Infection and Drug Resistance

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ORIGINAL RESEARCH

High Rates of Antimicrobial Resistance and Virulence Gene Distribution Among *Shigella* spp. Isolated from Pediatric Patients in Tehran, Iran

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Mohammadmahdi Karimi-Yazdi[1]
Zohreh Ghalavand[1]
Mahdi Shabani[2]
Hamidreza Houri[3]
Mehrzad Sadredinamin[1]
Marzieh Taheri[1]
Gita Eslami[1]

[1]Department of Microbiology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran; [2]Department of Immunology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran; [3]Foodborne and Waterborne Diseases Research Center, Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran

Background: *Shigella* continues to be important causes of acute pediatric diarrhea worldwide. *Shigella* produces numerous virulence factors involved in colonization and invasion into epithelial cells which eventually result in the disease. The present study was conducted to evaluate the prevalence of virulence genes and to investigate antibiotic resistance profiles among *Shigella* isolates obtained from pediatric patients in Iran.

Methods: A total of 141 *Shigella* isolates were collected between March 2017 and September 2018 from stool of children under 14 who were suspected to have shigellosis. *Shigella* isolates were identified using standard microbiological and serological tests and antimicrobial susceptibility testing was carried out via Kirby–Bauer disk diffusion method. In addition, the presence of seven virulence determinants including *ipaH, ipaB, ipaC, ipaD, ipgD, sen*, and *virA* were evaluated using PCR.

Results: *S. sonnei* (78.7%) was the most prevalent *shigella* spp. among children with shigellosis followed by *S. flexneri* (19.9%) and *S. boydii* (1.4%). Antimicrobial susceptibility testing revealed that most of the isolates were considered as multidrug-resistant (MDR) strains. Our findings also showed a high resistance rate against trimethoprim-sulfamethoxazole in *Shigella* isolates. The prevalence of *ipaH, ipaC, sen, ipaD, virA, ipaB*, and *ipgD* were 100%, 95.7%, 95.7%, 94.3%, 93.6%, 92.9%, and 80.8%, respectively.

Conclusion: The current study revealed that *S. sonnei* was the predominant species isolated from children with shigellosis in Iran. Our results also indicated a high distribution of type III secretion system effector protein-encoding genes and high multidrug-resistance among shigella spp. in Iran. Therefore, it is suggested that antimicrobial susceptibility testing be performed prior to antibiotic prescription.

Keywords: *Shigella*, virulence factors, type III secretion system, *ipaH*, *ipaB*, *virA*

Introduction

Shigellosis is an infectious disease ranging from mild diarrhea to severe dysentery. This infection has long been considered as a public health issue predominantly in developing countries due to overcrowding and poor sanitation.[1] According to the World Health Organization (WHO), approximately 191 million shigellosis cases were reported in 2010 worldwide.[2] As a matter of fact, infants and young children suffering from shigellosis have a more severe illness and a greater risk of death. Accordingly, it is estimated that approximately 28,000 deaths occur annually among children under five due to this acute bacterial infection.[3] Moreover, Rogawski et al recently reported that subclinical and non-diarrhoeal infection with *Shigella* has

Correspondence: Gita Eslami
Department of Microbiology, Shahid Beheshti University of Medical Sciences, Koodakyar St., Tabnak Blv., Yaman Av., Chamran Highway, P. Box: 19857-17443, Tehran, Iran
Tel +98 21 23872556
Email g_eslami@yahoo.com

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a substantial negative association with linear growth, which was sustained during the first two years of life in two-year-old children.[4] The major environmental factors contributing to shigellosis outbreaks are low hygienic and poor conditions, deficiency of drinking water, inadequate medical care, and malnutrition in the undeveloped regions.[5]

