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Profiling of Virulence-associated Factors in *Shigella* Species Isolated from Acute Pediatric Diarrheal Samples in Tehran, Iran

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Objectives: The genus *Shigella* comprises the most infectious and diarrheagenic bacteria causing severe diseases, mostly in children under five years of age. This study aimed to detect nine virulence genes (*ipaBCD*, *VirA*, *sen*, *set1A*, *set1B*, *ial*, *ipaH*, *stx*, and *sat*) in *Shigella* species (spp.) using multiplex polymerase chain reaction (MPCR) and to determine the relation of *Shigella* spp. from pediatric diarrheal samples with hospitalization and bloody diarrhea in Tehran, Iran.
Methods: *Shigella* spp. were isolated and identified using standard microbiological and serological methods. The virulence genes were detected using MPCR.
Results: Seventy-five *Shigella* spp. (40 *S. sonnei*, 33 *S. flexneri*, 1 *S. dysenteriae*, and 1 *S. boydii*) were isolated in this study. The prevalence of *ial*, *sen*, *sat*, *set1A*, and *set1B* was 74.7%, 45.4%, 28%, 24%, and 24%, respectively. All *S. flexneri* isolates, while no *S. sonnei*, *S. dysenteriae*, or *S. boydii* isolates, contained *sat*, *set1A*, and *set1B*. All isolates were positive for *ipaH*, *ipaBCD*, and *virA*, while one (1.4%) of the isolates contained *stx*. The highest prevalence of virulence determinants was found in *S. flexneri* serotype IIa. Nineteen (57.6%) of 33 *S. flexneri* isolates were positive for *ipaBCD*, *ipaH*, *virA*, *ial*, and *sat*. The *sen* determinants were found to be statistically significantly associated with hospitalization and bloody diarrhea ($p = 0.001$).
Conclusion: This study revealed a high prevalence of enterotoxin genes in *S. flexneri*, especially in serotype 2a, and has presented relations between a few clinical features of shigellosis and numerous virulence determinants of clinical isolates of *Shigella* spp.

Key Words: virulence gene, *Shigella*, pediatrics, diarrhea, gene profile, Iran

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INTRODUCTION

Shigellosis, or bacillary dysentery, continues to be a public health concern worldwide, mainly in the underdeveloped and developing regions with poor hygiene and limited access to clean drinking water [1,2]. The genus *Shigella* is divided into four serogroups—*S. dysenteriae* (serogroup A), *S. flexneri* (serogroup B), *S. boydii* (serogroup C), and *S. sonnei* (serogroup D) [3]. Shigellosis is an invasive illness of the human colon that leads to varied clinical symptoms ranging from mild watery diarrhea to severe colitis [4]. The pathogenesis of shigellosis is related to

various virulence factors located in the chromosome or large virulent *inv* plasmids [5]. Colonization—in which epithelial cell penetration and modification of the host response towards infection for dissemination from cell to cell occurs—is mediated by an invasion-associated locus (*ial*) and the invasion plasmid antigen H (*ipaH*) genes, respectively [6,7]. Chromosomal genes, *set1A* and *set1B*, encode the *Shigella* enterotoxin 1 (*ShET-1*), and are among the factors associated with the watery phase of diarrhea [8]. *Shigella* enterotoxin 2 (*ShET-2*) is involved in invasion and is located in large virulent plasmids [8].

ShET-1 and *ShET-2*, in addition to their enterotoxic activity, play an important role in the transport of electrolytes and water in the intestine [9]. Plasmid-encoded toxin (*pet*), secreted autotransporter toxin (*sat*), and *Shigella* IgA-like protease homologue (*SigA*) are members of the class 1 serine protease autotransporters of Enterobacteriaceae (SPATEs) [8,9]. *VirA* are located on large virulent plasmids and act as virulence determinants in intercellular spreading and invasion [8–10]. Two distinct shiga toxins, *stx-1* and *stx-2*, are encoded by chromosomal genes and expressed only by *S. dysenteriae* serotype 1 and are similar to the shiga-like toxins of enterohemorrhagic *Escherichia coli* (EHEC) [11]. These toxins lead to the expansion of vascular lesions in the kidney, central nervous system, and colon in a large number of cell types [12]. Because of the high toxicity of the shiga toxin, infections with *S. dysenteriae* serotype 1 commonly have life-threatening complications [13].

