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Distribution of genes encoding virulence factors and molecular analysis of *Shigella* spp. isolated from patients with diarrhea in Kerman, Iran

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ABSTRACT

Shigella is one of the important causes of diarrhea worldwide. *Shigella* has several virulence factors contributing in colonization and invasion of epithelial cells and eventually death of host cells. The present study was performed in order to investigate the distribution of virulence factors genes in *Shigella* spp. isolated from patients with acute diarrhea in Kerman, Iran as well as the genetic relationship of these isolates. A total of 56 isolates including 31 *S. flexneri*, 18 *S. sonnei* and 7 *S. boydii* were evaluated by polymerase chain reaction (PCR) for the presence of 11 virulence genes (*ipaH*, *ial*, *set1A*, *set1B*, *sen*, *virF*, *invE*, *sat*, *sigA*, *pic* and *sepA*). Then, the clonal relationship of these strains was analyzed by multilocus variable-number tandem repeat analysis (MLVA) method. All isolates were positive for *ipaH* gene. The other genes include *ial*, *invE* and *virF* were found in 80.4%, 60.7% and 67.9% of the isolates, respectively. Both *set1A* and *set1B* were detected in 32.3% of *S. flexneri* isolates, whereas 66.1% of the isolates belonging to different serogroup carried *sen* gene. The *sat* gene was present in all *S. flexneri* isolates, but not in the *S. sonnei* and *S. boydii* isolates. The result showed, 30.4% of isolates were simultaneously positive and the rest of the isolates were negative for *sepA* and *pic* genes. The *Shigella* isolates were divided into 29 MLVA types. This study, for the first time, investigated distribution of 11 virulence genes in *Shigella* spp. Our results revealed heterogeneity of virulence genes in different *Shigella* serogroups. Furthermore, the strains belonging to the same species had little diversity.

1. Introduction

Annually, approximately 164.7 million people are affected by diarrheal illness caused by *Shigella* spp. and 1.1 million people die each year from Shigellosis [1]. Shigellosis symptoms range from a mild diarrhea to severe life-threatening dysentery, with blood, mucus and pus in the stool [1]. *Shigella* invades epithelial cells and kills them, which in turn causes a severe inflammatory response [2]. The severity of the illness depends on the virulence of the infecting strains [1]. Several virulence factors have been associated with *Shigella* spp. Some virulence factors are involved in the invasion of intestinal cells such as invasion plasmid antigen H (*ipaH*) and invasion associated locus (*ial*) [3]. While *ipaH* gene with multiple copies is located both on plasmid and the chromosome, *ial* gene is only located on large virulence plasmid [3]. Two enterotoxins in *Shigella* called shigella enterotoxin 1 (ShET1) and shigella enterotoxin 2 (ShET2) alter electrolyte and water transport in the small intestine [2]. The ShET1 is a 55 KD protein, encoded by chromosomal genes *set1A* and *set1B*. The ShET2 is encoded by *sen* gene located on the large virulence plasmid [2]. Two plasmid-borne proteins, VirF and VirB (InvE), are the regulatory proteins that control expression of invasion genes [4].

In addition, some toxins as serin protease autotransporters of Enterobacteriaceae (SPATEs) are present in *Shigella* spp. The SPATE family has been categorized phylogenetically into 2 classes [5]. Members of the class 1 are toxic to epithelial cells, including toxins such as plasmid encoded toxin (Pet), secreted autotransporter toxin (Sat) and Shigella IgA-like protease homologue (SigA). Sat and Pet toxins have 52% similarity in amino acid and may have the same contribution in the pathogenesis [5]. Members of the class 2

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including members such as Pic (mucinase involve in colonization) and SepA (secreted protein which contribute to intestinal inflammation) are non-toxic SPATE. Both of these toxins are first reported in *Shigella flexneri* 2a [5,6].

Molecular typing methods such as multilocus variable-number tandem repeat analysis (MLVA) are increasingly used to determine genetic relatedness among bacterial pathogens for the purposes of epidemiological surveillance. Several studies have investigated variable-number tandem-repeat (VNTR) loci variation to discriminate different *Shigella* isolates [7–10]. Despite many reports about the prevalence and antimicrobial resistance of *Shigella* spp. from different parts of the world and Iran, investigations into *Shigella* spp. virulence genes are still rare worldwide and there is no report on this subject in Iran. Therefore we investigated the prevalence and distribution of 11 virulence genes on *Shigella* strains isolated from patients with diarrhea in Iran and also we determined genetic diversity and relationship of these isolates.