Shigellosis is caused by the members of *shigella* species including *Shigella dysenteriae, Shigella flexneri, Shigella sonnei*, and *Shigella boydii*.[6] *S. dysenteriae* and *S. flexneri* are principally responsible for epidemic and endemic shigellosis, respectively, with high transmission rates and significant mortality rates in low-income countries whereas *S. sonnei* and *S. boydii* generally cause a relatively mild self-limiting watery diarrhea.[7] The ability of *shigella* spp. to resist against high-level stomach acid and to produce several key virulence traits help the bacteria to survive in gastrointestinal tract, which makes it highly infectious with only 10–100 viable bacterial cells necessary to cause disease.[8] The predominant *shigella* strains producing shigellosis vary in different geographical locations. Accordingly, *S. flexneri* is isolated most frequently in resource-poor countries, while *S. dysenteriae* was traditionally responsible for large epidemics, and yet is now rarely identified. *S. boydii* frequently reported from India, and *S. sonnei*, which has historically been more frequently isolated in developed countries, are undergoing a significant and rapid expansion across developing regions in Latin America, Asia, and the Middle East.[7]

Species of the genus *Shigella* could express numerous virulence factors that contribute to the pathobiology of shigellosis. The *ipa* and *mxi-spa* loci code various mediators and virulence factors that play significant roles to facilitate cell invasion, intracellular diffusion, and cell death in the bowel epithelium.[7,9] The Ipa proteins have been demonstrated in the development of shigellosis and are acknowledged with respect to their associations with each other, their extracellular secretion, and target eukaryotic cells and their components.[3] The components of Ipa complex, e.g. IpaA, IpaB, IpaC, and IpaD, are required in the invasion process, which are injected upon contact with host cells using a type III secretion apparatus.[7]

Oral rehydration and antimicrobial therapy are recommended treatments for this illness; however, recent reports have determined that the rate of antimicrobial resistance for *Shigella* spp. is increasing.[10,11] The growing emergence of resistant clones of *Shigella* against antimicrobial agents used in clinical practice, including ciprofloxacin, azithromycin, and ceftriaxone, raises serious concerns for antimicrobial therapy of shigellosis.[12]

To the best of our knowledge, limited numbers of studies have been conducted to investigate antimicrobial resistance and virulence profiles among *Shigella* isolates from infected children in Iran. Therefore, the aim of the current study was to evaluate antibiotic resistance profiles and virulence-associated genes of *shigella* spp. isolated from children with shigellosis referred to Children's Medical Center in Tehran, Iran.

Materials and Methods

Bacterial Isolation and Serotyping

Diarrheal stool or rectal samples were obtained from children under 14 suspected to have shigellosis referred to Children's Medical Center in Tehran during 2018 and 2019. The samples were inoculated into MacConkey (MAC) (Mast, Bootle, UK) and xylose lysine deoxycholate media (XLD) (Mast, Bootle, UK) and then the media was incubated for 18 to 24 h at 37°C. Conventional biochemical tests, including triple sugar iron agar (TSI), ornithine decarboxylase (ODC), and O-Nitrophenyl-β-D-galactopyranoside (ONPG) were run for suspected colonies to identify *Shigella* isolates. In order to determine the *Shigella* serogroups, latex agglutination serotyping was carried out using the *Shigella* antisera purchased from Statens Serum Institut (Statens Serum Institut, Denmark) according to the manufacturer's protocol.

The *Shigella* isolates were kept in Trypticase Soy Broth (TSB) (Fluka, USA) supplemented with 15% glycerol and stored at −70°C for further experiments.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility pattern of all *Shigella* isolates was determined using Kirby-Bauer disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) 2018 recommendations.[13] We selected the antimicrobial agents based on CLSI 2018 and prescription of physicians in Iran. The antimicrobial agents were as follows: ampicillin (AMP), azithromycin (AZT), ciprofloxacin (CIP), nalidixic acid (NA), Trimethoprim-sulfamethoxazole (SXT), cefixime (CFM), cefotaxime (CTX), minocycline (MN), and levofloxacin (LEV) (Mast, Bootle, UK). In addition, the minimum inhibitory concentration (MIC) for the two most important antibiotics, including CIP and AZT was determined using microdilution broth method. *S. flexneri* ATCC 12022 was used

as a quality control in the antimicrobial susceptibility testing. In the present study, the multidrug-resistant (MDR) isolates were considered as those resistant to at least one member of three different classes of antibiotics.

