

VIRULOTYPING OF *SHIGELLA* SPP. ISOLATED FROM PEDIATRIC PATIENTS IN TEHRAN, IRAN

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(Received: 18 May 2016; accepted: 27 October 2016)

Shigellosis is a considerable infectious disease with high morbidity and mortality among children worldwide. In this survey the prevalence of four important virulence genes including *ial*, *ipaH*, *set1A*, and *set1B* were investigated among *Shigella* strains and the related gene profiles identified in the present investigation, stool specimens were collected from children who were referred to two hospitals in Tehran, Iran. The samples were collected during 3 years (2008–2010) from children who were suspected to shigellosis. *Shigella* spp. were identified throughout microbiological and serological tests and then subjected to PCR for virulotyping. *Shigella sonnei* was ranking first (65.5%) followed by *Shigella flexneri* (25.9%), *Shigella boydii* (6.9%), and *Shigella dysenteriae* (1.7%). The *ial* gene was the most frequent virulence gene among isolated bacterial strains and was followed by *ipaH*, *set1B*, and *set1A*. *S. flexneri* possessed all of the studied virulence genes (*ial* 65.51%, *ipaH* 58.62%, *set1A* 12.07%, and *set1B* 22.41%). Moreover, the pattern of virulence gene profiles including *ial*, *ial-ipaH*, *ial-ipaH-set1B*, and *ial-ipaH-set1B-set1A* was identified for isolated *Shigella* spp. strains. The pattern of virulence genes is changed in isolated strains of *Shigella* in this study. So, the *ial* gene is placed first and the *ipaH* in second.

Keywords: *Shigella*, shigellosis, virulence gene, gene profile, PCR

Introduction

Bacterial dysentery, red diarrhea, or shigellosis is a well-known infectious disease that spreads via contaminated foods and water. Shigellosis threatens children's health care via high rates of morbidity and mortality among pediatric populations worldwide and in particular developing countries. The infection of

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shigellosis is caused by non-motile, facultative anaerobic Gram-negative coccobacilli of *Shigella* spp. *Shigella* spp. are categorized into four species of *Shigella dysenteriae* (A serogroup), *Shigella flexneri* (B serogroup), *Shigella boydii* (C serogroup), and *Shigella sonnei* (D serogroup). The shigellosis is identified throughout its clinical symptoms including enteric cramps, nausea, diarrhea, vomiting, blood, and mucus in stool, dehydration, and fever. The spectrum of red diarrhea varies from mild to severe [1–10].

Shigella, as an important pathogenic member of *Enterobacteriaceae* family is able to suppress the host's immune system to have successful adhesion and colonization within the intestine milieu. The epidemiological studies reveal that shigellosis is very common among young children within the age range of up to 5 years old. Thus, the annual mortality rate in association with shigellosis is estimated as 1.1×10^6 patients in which only one-third of them are above 5 years old. Climate, improper medical health care system, inappropriate individual hygiene, contaminated food, incorrect sanitation, and the virulence potential of *Shigella* species are the considerable risk factors that predispose the infection of shigellosis among different geographic populations. [1–3, 5, 11–14].

The invasive infection of shigellosis is demonstrated through 1–2 days after the entrance of 10–100 bacterial cells of *Shigella* within human digestive tract. There are different bacterial virulence factors which may lead to clinical manifestations, such as intestinal inflammatory responses and watery diarrhea. The plasmid encoded gene of *ial* (invasion associated locus) enables *Shigella* bacteria to penetrate into the intestinal epithelial tissues while plasmid encoded *ipaH* gene (invasion plasmid antigen H) provides the capability of bacterial cell-to-cell movement and dissemination within epithelial cells of intestine. On the other hand, the chromosomal genes of *set1A* and *set1B* encode the iron-dependent protein of enterotoxin 1, which is known as a hexamer-ShET-1 protein. The ShET-1 protein, which is constituted of a subunit of A and five subunits of B, is responsible for clinical manifestation of watery diarrhea. The A subunit is secreted by *set1A* and the B subunits are produced by *set1B* [15–17].

The infectious disease of shigellosis is an important bacterial pathogenic infection with high rate morbidity among Iranian pediatric populations too. Detection and identification of pathogenic strains support us to control and reduce the rate of shigellosis outbreaks among children. Therefore, the application of an accurate and reliable diagnostic technique like polymerase chain reaction (PCR) is necessary [1, 3, 11, 18, 19].