Numerous studies have been conducted on the prevalence and antimicrobial resistance of *Shigella* species, both in Iran and other countries [13,14]. The aim of the present study was to detect nine virulence genes (*ipaBCD*, *VirA*, *sen*, *set1A*, *set1B*, *ial*, *ipaH*, *stx*, and *sat*) in *Shigella* species (spp.) using the multiplex polymerase chain reaction (MPCR) and to determine the relation of *Shigella* spp. from pediatric diarrheal samples with hospitalization and bloody diarrhea in Tehran, Iran.

MATERIALS AND METHODS

1. Clinical samples and laboratory identification

Seventy-five *Shigella* strains, including *S. sonnei* (n = 40), *S. flexneri* (n = 33), *S. dysenteriae* (n = 1), and *S. boydii* (n = 1), were used in this cross-sectional study. These strains were isolated from 946 non-duplicative stool samples from pediatric patients with diarrhea in Tehran, Iran, during an 18-month period from May 2015 to October 2016. The presence or absence of bloody diarrhea and any history of hospitalization were reported by the individual responsible for the clinical evaluation.

Cary-Blair transport medium (Oxoid, Basingstoke, Hampshire, UK) was used for sample transportation to the laboratory, where each sample was subjected to immediate testing. In the laboratory, all specimens were cultured in different differential media, including xylose lysine desoxycholate (XLD) agar and Hektoen enteric agar (HEA) (Merck, Darmstadt, Germany), and then incubated at 37°C for 24 hours. All grown colonies were identified using a conventional biochemical culture base and a microbiological API 20E kit (bioMerieux, Marcy l'Etoile, France). Serological tests were performed on the *Shigella* strains using the slide agglutination method [14]. The serotypes of all *Shigella* isolates were determined with commercially available polyclonal- and monoclonal-specific antisera (Denka Seiken, Tokyo, Japan) against all *Shigella* serotypes, including *S. sonnei* 1 and 2, polyvalent *S. flexneri*, *S. dysenteriae* 1, and polyvalent *S. boydii*. *S. boydii* ATCC 9207, *S. dysenteriae* ATCC 13313, *S. sonnei* ATCC 1202, and *S. flexneri* ATCC 9290 were used as quality controls in each test. All strains were stored in Luria-Bertani broth containing 50% glycerol at −80°C until use.

2. MPCR method

Each sample was subjected to MPCR amplification using 14 pairs (nine virulence genes and five species-specific genes) of different primers (Table 1 [15–18]); MPCR with various T_m details are shown in Table 1. MPCR was performed using a polymerase chain reaction (PCR) instrument with mastercycler gradient (PEQLAB, Erlangen, Germany) for the detection of various virulence- and species-specific genes (*set1A/set1B*, *ial/virA*, *sen/ipaBCD*, *sat*, *stx*, and *ipaH*). The overnight-grown colonies on the XLD agar plates were picked for template genomic DNA extraction by the boiling method. The total volume of the MPCR mixture was 20 μL, containing 0.5 μL extracted template DNA, 2.0 μL 10× PCR buffer, 0.5 μL $MgCl_2$ (50 mM), 0.5 μL deoxynucleotides (10 mM), 0.5 μL each virulence gene primer, 0.5 μL Taq DNA polymerase (5 U/μL) (Amplicon Co., Copenhagen, Denmark), and 13 μL ddH_2O (In *set1A/set1B*, 2 μL H_2O was added).

The MPCR conditions for the amplification of virulence genes included an initial denaturation at 94°C for 60 seconds, 35 cycles of denaturation at 94°C for 60 seconds, annealing at 58°C (variable) for 90 seconds, and extension at 72°C for 60 seconds, as well as a final extension at 72°C for 7 minutes. The reaction mixture was completed in a thermal gradient cycler (PEQLAB) for the detection of species-specific genes using the following MPCR procedure: pre-denaturation at 95°C for 1 minutes, 35 cycles with denaturation at 94°C for 35 seconds, annealing at 55°C for 90 seconds, extension at 72°C for 30 seconds, and final extension at 72°C for 7 minutes. The PCR products were subjected to electrophoresis using 1.0% agarose gel, stained with ethidium bromide, and observed under ultraviolet light.

