Distribution of genes encoding virulence factors of Shigella strains isolated from children with diarrhea in southwest Iran

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Abstract
This study aimed to investigate the distribution of virulence factor genes in Shigella strains isolated from children with diarrhea in the southwest, Iran. In this cross-sectional study, 1530 diarrheal stool specimens were collected from children aged under 15 years. The Shigella strains were identified by biochemical methods and polymerase chain reaction (PCR). Subsequently, all Shigella isolates were evaluated by PCR for the presence of nine virulence genes ipaH (responsible for dissemination from cell to cell), iai (responsible for epithelial cell penetration), sat (displays cytopathic activity in several intestinal cell lines), sigA (toxic to epithelial cells), pic (associated with colonization), pet (cytotoxic for epithelial cells), sepA (contribute to intestinal inflammation and colonization), virF and invE (regulatory proteins). A total of 91 isolates including 47 S. flexneri, 36 S. sonnei, and 8 S. boydii were identified. All isolates were positive for the ipaH gene. The other genes include iai, virF, invE, sigA, sat, sepA, pic and pet found in 84.6%, 72.5%, 68.1%, 62.6%, 51.6%, 39.5%, 37.3% and 28.5% of the isolates, respectively. The results showed a high distribution of virulence genes among Shigella strains in our region. It seems that for different Shigella spp. different virulence factors contribute to pathogenesis. The current study provided insights into some baseline information about the distribution of some virulence genes of Shigella isolates in Southwest Iran.

Keywords Shigella · Diarrhea · Virulence factor · Polymerase chain reaction

Introduction
Shigellosis is an acute gastroenteritis infection caused by Shigella species. It is one of the most common causes of morbidity and mortality, especially in children in developing countries [1]. Shigellosis is characterized by fever, abdominal cramps, mucoid stool, and bloody diarrhea [2]. The severity of Shigellosis is depended on the various virulence factors located in the chromosome or large virulent inv plasmids [3]. The invasion plasmid antigen H (ipaH) genes are present in multiple copies located on both a plasmid and the chromosome are responsible for dissemination in epithelial cells. The invasion-associated locus (ial) gene, which is located on a plasmid, is involved in cell penetration by Shigella [4]. Two regulatory proteins, virF and invE are involved in the control the transcription of invasion genes [5]. The serin autotransporters proteins of Enterobacteriaceae (SPATEs) are present in Shigella strains. The SPATEs family has been divided into two classes. Class I SPATEs members include the plasmid-encoded toxin gene (pet), secreted autotransporter toxin gene (sat), and Shigella IgA-like protease homolog gene (sigA), which are cytotoxic for epithelial cells. The protease involved in colonization of the intestine (pic) and the extracellular protein Shigella A (sepA) are members of class 2, which contribute to intestinal inflammation and colonization [6]. Shigella isolates harboring virulence genes can induce extensive mucosal damages and inflammation in intestinal cells, especially when these strains encode more than one of the mentioned virulence factors.

Despite many reports about the prevalence and antimicrobial resistance of Shigella from different parts of the world and Iran, investigations about the prevalence of virulence factors in Shigella spp. are still rare worldwide. Therefore, we investigated the distribution of genes encoding virulence factors of Shigella strains isolated from children with diarrhea in southwest Iran.
Materials and methods

Bacterial isolation

In this study, 1530 stool samples were collected from patients with diarrhea referring to the teaching hospitals in Ahvaz and Abadan, southwest of Iran, for 18 months from April 2017 to September 2018. Our study included patients with a history of fever, abdominal cramps, vomiting, watery and bloody diarrhea. Patients who had taken antibiotics during the 72 h prior to the time of sampling were excluded. Diarrhea was defined as having at least three loose or liquid stools without blood and mucus per day. Dysentery was characterized by inflammation of the intestine, frequent excretion (10–13 times a day) of slimy stools that often contain blood, pus, and mucus. All isolates were identified as *Shigella* strain by standard microbiological and biochemical tests as previously described [7]. Pure culture of bacterial isolates was kept in a micro-tube containing Tryptic Soy Broth (TSB) (Merck, Germany) with 30% glycerol at −70 °C until molecular analysis. All *Shigella* strains were subsequently confirmed by polymerase chain reaction (PCR) assay [7]. Oligonucleotides and genes used for the identification of *Shigella* species were described in Table 1.

