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PREVALENCE OF ENTEROTOXIN-ENCODING GENES AMONG DIVERSE *SHIGELLA* STRAINS ISOLATED FROM PATIENTS WITH DIARRHEA, SOUTHWEST IRAN

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Shigella spp. are a major cause of bacillary dysentery, particularly among children in developing countries such as Iran. This study aimed to investigate the presence of two important *Shigella* enterotoxins (ShET-1 and ShET-2), encoded by the *set* and *sen* genes, respectively, by polymerase chain reaction (PCR) assay among *Shigella* species isolated from children affected by shigellosis in Ahvaz, southwest of Iran. In this cross-sectional study, from June 2016 to April 2017, altogether 117 *Shigella* isolates were collected from fecal specimens of children aged <15 years with diarrhea in Ahvaz, southwest Iran. All isolates were identified by standard microbiological and molecular methods. The presence of enterotoxin genes was determined by PCR. The most prevalent isolate was *Shigella flexneri* (47.9%), followed by *Shigella sonnei* (41%) and *Shigella boydii* (11.1%), respectively. *Shigella dysenteriae* was not detected in patients' samples. The frequencies of *set1A, set1B*, and *sen* genes were 5.1% (6/117), 15.4% (18/117), and 76.9% (90/117), respectively. This study provides initial background on the prevalence and distribution of the *Shigella* enterotoxin genes in *Shigella* isolates in southwest of Iran. In addition, this study revealed a high prevalence of *sen* enterotoxin gene in *Shigella* species.

Keywords: *Shigella*, diarrhea, Iran, enterotoxin, PCR, *sen*, *set*

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Introduction

Shigellosis or bacillary dysentery caused by four *Shigella* species (*Shigella flexneri, Shigella dysenteriae, Shigella boydii*, and *Shigella sonnei*) is known as a main public health problem worldwide especially in low-hygienic regions [1–3]. Shigellosis is identified throughout different spectrum of clinical symptom from mild watery diarrhea to severe colitis. A major group affected by shigellosis are children under 5 years old [1, 4, 5]. Various epidemiological studies revealed that *S. flexneri* 2a and *S. sonnei* were predominant strains among *Shigella* spp. in both developing and developed countries [6, 7]. Based on the previous reports worldwide, 164.7 million people are annually infected by *Shigella* spp., in which 163 million of them are related to developing countries [8, 9].

There are different virulence factors in *Shigella* species. *Shigella* enterotoxin 1 (ShET1) and *Shigella* enterotoxin 2 (ShET2) are the two important virulence factors suggested to mediate early fluid secretion in the jejunum and cause of infection in the colon and create watery diarrhea due to shigellosis. The shared name is due to their similar properties as enterotoxins, as there is no homology between ShET1 and ShET2 [10].

ShET1 is encoded by *set1A* and *set1B* genes located on the *Shigella* chromosome and it is part of the *Shigella* Island 1 (SHI-1), and mainly present in *S. flexneri* 2a isolates. The two subunits form a 55 kDa holo-AB-type toxin complex in an A1–B5 configuration. The holotoxin secretion mechanism is similar to that of the cholera holotoxin, via the *secretory* pathway and type-II secretion. ShET1 is associated with the watery phase of diarrhea [11, 12].

Another enterotoxin named ShET2 is encoded by *ospD3* (*sen*), which is one of the three *ospD* genes found on the virulence invasion plasmid. This gene is present in all of *Shigella* serotypes and participates in invasion to host epithelial cells [13].

Khuzestan province in the southwest of Iran is an endemic area for *Shigella* infections in children [14]. Rapid diagnosis of *Shigella* strains is an effective way to control and to decrease the rate of shigellosis outbreaks among the children. Since using valid diagnostic techniques like polymerase chain reaction (PCR) is highly recommended [5], the aim of this study was to assess the prevalence of *Shigella* spp. and the presence of two important ShET-1 and ShET-2 enterotoxin genes by PCR method among *Shigella* isolates in children affected by shigellosis in Ahvaz, southwest Iran.

Materials and Methods

Ethics

This study was approved by the ethics committee of Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran (no.: IR.AJUMS.REC.1396.568).

Study design and specimen collection

This descriptive and cross-sectional study was conducted on 840 fecal specimens of children under 15 years old suffering from diarrhea during June 2016 and April 2017. All samples were obtained along with written consent from children's parents. These patients were referred to the medical diagnostic laboratories of the teaching hospitals of Abuzar and Golestan associated with the Ahvaz Jundishapur University of Medical Sciences in Ahvaz city, Iran. Patient information, such as age, gender, history of fever, vomiting, and bloody diarrhea, was recorded. Before antibiotics consumption, stool samples of all patients were collected in sterile containers and transferred to the Microbiology Department of Faculty of the Medicine for further investigation.