Hence, in this study the authors tried to detect and identify the distribution of four important virulence genes of *ial*, *ipaH*, *set1A*, and *set1B* among clinical samples.

Materials and Methods

Isolated bacterial strains

Diarrheal stool samples were taken from pediatric patients who were suspected to have shigellosis and referred to two pediatric hospitals in Tehran, Iran during 3 years (from 2008 to 2010).

The collected diarrheal stool samples were transferred from hospitals to laboratory in transport medium of Cary Blair for microbiological assays. Less than 5 h, the fecal specimens were inoculated into different culture media including Hektoen enteric agar, MacConkey agar, *Salmonella-Shigella* agar, and xylose lysine deoxycholate agar (Difco, Detroit, MI, USA) and then were incubated for 24 h at 37 °C. The positive colonies were checked for further microbiological tests like Gram staining, microscopic observations, morphological macroscopic characteristics of the colonies, and API20E test kit. The positive samples were processed for biochemical properties including lactose fermentation and motility [3].

Serotyping procedures

In parallel with microbiological and biochemical analyses, the isolated *Shigella* strains were identified by serological procedures. The bacterial strains were inoculated into trypticase soy agar (Difco, Detroit, MI, USA) and then were detected via commercial serological antisera. The bacterial serotyping was performed by slide agglutination with polyclonal antisera (Mast Group Ltd., Bootle, Merseyside) for observing the occurrence of agglutinations on the glass slides [3].

Genotyping of the virulence genes

The DNA molecules pertaining to isolated strains were extracted by boiling; a simple and easy method for DNA harvesting. In this study, four virulence related genes of *ial*, *ipaH*, *set1A*, and *set1B* in *Shigella* strains were selected for further investigations (Figures 1–4). The primers used in this study were previously designed (Table I) [17, 20–22].

Each individual pair of aforementioned primers was optimized distinctly. A single 20 µl-PCR reaction mixture was consisted of 9 µl (2×) master mix, 1 µl (50 pmol) related forward primer, 1 µl (50 pmol) related reverse primer, 1 µl (2 µg/µl) DNA template, and 8 µl sterile double distilled water. The amplification process was achieved by Eppendorf thermal cycler. The cycling program was

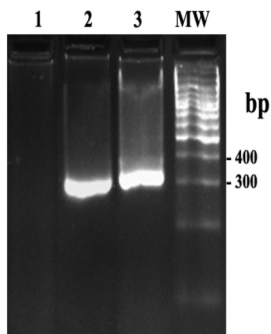


Figure 1. Detection of *ial* gene (320 bp) by PCR in *Shigella* strains. Lanes 2 and 3: positive representative strains; lane 1: negative representative strain. MW is molecular ladder (100 bp)

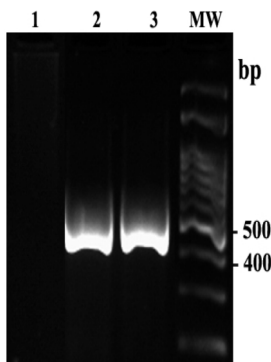


Figure 2. Detection of *ipaH* gene (430 bp) by PCR in *Shigella* strains. Lanes 2 and 3: positive representative strains; lane 1: negative representative strain. MW is molecular ladder (100 bp)

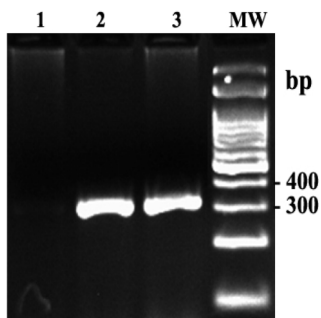


Figure 3. Detection of *set1A* gene (309 bp) by PCR in *Shigella* strains. Lanes 2 and 3: positive representative strains; lane 1: negative representative strain. MW is molecular ladder (100 bp)

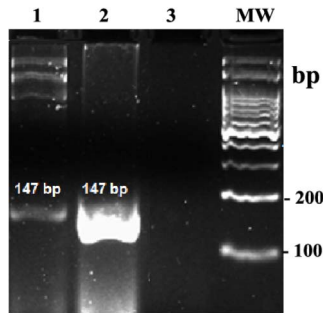


Figure 4. Detection of *set1B* gene (147 bp) by PCR in *Shigella* strains. Lanes 1 and 2: positive representative strains; lane 3: negative representative strain. MW is molecular ladder (100 bp)