Molecular identification of *Shigella* sp.

*Shigella* strains were evaluated for the prevalence of *rfpB*, *wbgZ*, *rfc*, and *hypothetical protein* genes. DNA extraction of isolates was performed by the boiling method [8]. PCR amplification was performed according to the previous study [7]. The sequences of species-specific primer are listed in Table 1. Amplification reaction was carried out in Thermal Cycler Gradient (Eppendorf, Germany). The PCR conditions were: 94 °C for 5 min, 35 cycles at 94 °C for 1 min, annealing (variable) for 1 min, 72 °C for 1 min, and a final step of 72 °C for 7 min. The PCR amplification products were electrophoresed on a 1.5% agarose gel stained with ethidium bromide and visualized in the gel documentation ( Protein Simple, CA, USA). We used *S. sonnei* (ATCC25931), *S. flexneri* (ATCC29903), *S. boydii* (ATCC8700), and *S. dysenteriae* (ATCC13313) as the positive controls, and PCR mixture without DNA template as the negative control.

PCR amplification of virulence genes

We performed PCR assays that targeted nine different virulence gene factors (*ipaH*, *ial*, *virF*, *invE*, *pet*, *sat*, *sigA*, *pic*, and *sepA*) using the primers described in Table 2. The total volume of the PCR mixture was 25 μL, containing 0.5 μL of DNA template, 1× PCR buffer, 2.5 Mm of MgCl2, 0.5 μL each virulence gene primer, 0.5 μL Taq DNA polymerase. The PCR conditions for the amplification of virulence genes included an initial denaturation at 94 °C for 60 s, 35 cycles of denaturation at 94 °C for 60 s, annealing (Table 2) for 60 s, and extension at 72 °C for 60 s, as well as a final extension at 72 °C for 7 min. The amplicons were separated in 1.5% agarose gel. Positive controls for each of the virulence gene were as follows: *S. flexneri* ATCC 12122 for *ipaH*, *S. flexneri* 2a strain 2457T for *sat*, *sepA* and *sigA*, enteroinvasive *Escherichia coli* (EIEC) strain 44825 for *invE*, *S. flexneri* ATCC 12122 for *virF*, enteroaggregative *Escherichia coli* (EAEC) strain 042 for *pet* and *pic*, EIEC strain 43893 for *ial*.

Statistical analysis

The descriptive statistic tests were performed in SPSS version 22. Correlation between the occurrence of virulence factor genes and multidrug resistance was calculated using Fisher’s exact test. A (*P* value <0.05) was considered statistically significant.
Results

Bacterial isolation

During our epidemiological survey, of 1530 fecal samples, 91 cases (5.9%) were positive for *Shigella* species. Of these, 54 cases (59.3%) isolated from children <5 years of age, 22 cases (24.1%) isolated from children in the age group 6–10 years, and 15 cases (16.5%) isolated from children in the age group 11–15 years. On presentation at the hospital, 1271 (83.1%) patients complained of abdominal pain, 932 (60.9%) patients had a history of fever, and 482 (31.5%) had a history of vomiting. Moreover, 1193 (77.9%) patients had suffered from watery diarrhea and 324 (21.2%) from dysentery. Of 91 confirmed *Shigella* strains, 40 (44.0%) and 51 (56.0%) were obtained from female and male patients, respectively. We found no significant differences in *Shigella* infection between female and male patients (*P* > 0.05).

