Distribution of Genes Encoding Virulence Factors of Shigella Strains Isolated from Children with Diarrhea in Southwest Iran.

maryam Afzali

infectious tropica disease research center health research inestitue ahvaz jundishapur university of medical sciences ahvaz,Iran

nabi jomehzadeh

Abadan faculty of medical sciences, Abadan, Iran.

khadijeh ahmadi (kh.ahmadi53@gmail.com)

Ahvaz Jondishapour University of Medical Sciences Abadan Faculty of Nursing and Midwifery Abadan

https://orcid.org/0000-0003-0012-2393

Research

Keywords: Shigella, diarrhea, virulence factor, PCR

Posted Date: May 28th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-29988/v1

Abstract

Background: This study aimed to investigate the distribution of virulence factor genes in *Shigella* strains isolated from children with diarrhea in the southwest, Iran.

Methods: In this cross-sectional study, a total of 91 *Shigella* spp. were isolated from diarrhea specimens of 1 530 children aged under 15 years in Ahvaz and Abadan, southwest Iran. The *Shigella* strains were identified by biochemical methods. Subsequently, *Shigella* spp. were identified by polymerase chain reaction (PCR).All *Shigella* spp evaluated by PCR for the presence of 9 virulence genes (*ipaH, ial, virF, invE, sat, sigA, pic, pet*, and *sepA*).

Results: 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 *ial, 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.

Conclusion: Our 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.

Background

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 a both plasmid and the chromosome is responsible for dissemination in epithelial cells [4]. 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 control the transcriptional of invasion genes [5]. The serin autotransporters of proteins Enterobacteriaceae (SPATEs) are present in *Shigella* strains. The SPATEs family has been divided into two classes. Class 1 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 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 Isolates

In this cross-sectional 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. Patients with a history of fever, abdominal cramps, vomiting, watery and bloody diarrhea were included in our study. All isolates were identified by standard biochemical tests [7]. All isolates that confirmed as *Shigella* spp. were stored in Tryptic Soy Broth (TSB) (Merck, Germany), containing glycerol (30%) at − 70 °C.

Molecular Confirmation of Shigella Strains

All *Shigella* strains were evaluated for the presence of the *ipaH* gene by PCR. DNA extraction of isolates was performed by the boiling method previously described [8]. The sequences of primers and annealing temperatures of the *ipaH* gene are shown in Table 1. PCR conditions were examined according to the protocol as described previously [6]. *S. flexnery* ATCC 12122 was used as a positive PCR control for the *ipaH* gene.

Table 1
Primers Used to Identify Virulence-Associated Genes of *Shigella* spp.

Target gene	Primer sequences	Product size (bp)	Annealing Temperature (°C)	References
ipaH	F-GAAAACCCTCCTGGTCCATCAGG R-GCCGGTCAGCCACCCTCTGAGAGTAC	619	58	6
sat	F-TCAGAAGCTCAGCGAATCATTG R-CCATTATCACCAGTAAAACGCACC	930	59	6
ial	F- CTGGATGGTATGGTGAGG R- GGAGGCCAACAATTATTTCC	320	59	6
virF	F- TCAGGCAATGAAACTTTGAC R- GGGCTTGATATTCCGATAAGTC	618	58	6
invE	F- CGATAGATGGCGAGAAATTATATCCCG R- CGATCAAGAATCCCTAACAGAAGAATCA	766	59	6
sigA	F- CCGACTTCTCACTTTCTCCCG R- CCATCCAGCTGCATAGTGTTTG	430	58	6
pet	F-GGC ACA GAA TAA AGG GGT GTT T; R-CCT CTT GTT TCC ACG ACA TAC	302	58	9
pic	F- ACTGGATCTTAAGGCTCAGGAT R- GACTTAATGTCACTGTTCAGCG	572	58	6
Sep A	F- GCAGTGGAAATATGATGCGGC R- TTGTTCAGATCGGAGAAGAACG	794	58	6

PCR assay for molecular identification of Shigella species

PCR was carried out on all *Shigella* strains to evaluate the prevalence of the *rfc, wbgZ, rfpB, hypothetical protein* genes. PCR was performed according to a previous study [7].