2. Materials and methods

2.1. Patients and samples

Between June 2013 and August 2014, a total of 56 *Shigella* strains including 31 *S. flexneri*, 18 *S. sonnei* and 7 *S. boydii* were isolated from 624 stool specimens of patients suffering from acute diarrhea in Kerman, south east of Iran. Only patients without a history of antimicrobial consumption were included in the study. All isolates were identified by standard biochemical tests [11] and serotyped using commercial antisera (MAST Group LTD, Merseyside, UK). Then *Shigella* isolates were kept in the TSB broth containing 30% glycerol at a temperature of −70 °C for further analysis.

2.2. Polymerase chain reaction (PCR) assay for virulence genes

For the detection of virulence genes, DNA template was obtained as method previously described by Ranjbar et al. [12]. The primers used to detect *ipaH*, *ial*, *set1A*, *set1B*, *sen*, *virF* and *invE* genes, and size of the amplified PCR products were as previously described [13,14]. Mastermix (Amplicon, Brighton, UK) was used and the reaction was carried out according to the manufacturer's instructions. Amplification was performed in thermocycler (Biometra-T gradient, Germany). Cycling conditions were as follows: initial denaturation at 95 °C for 7 min, followed by 30 cycles including denaturation for 1 min at 95 °C, annealing for 45 s and 72 °C for 45 s and a single final extension at 72 °C for 5 min. For SPATEs genes including *sat*, *sigA*, *pic* and *sepA* genes, multiplex PCR was performed according to the method described by Boisen et al. [5].

2.3. VNTR locus selection and amplification

The following Twelve VNTR loci were selected: ms06, ms07 (CVN001), ms09, ms11, ms21, ms23, ms32, sf5, ss20, ss24, ss25 and ss26 and PCR was performed according to the methods previously described [8–10]. After performing PCR, the size of each locus was determined on a 1.5% agarose gel and the number of repeats was calculated using the criteria of Ranjbar et al. [12]. Any difference in one or more VNTR loci was regarded as a distinct type.

2.4. Statistical analysis

For the analysis of data the SPSS (Statistical Product and Service Solutions, version 20.0) (SPSS Inc., Chicago, IL, USA) was used. The association of *Shigella* spp. with prevalence of virulence associated genes was assessed by the Chi-square and Fisher's exact test. A value of $p < 0.05$ was considered statistically significant.

3. Results

The prevalence of genes encoding virulence factor among *Shigella* spp. was shown in Table 1. All isolates were positive for *ipaH* gene, while 45 (80.4%) of the isolates were positive for *ial* gene. A total of 34 (60.7%) and 38 (67.9%) isolates were found to be positive for *invE* and *virF* genes, respectively. Ten (17.9%) isolates were positive for *set1A* and *set1B* genes and *sen* was detected in 37 (66.1%) of *Shigella* isolates. The *set1A* and *set1B* genes were only detected in *S. flexneri* isolates. All *Shigella* isolates harbored at least one SPATE proteins. The genes including *sigA*, *sepA*, *pic* and *sat* were detected in 36 (64.3%), 17 (30.4%), 17 (30.4%) and 31 (55.4%) of isolates respectively. There were statistically significant associations between the presence of *sigA*, *pic*, *sepA* and *sat* genes with *Shigella* species ($p < 0.05$). All *S. sonnei* and *S. boydii* isolates carried *sigA* gene, while only 11 (35.5%) isolates of *S. flexneri* harbored this gene. In addition, although all *S. sonnei* strains were negative for the presence of *sepA* and *pic* genes, 85.7% of *S. boydii* strains were positive for these genes. An interesting finding was the presence of *sigA*, *pic* and *sepA* genes simultaneously in 11 isolates (35.5%) of *S. flexneri* while these genes were not found in the 20 remaining isolates. Also 17 (30.4%) of 56 strains were positive for *sepA* and *pic* simultaneously and the rest of the strains carried neither *sepA* nor *pic* genes. All *S. flexneri* isolates harbored *sat* gene, but all the *S. sonnei* and *S. boydii* isolates were negative for this gene. Interestingly, while 9 *S. flexneri* isolates were positive for all virulence genes, 8 isolates were negative for all gene except for *sat* and *ipaH* genes.