Molecular Assay for Virulence Genes

Total genomic DNA was extracted using High Pure Isolation Kit (Roche, Mannheim, Germany) according to the manufacturer's instruction. The quality and quantity of extracted DNA was assessed via NanoDrup (Denovix, Wilmington, USA)

The presence of the virulence genes encoding T3SS effector proteins, including *ipaH, ipaB, ipaC, ipaD, ipgD, sen*, and *virA*, was investigated using polymerase chain reaction (PCR) using specific primers (Table S1 and Figures S1–S7). Each PCR reaction was performed in a total volume of 25 µL containing 12.5 µL of 2X master mix (Genet bio, Korea), 1µL (10 pM/µL) of each primer, 8.5 µL of distilled water, and 2 µL of DNA (10 ng) template. The cycling programs were preceded by 5 min at 95° C and consisted of 35 cycles of 94° C for 1 min, 1 min annealing at specific temperature for each primer set (Table S1), and 72° C for 1 min, followed by a final extension step at 72° C for 5 min. PCR amplicons were separated using 1.2% agarose gel and visualized by staining with safe stain (CinnaGen, Iran). *S. flexneri* ATCC 12022 was used as positive control.

Statistical Analysis

For data analysis, SPSS (version 20.0) was used (SPSS Inc., Chicago, IL, USA). The correlation between virulence-associated genes and antibiotic-resistant profiles was assessed running Chi-square exact test. *P-values*<0.05 were considered as statistically significant.

Results

Shigella Isolation and Speciation

In the present study, 59.6% (n=84) of the subjects were male and 40.4% (n=57) were female, ranging from 2 months to 14 years of age with the majority of the pediatric patients (56%, n=79) being under 5 years old. During an 18-month period, 141 isolates were identified as *Shigella* spp., revealing *S. sonnei* as the most prevalent species (78.7%, n=111), followed by *S. flexneri* (19.9%, n=28) and *S. boydii* (1.4%, n= 2). In the current study, no isolate was identified as *S. dysenteriae.*

Antimicrobial Resistance Profiles of *Shigella* spp

The majority (99.29%) of isolates exhibited the phenotypic resistance to at least one tested drug. A total of 101 (71.6%) *shigella* isolates were considered as MDR strains, including 86 (77.5%) out of 111 *S. sonnei*, 13 (46.4%) out of 28 *S. flexneri*, and 2 out of 2 *S. boydii* (100%). *S. sonnei* strains indicated a significantly higher prevalence of MDR compared with *S. flexneri* species ($\chi 2$, $P < 0.05$). The highest rate of resistance was against SXT (98.5%, 139/141) followed by NA (82.3%, 116/141), AMP (73.0%, 103/141), MN (65.9%, 93/141), CTX (59.6%, 84/141), CFM (57.4%, 81/141), and AZT (47.5%, 67/141) (Table 1). The lowest rate of resistance was observed for LEV (11.3%, 16/141) and CIP (5.7%, 8/141). The antibiotic-resistant profiles are shown in Table 2. The MIC_{50} of AZT and CIP for *S. sonnei* were 32 µg/mL

Table 1 Antimicrobial Resistance of *Shigella* Isolates from Pediatric Patients in Iran