According to species-specific PCR results, *S. flexneri* was the predominant species in all age groups (47 isolates; 51.6%), followed by *S. sonnei* (36 isolates; 39.6%) and *S. boydii* (8 isolates; 8.8%). The most prevalent *Shigella* species in developed and developing countries are *S. sonnei* and *S. flexneri*, respectively. *Shigella dysenteriae* were not isolated from any of the tested samples.

Frequency of virulence factors genes

All isolates were positive for the *ipaH* genes (responsible for dissemination from cell to cell). The detection of the virulence genes from 91 *Shigella* isolates 84.6% (*n* = 77) of isolates were positive for *ial* (responsible for epithelial cell penetration), whereas 72.5% (*n* = 66) and 68.1% (*n* = 62) were positive for the *virF* and *invE* genes (regulatory proteins). The data revealed that *sigA* (toxic to epithelial cells), *sat* (displays cytopathic activity in several intestinal cell lines), *sepA* (contribute to intestinal inflammation and colonization), *pic* (associated with colonization) and *pet* (cytotoxic for epithelial cells) genes were present in 62.6% (*n* = 57), 51.6% (*n* = 47), 39.5% (*n* = 36), 37.3% (*n* = 34) and 28.5% (*n* = 26) of the isolates, respectively. All *Shigella* isolates harbored at least one SPATE gene. All *S. flexneri* isolates harbored *sat* gene (*P* < 0.05), but all the *S. sonnei* and *S. boydii* isolates were negative for this gene. The prevalence of these genes among the *Shigella* spp. is shown in Table 3.
Discussion

Shigellosis is an acute invasive enteric infection. It is one of the important causes of morbidity and mortality in developing countries, especially among children younger than 5 years [9, 10]. In the current study, a total of 91 Shigella spp. were isolated from diarrheal specimens of children aged under 15 years in Ahvaz, Abadan, southwest Iran. The most frequent age group in our study was age 1–5 years (P < 0.05), which was consistent with previous studies [4, 11]. Children in this age group due to poor personal hygiene and lack of previous exposure and lower immune responses are more prone to shigellosis [9]. Epidemiological studies have shown that the distribution of four Shigella species is varied in different geographical areas, and S. flexneri was recognized as the major bacterial causative for diarrhea in many developing countries [3]. However, S. sonnei is the most commonly isolated species in many developed countries [12, 13]. In the current study, the prevalence rate of Shigellosis in diarrheal children was 5.9% and S. flexneri (51.6%) was the predominant species among Shigella species in our region, which is comparable with previous studies [3, 11, 14]. We also did not succeed in isolating S. dysenteriae, which was in accordance with some other studies conducted in Iran [15–17]. However, other researchers in our region have reported the S. dysenteriae as the less common species [18, 19]. S. dysenteriae is associated mainly with outbreaks and epidemics which often found in South Asia and sub-Saharan Africa [20].

Shigella invades epithelial cells of the colon and kills them. The genes related to the invasion of Shigella are located on the chromosome and plasmids [21]. Several virulence genes associated with Shigella pathogenesis have been identified. Identifying the virulence-associated genes in Shigella strains is useful to better understand its pathogenicity. In this study, we investigated the prevalence of nine virulence genes in Shigella isolates. The ipaH gene used as a diagnostic marker for Shigella detection because this gene is found both on the chromosome and plasmids. In our study, the ipaH gene was positive for all the isolates, whereas the ial gene was detected in 84.6% that is consistent with other studies [14, 22, 23]. It seems that because the ial gene is only located on the Inv plasmid, it is prone to be lost or deleted. In the current study, the prevalence of the invE and virF genes was 64.8% and 69.2%, respectively. These results are consistent with a previous study [23]. Since virF and invE genes are located on the plasmid, they are susceptible to elimination.