The specific primers and annealing temperatures of *Shigella* spp. genes are listed in Table 2. *S. sonnei* ATCC25931, *S. flexneri* ATCC29903, *S. boydii* ATCC8700, and *S. dysenteriae* ATCC13313 were used as a positive control.

Table 2
Primers used for identifying each *Shigella* spp.

Target gene	Primer sequences	Product size (bp)	Annealing Temperature (°C)	References
Hypothetical protein	F: GAGCACGGAAACAGAGAGCGCC R: GGTGCGTTCTTCCGGTGTTCTG	240	63	7
wbgZ	F: TCTGAATATGCCCTCTAC R: GACAGAGCCCGAAGAACCG	430	60	7
Rfc	F: TTTATGGCTTCTTTGTCG R: CTGCGTGATCCGACCATG	537	60	7
rfpB	F: TCTCAATAATAGGGAACACAGC R: CATAAATCACCAGCAAGGTT	211	59	7

Amplification of virulence factors genes

PCR was performed for all *Shigella* strains to evaluate the prevalence of the *ial, virF, invE, pet, sat, sigA, pic*, and *sepA* [6, 9]. The sequences of primers and annealing temperatures are shown in Table 1. The total volume of the PCR mixture was 25 µL, containing 0.5 µL of DNA template, 1X 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 seconds, 35 cycles of denaturation at 94 °C for 60 seconds, annealing (variable) for 60 seconds, and extension at 72 °C for 60 seconds, as well as a final extension at 72 °C for 7 min. After performing PCR, the size of each locus was easily determined on 1.5% gel agarose. Positive controls for each gene were as follows: *S. flexnery* ATCC 12122 for *virF*, EIEC strain 44825 for *invE*, EIEC strain 43893 for *ial, S. flexneri* 2a strain 2457T is for *sat, sepA* and *sigA*, EAEC strain 042 is the positive control for *pet* and *pic.*

Statistical analysis

The descriptive statistic tests were performed in SPSS version 16.00. 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

In this study, 5.9% (*n* = 91) of 1 530 stool samples were positive for *Shigella* spp. Of the 1 530 patients, 47.1% (*n* = 720) and 52.9% (*n* = 810) were males and females, respectively. The patients have had various clinical symptoms, including vomiting 31.5%) *n* = 482), fever 60.9%) *n* = 932), abdominal pain 83.1%)*n* = 1 271), watery diarrhea 77.9%) *n* = 1 193), and dysentery 21.2%) *n* = 324).

From a total of 91 *Shigella* spp., 56.0% (*n* = 51) and 44.0% (*n* = 40) were isolated from male and female patients, respectively. No significant differences in *Shigella* infection were found between male and female patient ($P > 0.05$). Distribution of *Shigella* spp. isolated from the 91 diarrheic children according to age were: 1–5 years, 59.3% (*n* = 54); 6–10 years, 24.1% (*n* = 22); 11–15 years, 16.5% (*n* = 15). Bloody diarrhea, mucoid diarrhea and watery diarrhea were found in 14.3% (n = 13), 7.7% (n = 7), 62.6% (n = 57) patients, respectively. Of these 91 positive samples, 51.6% (*n* = 47), 39.6% (*n* = 36) and 8.8% (*n* = 8) samples were identified as *S. flexneri, S. sonnei*, and *S. boydii* respectively. Distribution of *Shigella* strains according to age group and species are shown in Table 3.

Table 3
Distribution of Shigella spp. by age [n (%)].