Table 1. Primer sequences for detection of virulence genes

Gene targeted	Primer	Sequence	Product size (bp)	Reference	T_m
set1A	*ShET-1A*	F: 5′-TCACGCTACCATCAAAGA-3′	309	[15]	55
		R: 5′-TATCCCCCTTTGGTGGTA-3′			
set1B	*ShET-1B*	F: 5′-GTGAACCTGCTGCCGATATC-3′	147	[15]	55
		R: 5′-ATTTGTGGATAAAAATGACG-3′			
sat	*Sat1*	F: 5′-ACTGGCGGACTCATGCTGT-3′	387	[17]	55
		R: 5′-AACCCTGTAAGAAGACTGAGC-3′			
ial	*Ial1*	F;CTGGATGGTATGGTGAGG	320	[15]	58
		R;GGAGGCCAACAATTATTTCC			
ipaH	*Shig1*	F: 5′-TGGAAAAACTCAGTGCCTCT-3′	423	[16]	58
		R: 5′-CCAGTCCGTAAATTCATTCT-3′			
virA	*virA*	F-CTGCATTCTGGCAATCTCTTCACATC	215	[15]	58
		R-TGATGAGCTAACTTCGTAAGCCCTCC			
Stx	*Stx1*	F: 5′-CAGTTAATGTGGTTGCGAAG-3′	895	[15]	60
		R: 5′-CTGCTAATAGTTCTGCGCATC-3′			
sen	*ShET2*	F: 5′-ATGTGCCTGCTATTATTTAT-3′	799	[15]	60
		R: 5′-CATAATAATAAGCGGTCAGC-3′			
ipaBCD	*ipaBCD*	F: 5′-GCTATAGCAGTGACATGG-3′	612	[18]	60
		R: 5′-ACGAGTTCGAAGCACTC-3′			

Statistical analysis was then conducted for each of the virulence determinants. The analysis included cross-tabulation and the performance of the Pearson chi-square test of independence. Levels of significance were determined between the two clinical features (hospitalization and bloody diarrhea) and enterotoxin genes.

RESULTS

1. *Shigella* species

Of the 946 diarrheal samples, 75 isolates of *Shigella* spp. were obtained using conventional biochemical and microbiological tests. All isolates were confirmed by the *Shigella* genus-specific PCR. The prevalence of the *Shigella* species is shown in Table 2. The species-specific amplification test showed that 40, 33, 1, and 1 strains of *S. sonnei*, *S. flexneri*, *S. dysenteriae*, and *S. boydii*, respectively, were isolated from all the tested samples. The study was performed on children aged 1–15 years; as anticipated, children over one year of age were more affected by *Shigella* than the younger children were. The prevalence rate of *Shigella* spp. varied in different age groups; *S. sonnei* was identified in 12 (30.0%) isolates in the ≤ 5 years age group and 28 (70.0%) isolates in the < 5 years of age group, while *S. flexneri* was found in 17 (51.5%) isolates in the ≤ 5 year age group, but this difference was not statistically significant ($p = 0.16$). Of the total isolates, 45.3% and 54.7% of isolates were associated with males and females, respectively, but this distribution was not significant ($p = 0.19$).

2. Virulence factors

All isolates were positive for the *ipaH*, *ipaBCD*, and *virA*, while only one (1.4%) of all the isolates was positive for the *stx* (Table 2). The prevalence of the *ial*, *sen*, *sat*, *set1A*, and *set1B* was 74.7%, 45.4%, 28.0%, 24.0%, and 24.0%, respectively; the results are shown in Table 2. *set1A*, *set1B*, and *sat* were only detected in *S. flexneri* isolates. *stx* is carried by *S. dysenteriae*. The highest prevalence of virulence determinants was found in *S. flexneri*. One interesting finding was the simultaneous presence of the *ipaBCD*, *ipaH*, *virA*, and *ial* in 31 isolates (77.5%) of *S. sonnei*, while these genes were not found in the nine remaining isolates. In addition, 19 (57.6%) of the 33 *S. flexneri* isolates were simultaneously positive for the *ipaBCD*, *ipaH*, *virA*, *ial*, and *sat*. All *S. flexneri*, while no *S. sonnei*, *S. dysenteriae*, and *S. boydii*, isolates harbored *sat*, *set1A*, and *set1B*. Between the two *Shigella* enterotoxin genes, *sen*

Table 2. Prevalence and combination of virulence genes among 75 *Shigella* isolates