Antibiotics	*S. sonnei* No. (%), *n* = 111	*S. flexneri* No. (%), *n* = 28	*p*-value	*S. boydii* No. (%), *n* = 2	Total of *Shigella* Strains No. (%), *n* =141
CTX	70 (63.1)	12 (42.8)	**0.004**	2 (100.0)	84 (59.6)
NA	104 (93.7)	10 (35.7)	**0.00001**	2 (100.0)	116 (82.3)
AMP	74 (66.7)	27 (96.4)	**0.00001**	2 (100.0)	103 (73.0)
SXT	111 (100.0)	26 (92.8)	0.03	2 (100.0)	139 (98.5)
LEV	12 (10.8)	4 (14.3)	0.521	0 (00.0)	16 (11.3)
CFM	69 (62.1)	13 (46.4)	**0.023**	2 (100.0)	84 (59.6)
MN	86 (77.5)	7 (25)	**0.00001**	0 (00.0)	93 (65.9)
CIP	3 (2.7)	5 (17.8)	**0.0005**	0 (00.0)	8 (5.7)
AZT	65 (58.6)	2 (7.1)	**0.00001**	0 (00.0)	67 (47.5)
MDR	86 (77.5)	13 (46.4)	**0.00001**	2 (100.0)	101 (71.6)

Note: Statistically significant values (*P*<0.05) are presented in bold.

Abbreviations: AMP, ampicillin; AZT, azithromycin; CFM, cefixime; CIP, ciprofloxacin; CTX, cefotaxime; LEV, levofloxacin; NA, nalidixic acid; MDR, multidrug resistance; MN, minocycline; SXT, trimethoprim-sulfamethoxazole.

Table 2 Antibiotic Resistance Profiles of *Shigella* spp. from Pediatric Diarrhetic Patients

Antibiotics Profiles	Age						Total No.
	2 Months to 5 Years			6 to14 Years			
	S. sonnei No. (%)	*S. flexneri* No. (%)	*S. boydii* No. (%)	*S. sonnei* No. (%)	*S. flexneri* No. (%)	*S. boydii* No. (%)	
CIP/AZT/CFM/LEV/MN/CTX/NA/AM/SXT	4	_	_	5	_	_	9
AZT/CFM/CTX/MN/NA/AM/SXT/LEV	20	_	_	21	_	_	41
AZT/CFM/MN/CTX/NA/AM/SXT	_	_	_	3	_	_	3
SXT/CTX/NA/MN/AM/	2	5	1	_	1	_	9
SXT/MN/NA	17	_	_	7	_	_	24

Abbreviations: AMP, ampicillin; AZT, azithromycin; CFM, cefixime; CIP, ciprofloxacin; CTX, cefotaxime; LEV, levofloxacin; NA, nalidixic acid; MDR, multidrug resistance; MN, minocycline; SXT, trimethoprim-sulfamethoxazole.

and 2 µg/mL, and MIC90 of AZT and CIP for *S. sonnei* were 64 µg/mL and 2 µg/mL, respectively. In addition, MIC_{50} and MIC_{90} of AZT and CIP for *S. flexneri* were 2 µg/mL. Figure 1 displays the MIC distribution of AZT against clinical isolates of *Shigella* recovered from pediatric patients. Moreover, *S. sonnei* isolates showed a significantly higher resistance to various antibiotics, including CTX, NA, CFM, MN, CIP and AZT, compared with that observed for the *S. flexneri* isolates ($\chi 2$ analysis, $P < 0.05$).

Virulence Genes Profiles

The *ipaH* was detected as the most frequent virulence factor, which was found in all *Shigella* strains, while the *ipgD* was considered as the less common virulence determinant detected in 114 (80.8%) strains (Table 3). Overall, 13 virulence profiles were detected in the present study. A total of 113 isolates harbored all seven virulence genes whereas 13 isolates were shown to possess all virulence genes except for *ipgD*. Moreover, seven unique profiles were discovered in seven samples. Additionally, 130 strains were identified to harbor at least 5 virulence genes. The virulence profiles of the isolated *Shigella* are shown in Table 4.

Our experiments showed that five different virulence profiles were present among 28 *S. flexneri* (Table 4). In addition, two *S. boydii* isolates possessed all seven virulence genes.