Age group, year	*S. flexneri*	*S. sonnei*	*S. boydii*
1–5	26 (28.6%)	22 (24.2%)	6 (6.5%)
6–10	13 (27.6%)	7 (19.4%)	2 (2.1%)
11–15	8 (17.02%)	7 (19.4%)	0 (0%)
Total	47 (51.6%)	36(39.6%)	8(8.8%)

Frequency of virulence factors genes

All isolates were positive for the *ipaH* genes. The detection of the virulence genes from 91 *Shigella* isolates 84.6% (n = 77) of isolates were positive for *ial*, whereas 72.5% (n = 66) and 68.1% (n = 62) were positive for the *virF* and *invE* genes. The data revealed that *sigA, sat, sepA, pic* and *pet* genes found 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 4.

virulence genes	*S. flexneri*	*S. sonnei*	*S. boydii*	Total
pic	18(38.2%)	10(27.7%)	6(75%)	34(37.3%)
sepA	18(38.2%)	12(33.3%)	6(75%)	36(39.5%)
pet	11(23.4%)	15(41.6%)	0%	26(28.5%)
sigA	31(65.9%)	19(52.7%)	7(87.5%)	57(62.6%)
sat	47 (100%)	0 (0%)	0 (0%)	47 (51.6%)
ial	38(80.8%)	31(86.1%)	8(100%)	77(84.6%)
virF	26(55.3%)	29(80.05%)	7(87.5%)	62(68.1%)
invE	28(59.9%)	31(86.1%)	7(87.5%)	66(72.5%)

Table 4
Distribution of the virulence genes according to *Shigella* species.

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 [10, 11]. In the current study, a total of 91 *Shigella* spp. were isolated from diarrhea 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, 12]. Children in this age group due to poor poor personal hygiene and lack of previous exposure and lower immune responses are more prone to shigellosis [10]. *Shigella* invades epithelial cells of the colon and kill them. The genes related to the invasion of *Shigella* are located on chromosome and plasmid [13]. Several virulence genes associated with *Shigella* pathogenesis have been identified. Identifying the virulence-associated genes in *Shigella* strains would be useful to better understand its pathogenicity. In this study, we investigated the prevalence of 9 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 the plasmid. 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 studies that agree with other studies [14–16]. It seems that because of the *ial* gene is only located on the plasmid, it is prone to lose or deletion. In the current study, the prevalence of the *invE* and *virF* genes was 64.8% and 69.2%, respectively. These results are consistent with the previous study [16]. 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 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 [5,16]. 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 [16, 17]. This gene can cause damage to the intestinal epithelial cells and therefore plays a role in pathogenesis [9].

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 [16]. The *sepA* gene located on the virulence plasmid, and by 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 lade to fluid accumulation, the successful colonization of *Shigella* isolates in intestinal cells [18]. 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 [16].

Conclusion

In the present study, we provided some baseline information about the distribution of some virulence genes in clinical strains of *Shigella* spp. Southwest Iran. Our 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.

Declarations

Ethics Statement

The study was approved by the Research Ethics Committee of the Abadan School of medical sciences (Ethical code: IR.ABADANUMS.REC1398.073), Abadan, Iran. Written informed consent was obtained from all the children's parents.

Acknowledgments

This work was supported by the Vice-Chancellor for Research grant (Grant No.98U-603) of Abadan University of Medical Science. The authors of this manuscript would like to acknowledge the laboratory and nursing personnel of children and infants ward in teaching hospitals in Ahvaz, Abadan and khorramshahr, who assisted to collect the clinical specimens.

Authors' Contribution

Maryam Afzali developed the original idea and the protocol, performed the experiments, Khadijeh Ahmadi was involved in data collection, wrote the preliminary draft and analyzed the data, Nabi Jomehzadeh revised the manuscript.

Disclosure

The authors report no conflicts of interest in this work.

Availability of data and materials

All data generated or analysed during this study are included in this published article.