Virulence gene (*Shigella* species)	Isolates									Isolates with a combination of genes				
	ipaBCD	*VirA*	*sen*	*set1A*	*set1B*	*ipaH*	*ial*	*Stx*	*sat*	I	II	III	IV	V
S. sonnei (n = 40)	40 (100)	40 (100)	19 (47.5)	0 (0)	0 (0)	40 (100)	31 (77.5)	0 (0)	0 (0)	40 (100)	31 (77.5)	0 (0)	0 (0)	0 (0)
S. flexneri (n = 33)	33 (100)	33 (100)	15 (45.5)	18 (54.5)	18 (54.5)	33 (100)	25 (75.7)	0 (0)	21 (63.6)	33 (100)	25 (75.7)	19 (57.6)	10 (30.3)	5 (15.2)
1a (n = 11)	11 (100)	11 (100)	5 (45.5)	0 (0)	0 (0)	11 (100)	9 (81.8)	0 (0)	5 (45.4)	11 (100)	9 (81.9)	4 (36.3)	1 (9.0)	0 (0)
IIa (n = 22)	22 (100)	22 (100)	10 (45.4)	18 (81.8)	18 (81.8)	22 (100)	16 (72.7)	0 (0)	16 (72.7)	22 (100)	16 (72.7)	15 (68.1)	9 (40.9)	5 (22.7)
S. flexneri vs. *S. sonnei* (*p*-value)	NA	NA	< 0.862	< 0.000	< 0.000	NA	< 0.464	NA	< 0.000	NA	NA	NA	NA	NA
S. boydii (n = 1)	1 (100)	1 (100)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)
S. dysenteriae (n = 1)	1 (100)	1 (100)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	1 (100)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)
Total	75 (100)	75 (100)	34 (45.4)	18 (24)	18 (24)	75 (100)	56 (74.7)	1 (1.4)	21 (28)	75 (100)	53 (74.7)	19 (25.4)	10 (13.4)	5 (6.7)

Values are presented as number (%). NA, not available.
Combination of genes: I: *ipaBCD* + *ipaH* + *virA*; II: *ipaBCD* + *ipaH* + *virA* + *ial*; III: *ipaBCD* + *ipaH* + *virA* + *ial* + *sat*; IV: *ipaBCD* + *ipaH* + *virA*+ *ial* + *sat* + *sen*; V: *ipaBCD* + *ial* + *ipaH* + *virA* + *setIA* + *setIB* + *sen* + *sat*.
$p < 0.05$ was considered statistically significant.

was found to be statistically significant and associated with hospitalization and bloody diarrhea ($p = 0.001$), as shown in Table 3. The remainder of the calculations yielded values in which $p < 0.05$, and thus were considered to be statistically insignificant in this study.

DISCUSSION

In the current study, 75 *Shigella* isolates were obtained from all the tested stool samples. Conventionally identified isolates of *Shigella* were confirmed using *ipaH*-specific PCR assay. In our study, similar to Binet et al.'s study [19], *ipaH* was detected in all *Shigella* culture-positive specimens [20]. In accordance with these results, Vu et al. [21] showed that *ipaH* is carried by all four *Shigella* species as well as by enteroinvasive *E. coli* (EIEC). In agreement with Casabonne et al.'s [22] and Cruz et al.'s [23] results, the results of our study revealed that *virA* and *ipaBCD* were found to be positive in all the strains. *Shigella* attaches to the target region through the two receptors, hyaluronan receptor CD44 and integrin α5β1 [24]. *Shigella* attaches to CD44 through the *IpaB* determinant, while the *IpaBCD* complex interacts via α5β1 integrin receptor. Finally, invasion and cytoskeleton reformation occur by the binding of *Shigella* to the receptors [24,25].

VirA, *IcsA/VirG*, *SopA/IscP*, and *PhoN2* are important determinants for bacterial penetration into host cells and actin nucleation at one end of the bacterium [8]. Some of the virulence factors mentioned above are also situated in large virulent plasmids. Fifty-six (74.7%) *Shigella* strains were found to carry *ial*, in our study; these results are approximately consistent with those of Casabonne et al.'s [22] and Hosseini Nave et al.'s [26] studies. This contrast may be because *ial* is only located on the virulent plasmid and can cause deletion mutations [26]. *Sat* was described first in uropathogenic *E. coli* (UPEC), but has now also been found in *Shigella* spp. The prevalence of *sat* in *S. flexneri* has been found to be 21 (63.6%) [8]. This data conflicts with that of studies conducted in India [27] (56/65, 86.2%) and India 72/75 (96.0%) [28]. A large invasion plasmid gene (*sen*), which encodes *ShET-2*, has also been reported in numerous *Shigella* spp. In this study, 45.4% (34/75; 19 *S. sonnei* and 15 *S. flexneri*) isolates carried *sen* [29]. Similarly, *sen* has been detected in 37 (66.1%) *Shigella* isolates in Kerman, Iran [26]. Casabonne et al. [22] showed that of the 100 *Shigella* isolates, 29 *S. flexneri* and 11 *S. sonnei* carried the gene encoding *ShET-2*.