Other genes that possess virulence activities are SPATE genes, which encode for secreted autotransporters in gram-negative bacteria. There is little information about the distribution of SPATE genes in Shigella isolates. In the present study, the SigA gene has the highest frequency among class 1 SPATE genes, which is consistent with previous studies [5, 23]. Our results implied that sigA may play an important role in the pathogenesis of Shigella. In our study, the sat gene was found in 100% of S. flexneri strains. In agreement with our finding Hosseini Nave et al. and Roy et al. showed that sat were present almost in all S. flexneri strains, but it was not found in any of S. sonnei and S. boydii strains [23, 24]. This gene can cause damage to the intestinal epithelial cells and therefore plays a role in pathogenesis [25].

The pic and sepA genes were detected in 39.5% and 37.3% of isolates, respectively. Our results matched with the previous study from Kerman, Iran [23]. The sepA gene is located in the virulence plasmid, and close to the pic gene located on the chromosome. Due to storage or subculturing the plasmid might have been lost together with the sepA gene. These genes can cause fluid accumulation, and to the successful colonization of Shigella isolates in intestinal cells [26]. In our study, S. boydii isolates had high rates of class 2 SPATE genes (sepA and pic) that is in agreement with the previous study [23].

Conclusions

In the present study, we provided some baseline information about the distribution of some virulence genes in clinical strains of Shigella spp. in southwest Iran. These results showed a high distribution of virulence genes among Shigella strains in our region. It seems that for different Shigella spp. different virulence factors contribute to pathogenesis. It was found that the profile of these virulence genes correlated with serotype, period, and region.

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Authors contribution MA developed the original idea and the protocol, performed the experiments, KA was involved in data collection, wrote the preliminary draft and analyzed the data, NJ revised the manuscript.

Data availability All data generated or analyzed during this study are included in this published article.

Compliance with ethical standards

Conflict of interest None to declare.

Ethical approval The study was approved by the Research Ethics Committee of the Abadan Faculty of Medical Sciences (Ethical code: IR. ABADANUMS.REC1398.073), Abadan, Iran. Written informed consent was obtained from the guardians of the study participants. All data was anonymized before analyzing and reporting.

Data availability The data that support the findings of this study are available from the corresponding author upon reasonable request.
consent was obtained from all the children’s parents. Our manuscript has been already submitted to a pre-print platform (Research Square), providing the DOI (https://doi.org/10.21203/rs.3.rs-29988/v1) and licensing information.

References

1. Yang H, Chen G, Zhu Y, Liu Y, Cheng J, Hu L, Ye Y, Li J (2013) Surveillance of antimicrobial susceptibility patterns among Shigella species isolated in China during the 7-year period of 2005–2011. Ann Lab Med 33(2):111–115. https://doi.org/10.3343/alm.2013.33.2.111

2. Zhang J, Qian L, Wu Y, Cai X, Li X, Cheng X, Qu D (2013) Deletion of pic results in decreased virulence for a clinical isolate of Shigella flexneri 2a from China. BMC Microbiol 13(1). https://doi.org/10.1186/1471-2180-13-31

3. Shen Y, Qian H, Gong J, Deng F, Dong C, Zhou L, Guo H (2013) High prevalence of antibiotic resistance and molecular characterization of integrons among Shigella isolates in Eastern China. Antimicrob Agents Chemother 57(3):1549–1551. https://doi.org/10.1128/AAC.02102-12

4. Sousa MÁB, Mendes EN, Collares GB, Pêret-Filho LA, Penna PJ, Magalhães PP (2013) Distribution of genes encoding virulence factors in Shigella flexneri serotypes 1b clinical isolates from eastern Ma P (2017) Low distribution of genes encoding virulence factors and antimicrobial resistance patterns in Paediatric Medical Center, Ahvaz, Iran. J Microbiol 9(5):277

5. Fan W, Qian H, Wang Sh, Ying C, Zhang X, Cheng S, Gu B, Ma P (2017) Low distribution of genes encoding virulence factors in Shigella flexneri serotypes 1b clinical isolates from eastern Chinese populations. Gut Pathog 9(1):76. https://doi.org/10.1186/s13099-017-0222-9