Isolation and identification of Shigella *spp.*

All specimens were cultured on MacConkey and Hektoen enteric (HE) agar (Merck, Germany), and then incubated at 37 °C for 24 h. Small green to bluish green colonies on HE and colorless colonies on MacConkey agar were selected and examined using standard biochemical and microbiological tests such as Triple Suger Iron agar (Merck, Germany), Lysine Iron agar (Merck, Germany), Simmon's citrate (Merck, Germany), MR-VP, SH2 production, urease, indole production, and motility [15]. All of the isolates confirmed as *Shigella* spp. were stored at −80 °C in tripticase soy broth with 20% glycerol for further investigations. *Shigella* species were differentiated by PCR using specific primers.

DNA extraction

The boiling method was used to extract genomic DNA from *Shigella* isolates. A few bacterial colonies of *Shigella* strains grown overnight on nutrient agar (Merck, Germany) were resuspended in microtubes containing 500 µl of Tris-EDTA buffer, then the microtubes were placed in Incublock microtube

incubators (Denville Scientific, USA) for 5 min at 95 °C, and then centrifuged at 14,000 rpm for 10 min at 4 °C. The supernatant was used as the DNA template in the PCR assays. The DNA quantity and quality were assessed using NanoDrop Spectrophotometer PROMO (Thermo Scientific, USA) and electrophoresis on 1.5% gel agarose, respectively [16].

PCR for Shigella *spp. differentiation and enterotoxin genes*

Differentiation between *Shigella* spp. was performed by amplification of species-specific genes using primers for putative integrase for *Shigella* genus, *rfc* for *S. flexneri, wbgZ* for *S. sonnei, rfpB* for *S. dysenteriae*, and hypothetical protein for *S. boydii as* described in previous studies [17, 18]. Afterward, the existence of *Shigella* enterotoxins was assessed by amplification of sequences associated with *set1A, set1B*, and *sen* genes according to previous method described by Cruz et al. [7].

PCR assay was performed in thermocycler (Eppendorf, Germany) using the 2× Master Mix (1.5 mM $MgCl_2$, SinaClon, Iran). The primers oligonucleotides used in this study were obtained from Pishgam Co. (Iran). The reference strains of *S. flexneri* ATCC29903, *S. sonnei* ATCC25931, *S. boydii* ATCC8700, and *S. dysenteriae* ATCC13313 were used as positive control. The control positive for enterotoxins genes (*set1A, set1B*, and *sen*) was kindly donated by Dr. H. Hosseini Nave of the Department of Microbiology and Virology, School of Medicine, Kerman University of Medical Sciences, Kerman, Iran. The total volume of PCR reaction was 25 µl prepared as follows: 12.5 µl of 2× Master Mix, 1 µl of 10 pM each primer, 1 µl of template DNA, and distilled water to reach total volume of 25 µl. The cycling program was as follows: 1 cycle at 95 °C for 5 min, 35 cycles at 954 °C for 60 s, varying annealing temperatures for each gene for 45 s, and 72 °C for 45 s, and a final extension cycle at 72 °C for 5 min. The sterile deionized water was included with each PCR run as negative control. The primers' sequences, annealing temperature, and PCR product size are shown in Table I.

PCR products electrophoresis

The PCR products were separated by electrophoresis (80 V, 40 min) using a 1% agarose gel (SinaClon, Iran) in 1× Tris/Borate/EDTA buffer. An amount of 5 µl from each of the product was run on the agarose gel. Then, the gel was stained with ethidium bromide (0.5 µg/ml) (SinaClon) and visualized by UV illuminator device (ProteinSimple, USA) and all images were saved on hard disk. A DNA marker of 100 bp (Sinaclon) was used for comparative analysis.

Table I. List of oligonucleotide primers used in PCR method

Target gene	Primer sequences	Product size (bp)	Annealing temperature (°C)	References
Hypothetical protein	F: GAGCACGGAAACAGAGAGCGCC R: GGTGCGTTCTTCCGGTGTTCTG	240	63	[17]
Putative integrase	F: TCGCATTTCTCTCCCCACCACG R: CCGGATGTGTCTCGGGCAATC	159	63	[17]
wbgZ	F: TCTGAATATGCCCTCTAC R: GACAGAGCCCGAAGAACCG	430	60	[16]
rfc	F: TTTATGGCTTCTTTGTCG R: CTGCGTGATCCGACCATG	537	60	[16]
rfpB	F: TCTCAATAATAGGGAACACAGC R: CATAAATCACCAGCAAGGTT	211	59	[16]
set1A	F: TCACGCTACCATCAAAGA R: TATCCCCCTTTGGTGGTA	309	53	[7]
set1B	F: GTGAACCTGCTGCCGATATC R: ATTTGTGGATAAAAATGACG	147	55	[7]
sen	F: ATGTGCCTGCTATTATTTAT R: CATAATAATAAGCGGTCGC	799	55	[7]

Note: PCR: polymerase chain reaction.