Table I. Virulence-related genes and their primers [17, 20–22]

Virulence gene	Primer	5' → 3' nucleotide sequences
<i>ial</i>	ial	CTG GAT GGT ATG GTG AGG GGA GGC CAA CAA TTA TTT CC
<i>ipaH</i>	Shig1 Shig2	TGG AAA AAC TCA GTG CCT CT CCA GTC CGT AAA TTC ATT CT
<i>set1A</i>	ShET1A	TCA CGC TAC CAT CAA AGA TAT CCC CCT TTG GTG GTA
<i>set1B</i>	ShET1B	GTG AAC CTG CTG CCG ATA TC ATT TGT GGA TAA AAA TGA CG

constructed of a single 7-min cycle of initial denaturation at 95 °C, a 1-min template denaturation at 94 °C (30 cycles), a 1-min annealing at 52 °C (30 cycles), a 1-min extension at 72 °C (30 cycles), and a single 7-min cycle of final extension at 72 °C. Finally, the PCR productions were run on 2% gel electrophoresis. The fluorochrome of SYBR GREEN was used in gel electrophoresis (Figures 1–4).

Moreover, the amplified DNA nucleotide sequences were compared with gene sequences registered at gene database, National Center for Biotechnology Information (NCBI).

Results

According to the performed microbiological, biochemical, and serological tests in this study, all the subgroups involving *S. sonnei*, *S. flexneri*, *S. boydii*, and *S. dysenteriae* were detected within collected clinical samples of diarrheal stool. So, the most isolated subgroup was pertaining to *S. sonnei* (65.51%) and the least one was related to *S. dysenteriae* (1.7%).

Table II. Frequency of virulence genes in *Shigella* subgroups

Virulence gene	Location	Total (n = 58), n (%)	<i>S. sonnei</i> (n = 38), n (%)	<i>S. flexneri</i> (n = 15), n (%)	<i>S. boydii</i> (n = 4), n (%)	<i>S. dysenteriae</i> (n = 1), n (%)
<i>ial</i>	Plasmid	38 (65.51)	24 (63.16)	11 (28.95)	3 (7.89)	0 (0.00)
<i>ipaH</i>	Plasmid– chromosome	34 (58.62)	27 (79.41)	6 (17.65)	1 (2.94)	0 (0.00)
<i>set1A</i>	Chromosome	7 (12.07)	0 (0.00)	6 (85.71)	1 (14.29)	0 (0.00)
<i>set1B</i>	Chromosome	13 (22.41)	8 (61.54)	4 (30.77)	1 (7.69)	0 (0.00)

Table III. Four different patterns for virulence gene profiles including *ial* gene in association with isolated *Shigella* serogroups from clinical specimens

Virulence gene profile	Diarrheal stool samples				
	Total (n = 58), n (%)	<i>S. sonnei</i> (n = 38), n (%)	<i>S. flexneri</i> (n = 15), n (%)	<i>S. boydii</i> (n = 4), n (%)	<i>S. dysenteriae</i> (n = 1), n (%)
<i>ial</i> [1]	38 (65.51)	24 (63.16)	11 (28.95)	3 (7.89)	0 (0.00)
<i>ial-ipaH</i> [2]	23 (39.66)	18 (47.37)	4 (26.67)	1 (25)	0 (0.00)
<i>ial-ipaH-set1B</i> [3]	7 (12.07)	5 (13.16)	1 (6.67)	1 (25)	0 (0.00)
<i>ial-ipaH-set1B-set1A</i> [4]	2 (3.45)	0 (0.00)	1 (6.67)	1 (25)	0 (0.00)

The frequencies of virulence genes involving *ial*, *ipaH*, *set1A*, and *set1B* were detected (Table II) and virulence gene profiles regarding *Shigella* subgroups were determined (Table III).

For detecting aforementioned virulence genes, the PCR assay was performed thrice and obtained the same results (Figures 1–4). No, amplification was done relating to genes of non-*Shigella* strains. This confirms 100% reproducibility for PCR.