The conflict is likely because of the loss of the large plasmid that contains the gene in different *Shigella* serogroups and the number of samples. *Shigella* enterotoxin 1 (*ShET-1*) is encoded by *set* located on the chromosomes of several clinical strains of

Table 3. Assessing major *Shigella* virulence genes associated with main symptoms of shigellosis

Virulence gene		Hospitalization		OR (CI)	*p*-value	Bloody diarrhea		OR (CI)	*p*-value
		Positive	Negative			Positive	Negative		
SET.1A	Positive	3 (30.0)	15 (23.8)	0.7 (0.16–3.1)	NS	16 (88.9)	20 (36.4)	3.1 (2.97–6.1)	0.001**
	Negative	7 (70.0)	48 (76.2)			2 (11.1)	35 (63.6)		
SET.1B	Positive	3 (30.0)	15 (23.8)	0.7 (0.16–3.1)	NS	16 (88.9)	20 (36.4)	3.1 (2.97–6.1)	0.001**
	Negative	7 (70.0)	48 (76.2)			2 (11.1)	35 (63.6)		
SEN	Positive	10 (100)	24 (38.1)	1.1 (0.6–2.1)	0.001**	24 (70.6)	12 (30.8)	3.2 (1.9–7.2)	0.001**
	Negative	0	39 (61.9)			10 (29.4)	27 (69.2)		
SAT	Positive	3 (30.0)	18 (28.6)	0.9 (0.6-1.4)	NS	18 (85.7)	18 (34.6)	NR	NR
	Negative	7 (70.0)	45 (71.4)			3 (14.3)	34 (65.4)		

Values are presented as number (%).
OR, odds ratio; CI, confidence interval; NS, not significant; NR, not reported.
Analyzed by Fisher's exact test; **significant difference.

S. flexneri serotype 2 and rarely on those of other serotypes [28]. *ShET-1* has been found to stimulate fluid secretion into the intestine, thus, contributing to the watery phase of diarrhea [28,30]. In our study, 18 (24.0%) isolates were found to carry both *set1A* and *set1B*. Casabonne et al. [22], Vargas et al. [15], and Cruz et al. [23] showed that the prevalence of set1A and set1B was 7.0% (7/100), 3.92 (2/51), and 36.6 (11/30), respectively. In agreement with previous studies, the present study showed that *set1A* and *set1B* were detected only in *S. flexneri* strains [26].

stx is another virulence determinant related to *S. dysenteriae*; it is not excreted by the bacteria, but is released only during cell lysis [31]. Only one (1.4%) *S. dysenteriae* isolate carries *stx*. Bekal et al.'s [32] study detected *S. flexneri* isolates harboring the Shiga toxin 1-producing gene. In Gray et al.'s study [33], 21% of the isolates, including *S. flexneri* 2a, *S. flexneri* Y, and *S. dysenteriae* 4, were found to harbor and produce *stx*. Among *Shigella* enterotoxin genes, both *sen* and *set* enterotoxins are significantly associated with bloody diarrhea. In Cruz et al.'s study [23], *ShET-2* was found to contribute to intestinal injury and bloody diarrhea.

Farfán et al. [34] reported that the *ShET-2* coding *sen* is responsible for epithelial inflammation; in this research found a combination of the *ipaBCD*, *ipaH*, *virA*, and *ial* in 31 (77.5%) S. *sonnei* isolates. In addition, Zhang et al. [16] found that 19 (57.6%) of 33 *S. flexneri* isolates were positive for *ipaBCD*, *ipaH*, *virA*, *ial*, and *sat* simultaneously; however, only *sat*, *set1A*, and *set1B* were detected in *S. flexneri* strains. They also showed that 2, 123, 8, 12, and 53 of 198 *Shigella* isolates carried *Ia1/ipaH/virA*, *ia1/ipaH/vir/sen*, *ia1/ipaH/virA/setlA/sen*, *ia1/ipaH/virA/setlB/sen*, and *ia1/ipaH/virA/setlA/setlB/sen*, respectively [16]. Of the 100 *Shigella* isolates, 24 *S. flexneri* were found to carry *set* and *sen* in Casabonne et al.'s study [22].To the best of our knowledge, this is the first study on the distribution of virulence gene combinations, and these genes are related with hospitalization and bloody diarrhea among *Shigella* species in Tehran, Iran. In conclusion, this work has demonstrated the high prevalence of two enterotoxins, *ShET-1* and *ShET-2*, in *S. flexneri*, especially, among the hospitalized pediatric patients who were included in the study population. Among *Shigella* serotypes, *S. flexneri* serotype 2a was found to have a high number of virulence determinants. Bloody diarrhea and hospitalization were also found to be associated with the number of virulence determinants. Future studies should investigate the relations between shigellosis symptoms and virulence determinants in Iran.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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