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Original article

High frequency of antimicrobial resistance and virulence gene in *Shigella* species isolated from pediatric patients in an Iranian Referral Hospital

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Abstract. *Background: Shigella* is a main cause of gastroenteritis and it is responsible for 5 to 10% of diarrhea through the world. The aims of this study were to assess the antibiotic susceptibility pattern and the presence of 3 common virulence genes (*sigA, virF, invE*) of Shigella strains isolated from patients with gastroenteritis in Children's Medical Center Hospital, Tehran, Iran. *Methods:* Over a period of 15 months, all *Shigella* species collected from the patients with gastroenteritis were entered to the study. Susceptibility testing of all isolates towards different antibiotics was performed using the disk diffusion method and the prevalence of virulence genes was detected by polymerase chain reaction (PCR) technology. Results: Among a total of 183 *Shigella* strains, 128 *Shigella sonnei* (70%) and 55 *S. flexneri* (30%) were isolated. The resistance rate to the antibiotics in *S. sonnei* strains was higher than *S. flexneri*. The most sensitive antibiotics for *S. flexneri* strains were gentamicin (98%), amikacin (85%) and ciprofloxacin (82%), while high resistance rate to trimethoprim-sulfamethoxazole (96%), ampicillin (96%), nalidixic acid (64%) and cefotaxime (60%) was observed. The frequency of *invE, virF* and *sigA* gene in *S. flexneri* strains was 89 %, 93 % and 56 %, respectively; whereas they found in 93 %, 96 %, and 100 % of *S. sonnei* strains, respectively. *SigA* gene was identified significantly higher in the *S. sonnei* strains (100%). There was no significant difference between the presence of *virF* and *invE* genes among *Shigella* strains. *Conclusion*: The high presence of *sigA* gene in *S. sonnei* strains plays an important role in its pathogenesis, and the high frequency of *invE* and *virF* genes showed that this classical pathway regulating the expression of *Shigella* virulence factor genes could play a key role in the pathogenesis of this bacterium. (www.actabiomedica.it)

Key words: Shigella, children, antibiotics resistance, virulence factors

Introduction

Shigellosis continues to be a main public health problem worldwide, mainly in developing countries where it is endemic (1, 2) and it is considered as a prominent global cause of moderate to severe diarrhea in children (3) and adults (4). Children under five years of age face the biggest impact of the disease and 61% of deaths occur in children (5). Unfortunately, poor hygienic circumstances and low quality of water in developing countries enhance the incidence and prevalence of the disease (6). The genus *Shigella* includes four subgroups historically treated as species: *Shigella flexneri*, *Shigella boydii, Shigella sonnei*, and *Shigella dysenteriae* (7), among which, *S. flexneri* is the most prevailing species in developing nations followed by *S. sonnei*, whereas *S. boydii* and *S. dysenteriae* are less frequently isolated (8).

Although shigellosis is a self-limiting disease, antibiotics might decrease the duration of illness and

consequently reduce the person to person transmission (9). Unfortunately, irregular usage of antimicrobial agents in addition to horizontal gene transfer, have given rise to the increasing resistance of *Shigella spp.* and the growth of multi-drug resistance against common antibiotics worldwide (10, 11). *Shigella* capacity to cause disease is based on genes contained in an invasion plasmid pINV of 220 Kb, such as *ipaH, ipaBCD, ial, sen, virA, virB (invE), virF, icsA, sepA,* and *ipgD* and on chromosomal genes, *ipaH, iuc, sat, sigA, pic, set1A*, and *set1B* (12, 13).

The aim of this study was to investigate the antibiotic susceptibility pattern and the distributation of three common virulence genes (*sigA, virF, invE*) of Shigella species isolated from patients with gastroenteritis in Tehran, Iran.

Material and methods

The study was approved by the Ethical Committee from the Tehran University of Medical Sciences, Iran (IR.TUMS.CHMC.REC.1397.008). In our cross-sectional study over a period of 15 months, from September 2018 to February 2020, all *Shigella* species collected from the patients with gastroenteritis were entered to the study. The isolates were identified by standard biochemical tests (14). Susceptibility testing of all *Shigella* isolates towards different antibiotics was performed using the disk diffusion method based on Clinical Laboratory Standard Institute (CLSI) guidelines, 2019 (15). The antimicrobials tested were: nalidixic acid, amikacin, ampicillin, gentamicin, cefotaxime, ciprofloxacin, and trimethoprim sulfamethoxazole.