Results

In this study, a total number of 117 *Shigella* species were characterized from 840 fecal specimens by standard biochemical and microbiological tests. These specimens were collected from the children aged <15 years old suffering from diarrhea or dysentery during June 2016 and April 2017. Out of 840 cases, 660 (78.5%) were children under 10 years old. Considering the gender of patients, 308 (36.6%) were females and 532 (63.4%) were males.

For the confirmation of *Shigella* genus, PCR was carried out using specific primers for putative integrase gene (Figure 1) [17]. The results confirmed the presence of this gene in all 117 isolates. In addition, amplification of species-specific genes showed that most isolates belonged to *S. flexneri* (47.9%), followed by *S. sonnei* (41%) and *S. boydii* (11.1%), respectively (Table II). *S. dysenteriae* was not detected in any of the samples.

The distributions of the *set1A, set1B*, and *sen* genes in studied *Shigella* spp. are provided in Table II. The frequency of *sen* gene among 117 *Shigella* strains was most common with 76.9% (90/117), whereas the *set1A* and *set1B* genes were found only in 6 (5.1%) and 19 (16.2%) isolates, respectively. Among the toxin producers, 17 (30.3%) *S. flexneri* strains, 4 (8.3%) *S. sonnei*, and 4 (30.6%) *S. boydii* produced ShET-1 (A or B subunit positive), respectively. None of the

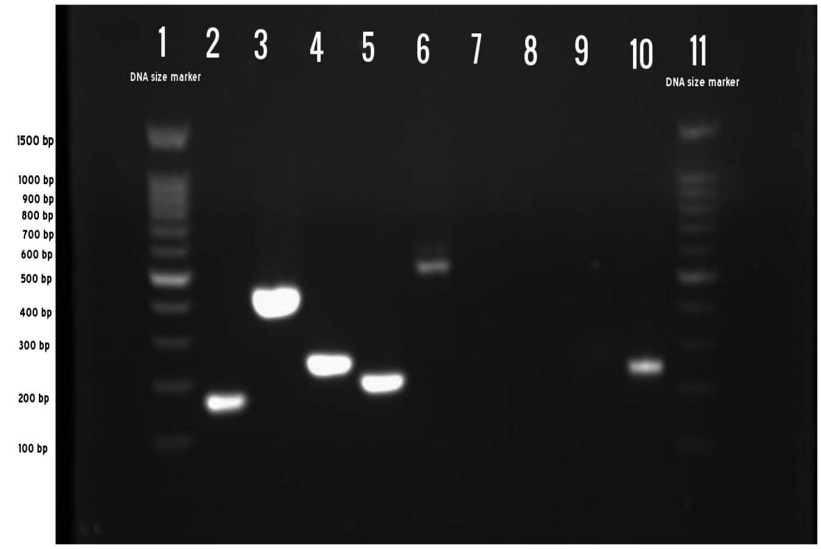

Figure 1. PCR assay profile with *Shigella* reference strains. Lane 1: 100 bp DNA marker; Lane 2: positive control (putative integrase – *Shigella* genus); Lane 3: positive control (*wbgZ* – *S. sonnei*); Lane 4: positive control (hypothetical protein – *S. boydii*); Lane 5: positive control (*rfpB* – *S. dysenteriae*); Lane 6: positive control (*rfc* – *S. flexneri*); Lanes 7–9: negative control; Lane 10: clinical sample (hypothetical protein – *S. boydii*); Lane 11: 100 bp DNA marker

isolates were simultaneously positive for both *set1A* and *set1B* genes. Furthermore, the *sen* gene was detected in 46 (82.1%) *S. flexneri*, 33 (68.7%) *S. sonnei*, and 11 (84.6%) *S. boydii*. Among the 117 *Shigella* strains, 20 (17%) isolates were positive for both the *set* and the *sen* genes. Our results show that the prevalence of *set1A* gene was low in all the *Shigella* species.