A total of 56 *Shigella* strains were divided into 29 MLVA types. *S. sonnei* isolates had less diversity than the other *Shigella* species and their virulence genes profile was almost identical in different MLVA types. *S. flexneri* isolates, showed high heterogeneity of virulence genes but most strains of the same MLVA type had similar virulence genes profile. In some cases, isolates of the same MLVA type had different virulence gene patterns.

4. Discussion

Although *Shigella* is one of the most common causes of diarrheal illness, information on the variety of virulence genes of these bacteria is limited. Indicating the existence of virulence-associated genes in *Shigella* would be useful to better understand its pathogenicity. This study, for the first time in Iran, investigated the distribution and prevalence of 11 virulence genes in *Shigella* isolates. The detection of the virulence genes from 56 isolates revealed that all isolates were positive for *ipaH* gene, whereas *ial* gene was detected in 80.4% that is consistent with several other studies [15–18]. This can be because of *ial* gene is only located on the plasmid and as a result is prone to lose or deletion, while *ipaH* gene is found both on the chromosome and the plasmid [18]. Recently in a study by Zhang et al. 17.1% of *S. sonnei* strains resistant to broad spectrum cephalosporins contained *set1A* or *set1B* genes or both of them [19]. However, in many other studies these genes were exclusively observed in *S. flexneri* strains [3,17,18,20]. Also, in the present study, *set1A* and *set1B* genes were only detected in *S. flexneri* strains. As mentioned in previous studies, *sen* was detected in different *Shigella* serogroups [3,17].

Few studies have investigated the prevalence of SPATEs genes in *Shigella* strains. Our results showed that SigA toxin may play an important role in the pathogenesis of *S. sonnei* strains. In contrast, probably Sat toxin has a major contribution in the virulence of *S. flexneri* strains. In agreement with our finding Roy et al. showed

Table 1
Prevalence of virulence-associated genes in *Shigella* spp.