6. Farajzadeh-Shiekh A, Savari M, Ahmadi K, Nave HH, Shahin M, Afzali M (2020) Distribution of genes encoding virulence factors and the genetic diversity of enteroinvasive Escherichia coli (EIEC) isolates from patients with diarrhea in Ahvaz, Iran. Infect Drug Resist 13:119. https://doi.org/10.2147/IDR.S235009

7. Jomehzadeh N, Afzali M, Ahmadi K, Salmanzadeh S, Mehr EJ (2020) Antimicrobial resistance patterns and prevalence of integrons in Shigella species isolated from children with diarrhea in south-west Iran. Asian Pac J Trop Med 13:1–5. https://doi.org/10.4103/1995-7645.280237

8. Ranjar B, Memariani M (2015) Multilocus variable-number tandem-repeat analysis for genotyping of Shigella sonnei strains isolated from pediatric patients. Gastroenterol Hepatol Bed Bench 8(3):225. https://doi.org/10.22037/gfthb.v8i3.651

9. Casabonne C, González A, Aquili V, Balagué C (2016) Prevalence and virulence genes of Shigella spp. isolated from patients with diarrhea in Rosario, Argentina. Jpn J Infect Dis 69(6):477–481. https://doi.org/10.7883/yoken.JJID.2015.459

10. Muthuirulandi Sethuvel D, Devanga Ragupathi N, Anandan S, Veeraraghavan B (2017) Update on: Shigella new serogroups/serotypes and their antimicrobial resistance. Lett Appl Microbiol 64(1):8–18. https://doi.org/10.1111/lam.12690

11. Jomezadeh N, Babamoradi S, Kalantar E, Javaherizadeh H (2014) Isolation and antibiotic susceptibility of Shigella species from stool samples among hospitalized children in Abadan, Iran. Gastroenterol Hepatol Bed Bench 7(4):218–223. https://doi.org/10.22037/gfthb.v7i4.578

12. Gupta A, Poleyak CS, Bishop RD, Sobel J, Mintz ED (2004) Laboratory-confirmed shigellosis in the United States, 1989–2002: epidemiologic trends and patterns. Clin Infect Dis 38(10):1372–1377. https://doi.org/10.1086/386326

13. Nógrády N, Király M, Borbás K, Tóth Á, Pászti J, Tóth I (2013) Antimicrobial resistance and genetic characteristics of integron-carrier Shigella isolated in Hungary (1998–2008). J Med Microbiol 62(10):1545–1551. https://doi.org/10.1099/jmm.0.058917-0

14. Cruz CB, Souza MC, Serra PT, Santos I, Balieiro A, Pieri FA, Nogueira PA, Orlandi PP (2014, 2015) Virulence factors associated with pediatric shigellosis in Brazilian Amazon. Biomed Res Int. https://doi.org/10.1155/2015/4539697

15. Eftekhar N, Bakhshi B, PourniapageTitle MR, Zarbakshh B, Rahbar M, Hajia M, Ghazvini K (2013) Genetic diversity of Shigella spp. and their integron content. Foodborne Pathog Dis 10(3):237–242. https://doi.org/10.1089/fpd.2012.1250

16. Moosavian M, Ghaderiyan GH, Moghaddam M, Navidifar T (2019) First investigation of the presence of SPATE genes in Shigella species isolated from children with diarrhea infection in Ahvaz, southwest Iran. Infect Drug Resist 12:795. https://doi.org/10.2147/IDR.S194740

17. Dallal MM, Motalebi S, Asl HM, Yazdi MK, Forushani AR (2020) Antimicrobial investigation on the multi-state outbreak of salmonellosis and shigellosis in Iran. Med J Islam Republic Iran 34:49. https://doi.org/10.34171/mjri.34.49

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