In order to detect virulence genes, DNA template was obtained as method previously described by Hosseini Nave *et al.* (16). PCR was performed to target the virulence genes (*sigA, virF, invE*) by using previously reported primers (Table 1).

Amplification was performed in a mixture consisting of 2.5 µl of the PCR buffer (10-times concentrated), 0. 5 µl of Mgcl2 (final concentration 200 µM), 0.5 µl of dNTPs (Fermentas, Vilnius, Lithuania, final concentration 2.5 mM), 0.5 µl of each primer, final concentration of 0.2 mM, 1.5 U of the Taq DNA polymerase (Bioron,Germany), 1 µl of DNA (final concentration 2 ng/µL) and DNase-, RNase-free deionised water (Biomedicals) to a final volume of 25 µl. Cycling conditions were carried out as follows: initial denaturation at 95° C for 7 min, followed by 30 cycles including denaturation for 5 min at 95° C, annealing for 45 s and a single final extension at 72° C for 15 min. The analysis of the amplified products was performed in 1% agarose (Sigma) and DNA bands were visualized by staining with gel red (Biotium), analysed under UV light and photographed using the GEL Doc 2000 documentation system (Bio-Rad).

Statistical Analysis

Data were analyzed using the SPSS version 16.0 software (SPSS Inc., Chicago, IL, United States) and the results were described by frequency (percentage) and mean ± standard deviation (SD). Univariate analysis was performed using the chi-squared test or Fisher's exact test, as appropriate. P-values were based

Table 1. Primers used in this study

Gene	Primer sequence (5' –3')	Size of product (bp)	Annealing temperature (° C)	Reference
sigA-forward	CCGACTTCTCACTTTCTCCCG	430	59	(21)
sigA-reverse	CCATCCAGCTGCATAGTGTTTG			
virF-forward	TCAGGCAATGAAACTTTGAC	618	56	(21)
virF-reverse	TGGGCTTGATATTCCGATAAGTC			
invE-forward	CGATAGATGGCGAGAAATTATATCCCG	766	60	(37)
invE-reverse	CGATCAAGAATCCCTAACAGAAGAATCAC			

on two-tailed test results, and P < 0.05 were considered statistically significant.

Results

In this study, 183 *Shigella* strains (2.6%) were isolated from a total of 7121 children with gastroenteritis referred to the Children's Medical Center Hospital, Tehran, Iran during the period of 15 months.

The most isolated species were *S. sonnei* with 128 cases (70%) and *S. flexneri* with 55 cases (30%). Among 183 patients whose stool culture was positive for *Shigella* bacteria, 93 patients were boys (51%) with a mean age of 5.7 years old (SD= 3.4 years, the age range of 1 to 16 years old). There was no significant difference between the distribution of *Shigella* bacteria in the children with gastroenteritis by age (p value=0.5) and sex (p value=0.87). The highest rate of *Shigella* isolates was isolated in autumn and from outpatients (n=108, 59%) and among the different wards of the hospital, emergency department had the highest rate of *Shigella* (35%).

The results of antibiotic susceptibility test showed high resistance rate of *Shigella* strains to ampicillin (p value ≥ 0.05) and trimethoprim sulfamethoxazole (p value=0.09). Generally, the resistance rate to cefotaxime, nalidixic acid, ciprofloxacin and ampicillin in *S. sonnei* strains was significantly higher than *S. flexneri* (Table 2).

The prevalence of *invE, virF* and *sigA* genes in *S. flexneri* strains was 89%, 93% and 56%, and in *S. sonnei* strains was 93%, 96% and 100%, respectively (Table 2). The *sigA* gene was significantly more detected in *S. sonnei* strains (p value ≤ 0.0001). However, no significant difference was observed between *virF* and *invE* gene detection in *Shigella* strains.

Discussion

Gastroenteritis is considered as one of the most vital diseases all around the world and it is more severe and dangerous among children, the elderly, and people who are malnourished or live in poor conditions. *Shigella* is a main cause of gastroenteritis throughout the world and it is responsible for 5 to 10% of diarrhea through the world (17). It can be considered as a major pathogen in developing countries with lower levels of hygiene (17, 18).