The molecular weights of the genes including *ial* (320 bp), *ipaH* (430 bp), *set1A* (309 bp), and *set1B* (147 bp) were determined (Figures 1–4). The partial coding sequence (cfs) genes belonging to *set1A* (accession numbers: KP116116.1, KP116115.1, KP116114.1, and KP116113.1) in *S. flexneri*, and *ipaH* (accession numbers: KP116110.1, KP116109.1, and KP116108.1) in *S. sonnei* were submitted to NCBI.

Discussion

Shigellosis as an important invasive enteric infectious disease may appear in different forms of sporadic, epidemic, and pandemic. According to geographical area, the rate of shigellosis and the causative subgroups are different; but the

reported records show a considerable increase of the infection within developing countries [15, 23].

Sangeetha et al. have shown that *S. flexneri* was ranking first as the most important bacterial agent regarding to shigellosis among children. This situation has been reported from other countries like Brazil, China, and Egypt [2, 6, 15].

In our previous report, *S. sonnei* was the predominant bacterial agent of shigellosis in Iran which was similar to some countries such as Canada and the United States of America. However, in some recent reports *S. flexneri* was identified as first [6, 24–27].

As mentioned before, the pathogenicity of *Shigella* spp. is in association with the presence of several virulence genes including *ial*, *ipaH*, *set1A*, and *set1B*. Therefore, in the present investigation the presence of the four aforementioned virulence genes were studied [2, 15, 17, 28, 29].

As the genetic diversity of *Shigella* subgroups varies in different countries and the knowledge about *Shigella* subgroups' virulence gene profiles is limited, we tried to represent a pattern in association with virulence gene profiles including *ial*, *ipaH*, *set1A*, and *set1B* [2, 30].

Farshad et al., Sousa et al., Thong et al., Bin Kingombe et al., Vargas et al., and Sangeetha et al. have indicated that *ipaH* was identified in all isolated *Shigella* spp. in their studies; while these results are not in accordance with our outcomes [6, 15, 17, 27, 28, 31].

In our study, the *ial* gene was the predominant gene among other studied bacterial strains. 63.16% of *S. sonnei* encompassed the *ial* gene. This result shows the high capacity of invasive property by *ial* gene among isolated strains which are charged by plasmids.

The *ipaH* gene was ranked in second place among isolated strains in this study. This outcome does not match the results showed by aforementioned authors. *S. flexneri* has the highest level (79.41%) regarding to *ipaH* gene [6, 15, 17, 27, 31].

As results show in our study, *S. sonnei* (47.37%) ranked first for possessing *ipaH* gene too; while no *ipaH* gene was recognized among *S. dysenteriae*.

The *set1* gene which regulates the secretion of enterotoxin was determined in isolated subgroups. *set1B* virulence gene was recognized in all isolated subgroups exclusive *S. dysenteriae*. The *set1B* is related to watery diarrhea and dehydration symptoms. *set1A* was identified neither in *S. sonnei* nor *S. dysenteriae*. The identified virulence gene profiles confirm the clinical conditions of the patients [2, 21, 32, 33].

da Cruz et al. have shown that the presence of *set1A* was more frequent than *set1B* in the isolated *Shigella* strains in their study, while Thong et al. have

reported an equal percentage of the presence of *set1A* and *set1B* genes among the isolated bacteria in their survey [2, 17].

According to Noriega et al. and Vargas et al. surveys, our results show that *S. flexneri* possesses the most number of *set1A* and *set1B* genes as individuals. Furthermore in our research, the predominant *Shigella* subgroup isolated from 58 clinical specimens was *S. sonnei*. It was followed by *S. flexneri*, *S. boydii*, and *S. dysenteriae*. Several studies show that *S. sonnei* is the most important causative agent of shigellosis in modern and industrialized countries. This pattern confirms a clear improvement in public health and individual hygiene [11, 15, 24, 34].

In conclusion, our study represents a new pattern of frequencies in association with virulence genes of *ial*, *ipaH*, *set1A*, and *set1B*. Our investigation shows the highest frequency of *ial* gene among *Shigella* strains and it is followed by *ipaH*, *set1B*, and *set1A*. Moreover, PCR typing is an appropriate method to find out virulence gene profiles regarding isolated bacteria. It helps scientist to put puzzle pieces to have a complete genetic data in association with different *Shigella* strains that cause shigellosis around the world. Finally, a clear virulence gene profile may lead to have an accurate diagnosis to have a definite treatment relating to different pathogenic strains.

Conflict of Interest

The authors declare no conflict of interest.

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