Isolation, Identification And Molecular Characterization Of Shigella Spp. From Tiruchirappalli District, Tamil Nadu India


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Abstract

The present study aimed at isolation and identification of Shigella spp. from human stool and characterization of the samples serovars using biochemical, molecular characterization techniques. To study the molecular characteristics of 59 Shigella spp. isolated during May 2014 to April 2015 from rectal swabs of patients with watery and/or bloody diarrhoea in a new area of Tiruchirappalli district. A total of 25 samples were collected of which 16% were positive to Salmonella serovars. Members of the genus Shigella, namely S. flexneri, S. dysenteriae, S. sonnei and S. boydii have caused and continue to be responsible for mortality and/or morbidity in high risk populations such as children under five years of age. Yearly episodes of shigellosis globally have been estimated to be 164.7 million and of these, 163.2 million were in developing countries and the remaining in industrialized nations. The mortality rate was approximately 0.7%. Shigellosis is usually a self-limiting infection, however when it subsides, the intestinal ulcers heal with scar tissue formation. Uncomplicated recovery is usual and the organisms rarely cause other types of infections. Adversely, in 3 to 50% of cases, depending on the virulence of the strain, the nutritional and immune status of the host, the initial infection may be followed by neurological complications or kidney failure. Serious complications do occur at greatest frequencies in malnourished infants, toddlers, older adults and immunocompromised individuals [1, 2]. Virulence genes responsible for the pathogenesis of shigellosis may be located in the chromosome or on the inv plasmid borne by the organism. Here, we describe the application of a multiplex PCR (mPCR) design for simultaneous detection of four virulence genes (set1A, set1B, ial and ipaH) in Shigella spp. and to determine the prevalence of these virulence genes in a random selection of Shigella strains.

I. Introduction

Virulence genes responsible for the pathogenesis of shigellosis may be located in the chromosome or on the inv plasmid borne by the organism. They are often multifactorial and coordinately regulated, and the genes tend to be clustered in the genome. Previously reported PCR-based detection methods concentrated mainly on the ipaH gene alone [3, 4] or on ipaH and ial genes in two separate PCR assays [5, 6]. As ial is found on the large inv plasmid which is prone to loss or deletions, this gene-based detection may give false negative results. ipaH, on the other hand, is present on both the Shigella chromosome and on a large plasmid and hence, it is a more stable gene to detect. However, the sole presence of ipaH is not an absolute indicator of virulence as loss or deletion of the plasmid renders the bacterium noninvasive and therefore, avirulent. set1A and set1B are chromosomal genes...
encoding *Shigella* enterotoxin 1 (ShET1), which cause the watery phase of diarrhoea in shigellosis [7.8]. *ial* and *ipa* H are responsible for directing epithelial cell penetration by the bacterium and for the modification of host response to infection, respectively [9-11]. Here, we describe the application of a multiplex PCR (mPCR) design for simultaneous detection of four virulence genes (*set1A*, *set1B*, *ial* and *ipaH*) in *Shigella* spp. and to determine the prevalence of these virulence genes in a random selection of Malaysian *Shigella* strains.

II. Materials and Methods

A total of 12 *Shigella* strains were used in this study. All bacteriological examinations were performed at the KAPViswanathan Medical college hospital in the department of Microbiology Trichy, Tamilnadu. The samples were collected from seven different government hospitals in and around Tiruchirappalli district after receiving permission from institutional ethical committee. All the strains were checked on Salmonella-*Shigella* (SS) agar before being transferred to Luria Bertani (LB) agar plate, incubated overnight at 37°C for subsequent screening of virulence-associated genes. All strains were stored at -20°C in LB broth containing 15% glycerol.

**Multiplex PCR assay**

Multiplex PCR is a highly sensitive and specific molecular biology technique for the detection of target DNA in various clinical specimens. Boiled suspensions of bacterial cells was used as DNA template. Previously described primers, obtained from Integrated DNA Techs, USA, for detection of the four virulence genes were applied to the template [12, 13]. (Table1). Prior to combining all the four primer sets in an mPCR, each pair of primers was optimized singly in separate PCR assays. A typical 25-μl PCR reaction mixture for every primer set consisted of 1x PCR buffer B (Promega, USA), 4 mM MgCl₂, 130 μM of each deoxynucleotide (dNTP), 0.5 μM of each primer, 1 U of *Taq* DNA polymerase (Promega, USA) and 2 μl of DNA template. Amplifications were carried out using a Robocycler Gradient 40 Temperature Cycler (Strategene Cloning Systems, USA). The cycling conditions were an initial denaturation at 95°C for 5 min, template denaturation at 95°C for 50 s, annealing at 55°C for 1.5 min, and extension at 72°C for 2 min for a total of 30 cycles, with a final extension at 72°C for 7 min.