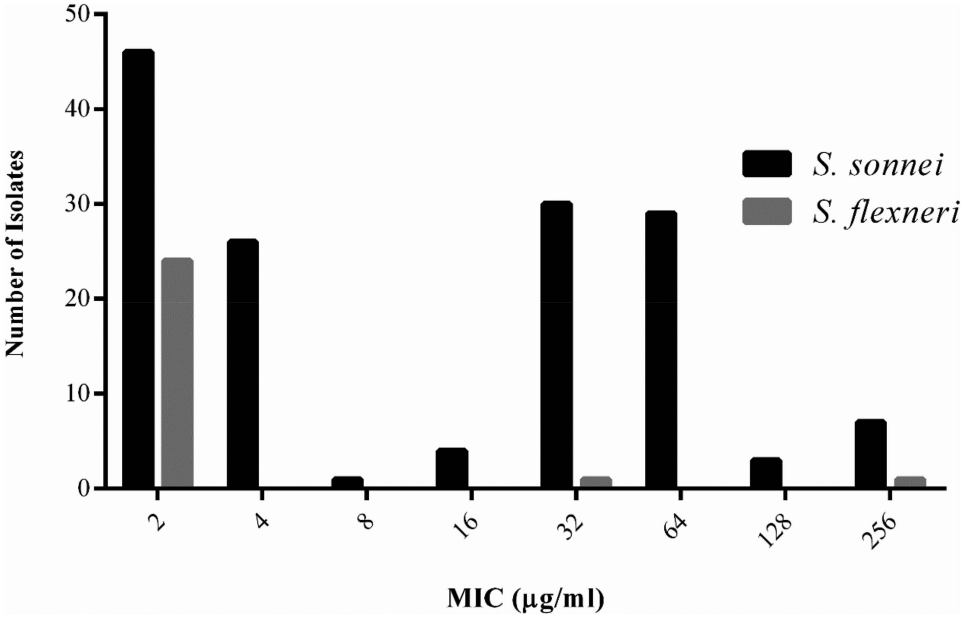

Figure 1 The minimum inhibitory concentration (MIC) of azithromycin for clinical isolates of *Shigella* recovered from pediatric patients in Tehran, Iran.

Table 3 Distribution of Virulence Factor Genes in 141 *Shigella* spp. Isolated from Pediatric Patients

Virulence Genes	S. sonnei No. (%), n = 111	S. flexneri No. (%), n = 28	S. boydii No. (%), n = 2	Total of Shigella Strains No. (%), n = 141
ipaH	111 (100.0)	28 (100.0)	2 (100.0)	141 (100.0)
ipaB	101 (90.1)	28 (100.0)	2 (100.0)	131 (92.9)
ipaC	105 (94.6)	28 (100.0)	2 (100.0)	135 (95.7)
ipaD	103 (92.8)	28 (100.0)	2 (100.0)	133 (94.3)
ipgD	88 (79.3)	24 (85.7)	2 (100.0)	114 (80.8)
Sen	107 (96.4)	26 (92.8)	2 (100.0)	135 (95.7)
virA	103 (92.8)	27 (96.4)	2 (100.0)	132 (93.6)

Discussion

Due to the limited information available about antibiotic-resistant and virulence factor profiles of *Shigella* in Iran, the present epidemiological study was conducted to characterize antimicrobial susceptibility and virulence-associated genes of *shigella* isolates from pediatric patients in Tehran, Iran. In the current study, the serotyping findings indicated that *S. sonnei* was the most predominant species, which is consistent with the findings reported by Yaghoubi et al[14] in Tehran, Iran. However, Moosavian et al in Ahvaz[15] and Hosseini et al in Kerman[16] previously reported that *S. flexneri* was the most common species. These variations could be due to the differences between these studies in terms of geographical and socio-economic conditions as well as the groups of individuals studied.

