (1982) that *Shigella* spp. and EIEC strains carry a virulence plasmid of approximately 200 kb that is essential for their invasiveness. Similarly Dutta et al. (2001) mentioned that the *ipaH* locus is a multicopy element present on both the chromosome and the invasion plasmid, thereby allowing detection of the pathogens despite a possible loss of the plasmid. Several PCR protocols have been used for the detection of *Shigella* spp. and related organisms in faecal, food and environmental specimen. Recently, Thiem et al. (2004) reported that the developed molecular diagnostic methods can overcome some of the shortcomings of traditional culture methods. In the present study an attempt was made to characterize *Shigella* isolates by targeting *ipaH* gene.

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As many as 511 (311 human stool, 100 cattle and 100 poultry faecal) samples were collected from Pantnagar and nearby areas and were examined for the presence of *Shigella* species. These faecal specimens were processed according to the protocol described in the manual (CDC and WHO, 2003). All 8 *Shigella* isolates identified to be *Shigella* spp. by cultural and biochemical tests were cultured on nutrient agar slant and kept at 4°C for further use.

Extraction of DNA from eight pure cultures of *Shigella* spp. isolated from faecal samples was undertaken using Wizard®Genomic DNA purification kit (Promega) following the manufacturer’s instructions supplied along with the kit. The pure bacterial culture was streaked on MLA and incubated at 37°C for 24 h. Then, 1.5 ml of an overnight broth culture was taken and centrifuged for 2 minutes at 12000 rpm. The pellet so obtained was re-suspended thoroughly in 180 µl of lysis solution (AL) and then 25 µl of proteinase K (20 mg/ml) was added and then incubated at 55°C for 30 minutes. The lysate so obtained was transferred into HiEluteMiniprep Spin Column and centrifuged at 12000 rpm for 1 minute. Finally, 200 µl of elution buffer (ET) was pipetted into the column directly without spilling to the sides and incubated for 5 minutes at room temperature. Then it was centrifuged at 12000 rpm for 1 minute. The spin column was removed and the concentration of collected DNA was adjusted to 50 ng/µl using UV spectrophotometer (Sambrook *et al*., 1989) and then stored at -20°C for future use.

*Shigella* specific primers targeting a 619 bp fragment of the *ipaH* gene was used in the present study (Farshad *et al*., 2006). The primers were synthesized by M/S Bangalore Genei. The details of the *ipaH* gene primer used in the present study are Forward (5'-3') GTT-CCT-TGA-CCG-CCT-TTC-TAC-GT and Reverse (5'-3') GCC-GGT-CAG-CCA-CCC-TCT-GAG-AGT-AC.

The method as described by Sharma *et al*.(2009) was followed for PCR with suitable modifications. The optimized PCR was setup in a 25 µl volume reaction mixture consisting of 10x PCR assay buffer [160 mM (NH₄)₂SO₄, 670 mM Tris-HCl, pH 8.8, 0.1% tween-20, 25 mM MgCl₂² (2.5 µl), dNTP mix [200 µM each] (0.5 µl), primers [Forward and Reverse] [100 pmol/µl] (0.5 µl each), Taq polymerase [5 U/ µl] (0.2 µl), genomic DNA [50 ng/µl] (2 µl) and sterile triple distilled water (q.s 25 µl).

The cycling conditions were optimised for PCR reaction mixture which consisted of an initial denaturation (94°C for 5 min), followed by 35 cycles of denaturation (94°C for 30 sec), primer annealing (55°C for 30 sec), and extension (72°C for 30 sec). A final extension at 72°C was given for 5 min. Then the PCR products were held at 4°C until electrophoresis.

The amplified PCR products were analysed using horizontal submarine gel electrophoresis with the gel concentration of 2% agarose. A separate well charged with 100 bp ladder was allowed to run simultaneously. After sufficient migration, the amplified product was visualised and confirmed over a gel documentation system. The relative molecular weight of the amplified product was calculated against 100 bp ladder.

All 8 *Shigella* isolates identified to be *Shigella* spp. by cultural and biochemical tests were confirmed by PCR assay. The PCR was standardized with primer constructed from reported specific sequence of *ipaH* gene of *Shigella*. Experiments were conducted to optimize the components of PCR mixture and thermocycling conditions. Electrophoretic analysis of the PCR product revealed specific amplification of a 619 bp fragment in all the eight isolates of *Shigella* (Fig. 1).

![Fig. 1. PCR products of amplicon size of 619 bp amplified from genomic DNA of *Shigella* isolates](image)

Lane M : 100 bp; Lane 1 : HS-58; Lane 2 : HS-85; Lane 3 : HS-108; Lane 4 : HS-152; Lane 5 : HS-238 DNA ladder; Lane 6 : HS-273; Lane 7 : HS-305; Lane 8 : HS-309; Lane C : Negative control
The *ipaH* gene was carried by all the species of *Shigella* as well as by entero-invasive *E. coli* (EIEC). Therefore, by using PCR alone, *Shigella* cannot be differentiated from EIEC. Moreover, different serogroups of *Shigella* also cannot be differentiated from each other. But since, EIEC is lactose fermenter and *Shigella* is non lactose fermenter, they were easily differentiated by culture methods.

Similar findings were also reported by Phantouamath *et al.* (2003) who mentioned that apart from *Shigella* spp. the only other organisms that are known to have *ipaH* are EIEC. Toma *et al.* (2003) also reported that the *ipaH* primers used in the novel multiplex PCR assay covered a larger genomic region, as *ipaH* was also detected in all EIEC cases in their study. Sansonetti *et al.* (1982) reported that *Shigella* and EIEC strains carry a virulence plasmid of approximately 200 kb that is essential for their invasiveness. Similar findings were also reported by Song *et al.* (2005) who mentioned that EIEC is closely related to *Shigella* spp. and the *ipaH* gene encoding the invasive plasmid antigen is carried by both pathogens.

In conclusion, the simplex PCR assay was standardized to detect *ipaH* gene, which is essential for detection of *Shigella* spp. at genus level. In the present study, all the isolates identified as *Shigella* spp. by cultural and biochemical methods were found to contain *ipaH* gene which is responsible for their invasive property. Thus, by using molecular techniques along with cultural and biochemical tests, *Shigella* spp. can be detected.

References

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