ID	*Shigella* species	Virulence genes											MLVA type
		ipaH	*ial*	*invE*	*virF*	*sen*	*set1A*	*set1B*	*sigA*	*sepA*	*pic*	*sat*	
S1	*sonnei*	+	+	+	+	+	−	−	+	−	−	−	M1
S2	*sonnei*	+	−	−	−	+	−	−	+	−	−	−	M1
S3	*sonnei*	+	+	+	+	+	−	−	+	−	−	−	M1
S4	*sonnei*	+	+	+	+	+	−	−	+	−	−	−	M1
S5	*sonnei*	+	+	+	+	+	−	−	+	−	−	−	M1
S6	*sonnei*	+	+	+	+	+	−	−	+	−	−	−	M1
S7	*sonnei*	+	+	+	+	+	−	−	+	−	−	−	M1
S8	*sonnei*	+	+	+	+	+	−	−	+	−	−	−	M1
S9	*sonnei*	+	+	−	−	−	−	−	+	−	−	−	M1
S10	*sonnei*	+	+	+	+	+	−	−	+	−	−	−	M1
S11	*sonnei*	+	+	+	+	+	−	−	+	−	−	−	M3
S12	*sonnei*	+	+	+	+	+	−	−	+	−	−	−	M3
S13	*sonnei*	+	+	−	+	+	−	−	+	−	−	−	M3
S14	*sonnei*	+	+	+	+	+	−	−	+	−	−	−	M5
S15	*sonnei*	+	+	+	+	+	−	−	+	−	−	−	M5
S16	*sonnei*	+	−	−	+	+	−	−	+	−	−	−	M15
S17	*sonnei*	+	−	−	−	−	−	−	+	−	−	−	M16
S18	*sonnei*	+	+	+	+	+	−	−	+	−	−	−	M17
S19	*flexneri*	+	+	+	+	+	+	+	+	+	+	+	M2
S20	*flexneri*	+	+	+	+	+	+	+	+	+	+	+	M2
S21	*flexneri*	+	+	+	+	+	+	+	+	+	+	+	M2
S22	*flexneri*	+	+	+	+	+	+	+	+	+	+	+	M2
S23	*flexneri*	+	+	+	+	+	+	+	+	+	+	+	M2
S24	*flexneri*	+	+	−	−	−	−	−	−	−	−	+	M4
S25	*flexneri*	+	+	−	+	−	−	−	−	−	−	+	M4
S26	*flexneri*	+	−	−	−	−	−	−	−	−	−	+	M4
S27	*flexneri*	+	−	−	−	−	−	−	−	−	−	+	M6
S28	*flexneri*	+	−	−	−	−	−	−	−	−	−	+	M6
S29	*flexneri*	+	+	−	+	+	−	−	−	−	−	+	M7
S30	*flexneri*	+	−	−	−	−	−	−	−	−	−	+	M7
S31	*flexneri*	+	+	+	+	+	+	+	+	+	+	+	M8
S32	*flexneri*	+	+	+	+	+	+	+	+	+	+	+	M8
S33	*flexneri*	+	−	−	−	−	−	−	−	−	−	+	M9
S34	*flexneri*	+	−	−	−	−	−	−	−	−	−	+	M9
S35	*flexneri*	+	+	+	+	+	−	−	−	−	−	+	M10
S36	*flexneri*	+	+	−	−	−	−	−	−	−	−	+	M10
S37	*flexneri*	+	+	+	+	+	+	+	+	+	+	+	M11
S38	*flexneri*	+	+	+	+	+	−	−	−	−	−	+	M11
S39	*flexneri*	+	+	−	−	−	−	−	−	−	−	+	M18
S40	*flexneri*	+	+	+	−	+	−	−	−	−	−	+	M19
S41	*flexneri*	+	+	−	−	−	−	−	−	−	−	+	M20
S42	*flexneri*	+	+	+	+	+	−	−	+	+	+	+	M21
S43	*flexneri*	+	−	−	−	−	−	−	−	−	−	+	M22
S44	*flexneri*	+	−	−	−	−	−	−	−	−	−	+	M23
S45	*flexneri*	+	+	+	+	+	−	−	−	−	−	+	M24
S46	*flexneri*	+	+	+	−	−	−	−	−	−	−	+	M25
S47	*flexneri*	+	+	+	+	−	−	−	−	−	−	+	M26
S48	*flexneri*	+	+	−	+	−	+	+	+	+	+	+	M27
S49	*flexneri*	+	+	+	+	+	+	+	+	+	+	+	M28
S50	*boydii*	+	+	+	+	+	−	−	+	+	+	−	M12
S51	*boydii*	+	+	−	+	+	−	−	+	+	+	−	M12
S52	*boydii*	+	+	−	−	−	−	−	+	+	+	−	M13
S53	*boydii*	+	+	+	+	+	−	−	+	+	+	−	M13
S54	*boydii*	+	+	+	+	+	−	−	+	−	−	−	M14
S55	*boydii*	+	+	+	+	+	−	−	+	+	+	−	M14
S56	*boydii*	+	+	+	+	+	−	−	+	+	+	−	M29

that *sat* was present almost in all *S. flexneri* strains, but it was not found in any of *S. sonnei* strains [2]. However, the findings of the current study do not support the previous research by Ruiz et al. that showed this gene was present in *S. sonnei* isolates as well as *S. flexneri* [21].

MLVA is a simple method, with high repeatability and low cost, which can even be replaced by methods such as pulsed-field gel electrophoresis and multilocus sequence typing [9]. In this study, 56 isolates were divided into 29 MLVA types. AS expected *S. sonnei* isolates had less diversity than other *Shigella* spp. since *S. sonnei* has only one serotype. Although our selected loci exhibited less diversity than those of other studies [7–10], in our MLVA scheme, we used VNTR loci which can be easily estimate by eye on agarose gels. Hence, this method can be performed for epidemiological purposes in many laboratories with simple molecular biology equipment.

In current study some isolates with the same MLVA type had different virulence gene patterns. It might be as a result of many virulence genes located on the large *inv* plasmid which this extra-chromosomal element is prone to loss or deletions during growth at in-vitro conditions.

5. Conclusion

Diversity was observed in the prevalence of virulence genes that

can result in different severities of the disease. Virulence genes profile was correlated with serogroup. It seems that for different *Shigella* serogroups different virulence factors contribute in pathogenesis. Furthermore, the strains belonging to the same species had little diversity and this could be due to that the *Shigella* isolates were taken from a limited geographical area and a larger sample size is required to make a firm conclusion on the heterogeneity in *Shigella* isolates.

Conflict of interest

There are no conflicts of interest.

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