Our study revealed that 15% of *Shigella* isolates were non-susceptible to all of the tested antibiotics. A majority of *S. flexneri* (92.8%) and all of *S. sonnei* were resistant to SXT, a finding which was similar to those reported by other groups in Iran[15,17,18] and in other countries[19,20] and opposite to that reported by Moosavian et al in Iran.[15] Moreover, in the current study *S. flexneri* showed high resistance to AMP that is consistent with those previously reported by Iranian authors.[16,21,22] Moreover, a high-level resistance was observed against AMP, NA, MN, CTX, CFM, and AZT among *Shigella* isolates. Until a few years ago, AMP and SXT were the first choice for treatment of shigellosis and were used to improve the dysenteric syndrome; however, the prescription of these drugs is currently not suggested anymore due to development of resistant strains.[23]

Furthermore, in the current study, medium-level resistance was observed against AZT, that is consistent with that found by Barak et al in Ardebil, Iran[18] but different from some reports in other studies.[20,24] In our study, the high level of antibiotic susceptibility of *shigella* spp. to CIP is in agreement with those reported in some studies in Iran and other countries.[22,25,26] In shigellosis, depending on the severity of symptoms, the medical specialist may prescribe antibiotics to deal with it. CIP is a preferred treatment for adults, but because of potential toxicity for children below 18 years of age, it is relatively unsafe to use for this age group. Accordingly, AZT is currently the mainstay antibiotic for the treatment of shigellosis in children in Iran;[24] however, in the

Table 4 Virulence Determinant Profiles of the *Shigella* spp. Isolated from Pediatric Patients

Shigella spp.	ipaH	ipaB	ipaC	ipaD	ipgD	Sen	virA	No. (%)
S. sonnei, n = 111	+	+	+	+	+	+	+	86 (77.5)
	+	+	+	+	–	+	+	12 (10.8)
	+	+	–	+	–	+	+	2 (1.8)
	+	–	+	–	–	+	–	3 (2.7)
	+	–	–	–	–	+	+	2 (1.8)
	+	–	–	+	–	–	–	2 (1.8)
	+	+	+	–	+	–	–	1 (0.9)
	+	–	+	+	+	+	+	1 (0.9)
	+	–	+	–	–	+	+	1 (0.9)
	+	–	+	–	–	–	–	1 (0.9)
S. flexneri, n = 28	+	+	+	+	+	+	+	24 (85.7)
	+	–	+	+	–	+	+	1 (86.0)
	+	+	+	+	–	–	+	1 (3.5)
	+	+	+	+	–	+	+	1 (3.5)
	+	+	+	–	–	–	+	1 (3.5)
S. boydii, n = 2	+	+	+	+	+	+	+	2 (100.0)

present study, approximately half of *shigella* spp., especially *S. sonnei*, were found to be resistant to AZT. According to the WHO, AZT is recommended as a second-line treatment in patients who have severe cases of shigellosis.[27]

Unfortunately, high rates of MDR profiles were observed in the current study with 88 (62.4%) isolates resistant to at least four antibiotics. The misuse and unreasonable administration of antibiotics could induce the development of antibiotic resistance.[28] To reduce the emergence of resistance, antibiotics should be avoided for treating *Shigella* infections, except in patients with severe shigellosis or cases at risk for systemic infection.[29] In our study, nine (6.3%) *S. sonnei* were non-susceptible to all of the tested antibiotics. Our analysis also showed that the multidrug resistance was more frequent among *S. sonnei* as compared to *S. flexneri*. Several studies have described that the antimicrobial resistance levels are different between *Shigella* spp.[19,30] Thus, healthcare providers are suggested to order susceptibility testing so as to determine which antibiotics are preferred rather than pursuing the empirical treatment.[31]