Discussion

The previous studies have indicated that humans and primates are only natural hosts of *Shigella* spp. This bacterium with high adaptation with human is a major cause of bacillary dysentery throughout the world, and due to minimal infectious dose (less than 200 bacterial cells), transmission of disease is facilitated in areas with unsuicircumstances such as lack of sanitation [19, 20].

Molecular studies about *Shigella* enterotoxin genes are still insufficient worldwide and to our knowledge there are no published data on this subject in southwest of Iran. The assessment of *Shigella* virulence markers helps better understand its pathogenicity. In this study, we investigated *Shigella* isolates

Table II. Prevalence of the *set1A, set1B*, and *sen* genes in *Shigella* spp. isolated from children with diarrhea

Species	Total no. of isolates	*set1A* gene	*set1B* gene	*sen* gene	None	*set1A* + *set1B* + *sen* genes	*set1A* + *sen* genes	*set1B* + *sen* genes
S. flexneri	56 (47.9%)	5 (8.9%)	12 (21.4%)	46 (82.1%)	8 (14.3%)	2 (3.6%)	3 (5.3%)	10 (17.8%)
S. sonnei	48 (41%)	0 (0%)	4 (8.3%)	33 (68.7%)	12 (25%)	0 (0%)	0 (0%)	3 (6.2%)
S. boydii	13 (11.1%)	1 (7.6%)	3 (23%)	11 (84.6%)	2 (15.4%)	0 (0%)	0 (0%)	2 (15.4%)
	117 (100%)	6 (5.1%)	19 (16.2%)	90 (76.9%)	22 (18.8%)	2 (1.7%)	3 (2.5%)	15 (12.8%)

collected from children with diarrhea for the presence of *set1A*, *set1B*, and *sen* enterotoxin genes.

Our findings showed that *S. flexneri* (47.9%) is the most important cause of shigellosis among children in Ahvaz, southwest of Iran. Our results were in accordance with the previous studies in different parts of Iran, such as Babol, Abadan, and some countries like Brazil, China, Egypt, and India [7, 21–24]. In addition, there are some studies that had reported *S. sonnei* as the most prevalent species. These differences may be related to higher hygiene in these regions [25]. In this study, we also assessed the presence of two chromosomal virulence genes (*set1A* and *set1B*) and one plasmid virulence gene (*sen*) that encode shET1 and shET2, respectively. Both enterotoxins are significantly associated with bloody diarrhea [26].

This study and some studies in recent years are conflict with prior studies that *set1* genes were almost exclusively found in *S. flexneri* serotypes 2 (2a and 2b) and rarely in other serotypes or species [26–29]. More studies in future may help the confirmation of SHI-1 island as location for *set1* gene in different species of *Shigella*.

We detected *set1* genes in 30.3% of *S. flexneri*, 8.3% of *S. sonnei*, and 30.6% of *S. boydii*. Ranjbar [5] in Tehran showed that the prevalence of *set1* gene was 66.6%, 21%, 50%, and 0% in *S. flexneri, S. sonnei, S. boydii*, and *S. dysenteriae*, respectively. According to Medeiros's [30] results, 66.7% of *S. flexneri*, 11.1% of *S. sonnei*, 25% of *S. boydii*, and 0% of *S. dysenteriae* carried *set1* genes. In the study by Roy et al. [31] in India, a total of 153 *Shigella* isolates were analyzed for the presence of *set* and *sen* genes. They found *set* gene only in *S. flexneri* isolates but not in other species and *sen* gene was well distributed among all species [31]. In this study, both ShET-1 and ShET-2 were found in 26.7% of *S. flexneri* isolates. Similarly, Niyogi et al. [27] also reported that 26% of *S. flexneri* isolates produced both ShET-1 and ShET-2.

Interestingly, most isolates had only one subunit of ShET-1, and B subunit was more frequent than A subunit (Table II). The contingency analysis in Cruz et al.'s [7] study showed that there is a relationship between existence of *set1B* gene in *Shigella* isolates and dehydration symptoms in children. However, answer to the question about whether a single subunit would affect the pathogenicity of ShET-1 needs further study for verification.

In this study, the prevalence of *sen* gene was higher (78.7%) than other two genes that was in accordance with reports of the previous studies [12, 30, 32].

The conflict of various researches is likely because of the loss of the large plasmid that contains the gene in different *Shigella* species, differences in the distribution of species, and the sample size.

Conclusions

In this study, we afforded some baseline information about the distribution of some virulence genes in clinical strains of *Shigella* spp. in Ahvaz city, southwest Iran. We found that the prevalence of *sen* virulence gene is high among *Shigella* species in southwest Iran.

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Conflict of Interest

The authors declare no conflict of interest.

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