References

1. Yang H, Chen G, Zhu Y, et al. Surveillance of antimicrobial susceptibility patterns among Shigella species isolated in China during the 7-year period of 2005-2011. *Annals of laboratory medicine.* 2013;33(2):111-115.
2. Zhang J, Qian L, Wu Y, et al. Deletion of pic results in decreased virulence for a clinical isolate of Shigella flexneri 2a from China. *BMC microbiology.* 2013;13(1):31.
3. Shen Y, Qian H, Gong J, et al. High prevalence of antibiotic resistance and molecular characterization of integrons among Shigella isolates in Eastern China. *Antimicrob Agents Chemother.* 2013;57(3):1549-1551.
4. Sousa MÂB, Mendes EN, Collares GB, Péret-Filho LA, Penna FJ, Magalhães PP. Shigella in Brazilian children with acute diarrhoea: prevalence, antimicrobial resistance and virulence genes. *Mem Inst Oswaldo Cruz.* 2013;108(1):30-35.
5. Fan W, Qian H, Shang W, et al. Low distribution of genes encoding virulence factors in Shigella flexneri serotypes 1b clinical isolates from eastern Chinese populations. *Gut Pathog.* 2017;9(1):76.
6. Farajzadeh-Sheikh A, Savari M, Ahmadi K, Nave HH, Shahin M, Afzali M. 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.* 2020;13:119.
7. Jomehzadeh N, Afzali M, Ahmadi K, Salmanzadeh S, Mehr FJ. Antimicrobial resistance patterns and prevalence of integrons in Shigella species isolated from children with diarrhea in southwest Iran. 2020.
8. Ranjbar R, Memariani M. Multilocus variable-number tandem-repeat analysis for genotyping of Shigella sonnei strains isolated from pediatric patients. *Gastroenterol Hepatol Bed Bench.* 2015;8(3):225.
9. Boisen N, Ruiz-Perez F, Scheutz F, Krogfelt KA, Nataro JP. High prevalence of serine protease autotransporter cytotoxins among strains of enteroaggregative Escherichia coli. *Am J Trop Med Hyg.* 2009;80(2):294-301.
10. Casabonne C, González A, Aquili V, Balagué C. Prevalence and virulence genes of Shigella spp. isolated from patients with diarrhea in Rosario, Argentina. *Jpn J Infect Dis.* 2016;69(6):477-481.

11. Muthuirulandi Sethuvel D, Devanga Ragupathi N, Anandan S, Veeraraghavan B. Update on: Shigella new serogroups/serotypes and their antimicrobial resistance. *Letters in applied microbiology.* 2017;64(1):8-18.
12. Jomezadeh N, Babamoradi S, Kalantar E, Javaherizadeh H. Isolation and antibiotic susceptibility of Shigella species from stool samples among hospitalized children in Abadan, Iran. Gastroenterol Hepatol Bed Bench. 2014;7(4):218-23.
13. Peng J, Yang J, Jin Q. The molecular evolutionary history of Shigella spp. and enteroinvasive Escherichia coli. *Infect Genet Evol.* 2009;9(1):147-152.
14. Yaghoubi S, Ranjbar R, Dallal MMS, Fard SY, Shirazi MH, Mahmoudi M. Profiling of virulence-associated factors in Shigella species isolated from acute pediatric diarrheal samples in Tehran, Iran. *Osong public health and research perspectives.* 2017;8(3):220.
15. Cruz CBNd, Souza MCSd, Serra PT, et al. Virulence factors associated with pediatric shigellosis in Brazilian Amazon. *Biomed Res Int.* 2014;2014.
16. Nave HH, Mansouri S, Emaneini M, Moradi M. Distribution of genes encoding virulence factors and molecular analysis of Shigella spp. isolated from patients with diarrhea in Kerman, Iran. *Microbial pathogenesis.* 2016;92:68-71.
17. Roy S, Thanasekaran K, Dutta Roy AR, Sehgal SC. Distribution of Shigella enterotoxin genes and secreted autotransporter toxin gene among diverse species and serotypes of Shigella isolated from Andaman Islands, India. *Trop Med Int Health.* 2006;11(11):1694-1698.
18. Dautin N. Serine protease autotransporters of enterobacteriaceae (SPATEs): biogenesis and function. *Toxins.* 2010;2(6):1179-1206.