Each *Shigella* species possess a large virulence plasmid and single circular chromosome. There are several lines of evidence indicating that type III secretion system (T3SS) as well as *ipa* and *ipg* genes are necessary for invasion to epithelial cells and development of shigellosis. These factors are encoded in the *ipa-mxi-spa* region by the virulence plasmid.[7] The *IpaH*, which encodes invasion plasmid antigen H (IpaH), is commonly used for molecular identification of *Shigella* spp. using PCR assays. There are 4 to 10 copies of *ipaH* in the chromosome of the bacterium as well as in invasion plasmids. In our study, all isolates harbored *ipaH*, which was described previously in other studies.[6,32,33] *ipaB, ipaC*, and *ipaD* are essential virulence factors to develop shigellosis, because they are necessary for invasion and intracellular survival. Furthermore, they regulate secretion and translocation of some other effector proteins and play a principle role in the intracellular actin polymerization and depolymerization.[32] Most of *Shigella* isolates in the current study possessed *ipaB, ipaC*, and *ipaD* and these genes were positive in all *S. flexneri* and *S. boydii* isolates. Another T3SS effector is invasion plasmid gene D (*ipgD*) that stops T cell migration during infection and then blocks the release of ATP to reduce inflammation. It seems that *ipgD* plays an important role in evading the immune system.[34] Our study also showed that *ipgD* was detected at the lowest rate among clinical isolates of *Shigella* spp., which was more frequent in *S. flexneri* than in *S. sonnei* isolates. This finding is in accordance with the results reported by Lluque et al.[32] The *sen* gene encodes enterotoxin 2 (ShET2) and is located in a 140 MDa plasmid in *Shigella* strains. It has long been described that ShET2 is responsible for water and electrolyte fluxes in early watery phase of shigellosis in intestine. Therefore, the symptoms of dehydration are closely associated with ShET2 activity.[35] In the study conducted by Yaghoubi et al, it was reported that only a few clinical isolates of *S. sonnei* possess ShET2, which is in contrast to our finding.[14] The *virA* in *shigella* spp. is located in the virulence plasmid and is involved in intracellular spreading and invasion of bacteria, and is responsible for the formation of entry region.[36] In many previous studies, it was reported that all *Shigella* isolates harbored *virA*[14–37] and, correspondingly, a high rate of *virA* was detected in our samples.

In addition, our results revealed that 130 (92.2%) cases had at least 5 T3SS effector protein-encoding genes, which suggests the ability of these *Shigella* isolates to induce inflammatory statement and diarrhea. However, it should be pointed out that the pathogenicity of *Shigella* spp. is also associated with the immunity of patients and the number of infected bacteria.[38]

Our study has also some limitations that should be taken into consideration prior to any generalization, including requirement of validation by appropriate techniques addressing virulence factor expression in clinical isolates of Shigella, which can provide a deeper insight into the actual function of the genes.

In the current study, we provided some information about the prevalence of *Shigella* spp. and also the distribution of some relevant virulence genes among pediatric patients in Tehran, Iran. Our results showed that *S. sonnei* was the predominant species among children. Most of the *Shigella* isolates have all of the seven T3SS effector genes. Among the tested antibiotics, CIP and LEV were found to be the most effective for both *S. sonnei* and *S. flexneri*; however, CIP is contraindicated in young children due to its adverse effects. Consequently, AZT is still recommended as a good choice for the treatment of *S. flexneri* severe pediatric shigellosis in Iran. Accordingly, accurate identification of *Shigella* species and antimicrobial susceptibility testing are recommended to avoid empirical treatment.

Abbreviations

ipa, invasion plasmid antigen; ShET-2, *Shigella* enterotoxin-2; *ipgD*, invasion plasmid gene D; MDR, multidrug-resistant; MIC, minimum inhibitory concentration; PCR, polymerase chain reaction; T3SS, Type 3 secretion system.

Ethics Approval and Consent to Participate

This study was approved by the Ethics Committee at the School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran. Informed consent was obtained from all enrolled patients from parents and/or legal guardians.

Data Sharing Statement

The datasets analyzed during this study are available from the corresponding author on reasonable request.

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Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no competing interests.

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