**Table.1 Primers used to identify various virulence-associated genes of *Shigella* app.**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Virulence gene</th>
<th>Nucleotide sequences (5’ – 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ShET1A</td>
<td><em>set1A</em></td>
<td>TCA CGC TAC CAT CAA AGA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TAT CCC CCT TTG GTG GTA</td>
</tr>
<tr>
<td>ShET1B</td>
<td><em>set1B</em></td>
<td>GTG AAC CTG CTG CCG ATA TC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ATT TGT GGA TAA AAA TGA CG</td>
</tr>
<tr>
<td><em>ial</em></td>
<td><em>ial</em></td>
<td>CTG GAT GGT ATG GTG AGG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GGA GGC CAA CAA TTA TTT CC</td>
</tr>
<tr>
<td><em>Shig1</em></td>
<td><em>ipaH</em></td>
<td>TGG AAA AAC TCA GTG CCT CT</td>
</tr>
<tr>
<td><em>Shig2</em></td>
<td></td>
<td>CCA GTC CGT AAA TTC ATT CT</td>
</tr>
</tbody>
</table>

**Detection of Virulence genes**

Based on the results of individual priming, an mPCR was designed. Various parameters such as concentrations of primers (0.5–0.8 μM), MgCl₂ (2 to 4 μM), *Taq* DNA polymerase (0.6 to 4 U) and
dNTPs (100–150 μM) and buffer strength (1.4X to 2.4X) were tested. The simultaneous gene amplifications were performed in a reaction volume of 25 μl consisting of 1.8X PCR buffer B (Promega, USA), 4 mM MgCl₂, 130 μM of each dNTP, 0.3 μM of each ShET1B primer, Shig1 and Shig2 primers, 0.5 μM of each ShET1A and ial primers, 1U of TaqDNA polymerase (Promega, USA) and 2 μl of DNA template. All the reaction mixtures were overlaid with 20 μl of sterile mineral oil. Amplifications were similarly carried out as above. After initial screening, strain TH13/00 (S. flexneri 2a) was chosen as a positive control for PCR assays. A negative control using sterile distilled water as template was included in every PCR assay. The DNA fragments were separated in 2% agarose gel.

**Specificity of Multiplex PCR Assays**

The specificity of the two multiplex PCR assays was determined by using standard reference and ATCC strains of DEC. The strains were subjected to both multiplex PCRs, and the results were compared with those obtained by monoplex PCR assays. Both multiplex PCR assays showed 100% specificity in identifying the reference strains. Non-specific bands were not visualized.

**III. Results and Discussion**

Initial attempts to amplify equally all the four genes in a single reaction using the reaction condition in monoplex PCR were not successful. A common practice in multiplex PCRs involving any non-amplification of a required gene ('weak locus') is to increase the amount of primers of the gene at same time with a decrease of the amount of primers for all the loci that can be amplified, especially those with strong amplifications. All the 12 strains of Shigella spp. tested showed the presence of virulence Conversely; only two strains had both set1A and set1B genes, and ial gene.

Ethidium bromide-stained agarose gel showing PCR products. Lane 1 set1A gene product; (size of amplicon (bp) 309); lane 2 set1B gene product(size of amplicon (bp) 147); lane 3, ial gene product(size of amplicon (bp) 323): lane 4, ipaH gene product(size of amplicon (bp) 423): lane 5, mPCR product

The present mPCR system encompasses the presence of virulence genes found in the Shigella chromosome and on the large inv plasmid. Hence, it can determine if the pathogenesis of a particular strain is attributable to its chromosome or the plasmid, or if the strain is still invasive or otherwise, in a single reaction. In the present study showed that both set1A and set1B were present exclusively in S. flexneri 2a. The complete correlation between the presence of both set1A and set1B showed that both genes were indeed found in tandem in the Shigella genome. Multiplex PCR is more tedious and difficult to achieve than monoplexes, the ease of screening a large
number of specimens. The present mPCR system encompasses the presence of virulence genes found in the *Shigella* chromosome and on the large inv plasmid. In this study more numbers of *Shigella* strains positive for the presence of *set1A, set1B, ial* and *ipaH* genes.

IV. Conclusion

In conclusion, the multiplex PCR assays presented in this study correctly determined the presence of corresponding *Shigella* species virulence genes in all strains tested. The Multiplex PCR assays offer a practical possibility for rapid identification of *Shigella* spp. and could be used in the routine diagnostic laboratory. They can save considerable time and effort involved in testing for various virulence factors.

Bibliography