In our study, 70% of the strains were *S. sonnei* and 30% of them were confirmed as *S. flexneri*. *S. flexneri* is responsible for most of the shigellosis burden in developing countries worldwide, while *S. sonnei* occurs predominantly in developed countries and in countries shifting from low- to middle-income (19, 20). The reason for this discrepancy is not clear; however, efforts to increase local health have drastically reduced the prevalence of the disease and even changed the distribution pattern of *Shigella* species (21, 22). This pattern change has also been observed in countries such as Brazil (23), South America (24) and China (25), which is similar to the results of studies conducted in our country in the cities of Tehran (22, 26-28), Babol (29) and Abadan (30).

In developing countries where the prevalence of shigellosis is usually reported endemically, evaluating the pattern of antibiotic resistance can be very effective in prescribing appropriate drugs. The resistance rate to the antibiotics studied in our study in *S. sonnei* strains was higher than *S. flexneri*, which was in consistent with previous studies (22, 31). *Shigella* strains showed high sensitivity to aminoglycosides in the present study. The most sensitive antibiotics for *S. flexneri* strains were gentamicin (98%), amikacin (85%) and ciprofloxacin (82%). While high resistance pattern to trimethoprim-sulfamethoxazole (96%),

Table 2. Antibiotic susceptibility pattern and frequency of the virulence genes in Shigella isolates

	Antibiotics							Genes		
Bacteria	Amikacin	Gentamycin	Ampicillin	Cefotaxime	Trimethoprim Sulfamethoxazole	Nalidixic acid	Ciprofloxacin	*invE*	*sigA*	*virF*
S. sonnei	127 (100%)	122 (96.1%)	6 (4.7%)	6 (4.7%)	0	3 (2.3%)	72 (56.7%)	119 (93%)	128 (100%)	123 (96.1%)
S. flexneri	46 (85.2%)	53 (98.1%)	2 (3.7%)	22 (40%)	2 (3.6%)	20 (36.4%)	40 (81.6%)	49 (89.1%)	31 (56.4%)	51 (92.7%)

ampicillin (96%), nalidixic acid (64%) and cefotaxime (60%) was observed. Several studies around the world have reported increased resistance of *Shigella* species to common antibiotics such as trimethoprim, sulfamethoxazole, and ampicillin (23, 32, 33).

Virulence of *Shigella* depends on the presence of a large virulence *inv* plasmid, carrying an operon that encodes the type III-secretion-system (T3SS) responsible for bacterial entry (34, 35). In our study, *sigA* gene was significantly identified more frequent in *S. sonnei* strains (p value ≤ 0.0001). The high frequency of *sigA* gene in *S. sonnei* strains plays a key role in its pathogenesis, which is consistent with previous studies (7, 16, 36). However, no significant difference was observed between *virF* and *invE* genes identification in *S. flexneri* and *sonnei* strains. When *Shigella* growth conditions are suitable for invasion, a transcription cascade begins by activating the *virF* gene to express the AraC-like protein *virF*, which in turn activates the transcription of the *invE* regulatory gene (36). The high abundance of these genes indicated that this classical regulatory pathway of *Shigella* virulence gene expression might play a major role in its pathogenesis.

In conclusion, due to the overuse of antibiotics and the consequent increase in drug resistance, some antibiotics should be removed from the list of drugs for the treatment of *Shigella*, which include ampicillin and trimethoprim sulfamethoxazole. The lowest pattern of resistance in the present study was observed to gentamicin, amikacin and ciprofloxacin. The high presence of *sigA*, *invE* and *virF* genes showed that this classical regulatory pathway of *Shigella* virulence factor gene expression can play a major role in the pathogenesis of this bacterium.

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Ethical Approval statement: The study was approved by the Ethical Committee from the Tehran University of Medical Sciences, Iran (IR.TUMS.CHMC.REC.1397.008).

Authors' Contribution: SM1 and RHS involved in writing of the manuscript. BP, MGH, and MRA participated in data collection. SM1 and SM2 involved in funding and interpretation of data and supervised the project. BP supervised the project and involved in interpretation of data. All authors discussed the results and contributed to the final manuscript.

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