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Original Article

Determination of ESBLs, pAmpC-beta-lactamase genes, and plasmid replicon types among *Shigella* species from different cities in Iran

Seyyed Amin Ayatollahi Mousavi[1,2], Mahla Mohammadian[3], Hossein Hosseini Nave[1,4], Parvin Mohseni[5*], Davood Kalantar-Neyestanaki[1,4*]

1. *Medical Mycology and Bacteriology Research Center, Kerman University of Medical Sciences, Kerman, Iran.*
2. *Department of Medical Mycology and Parasitology, Afzalipour Faculty of Medicine, Kerman University of Medical Sciences, Kerman, Iran.*
3. *Student Research Committee, Kerman University of Medical Sciences, Kerman, Iran.*
4. *Department of Medical Microbiology (Bacteriology and virology), Afzalipour Faculty of Medicine, Kerman University of Medical Sciences, Kerman, Iran.*
5. *Department of Pathobiology, Faculty of Veterinary Medicine, Shahid Bahonar University of Kerman, Kerman, Iran.*

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ABSTRACT

Shigella species (spp) are the common gram-negative bacilli isolated from patients with diarrhea. Treatment of infections caused by this genus of bacteria remains a global challenge due to increasing resistance to antibiotics. This study aimed to assess the prevalence of ESBLs, plasmid-mediated AmpC-beta-lactamase (pAmpC) genes, and plasmid replicon types among 210 clinical isolates of *Shigella* spp, collected from different cities across Iran. Antibacterial susceptibility of the isolates to antibiotics, as well as ESBLs production, were assessed in accordance with Clinical & Laboratory Standards Institute (CLSI) guidlines. ESBLs, pAmpC genes, and plasmid replicon types of the isolates were detected using PCR and multiplex PCR methods. The highest rate of antibiotic resistance was observed with trimethoprim-sulfamethoxazole, while the lowest rate of resistance was observed with cefoxitin. Fifty-four percent of the isolates were considered ESBL-producers. Beta-lactamase genes, including $bla_{\text{CTX-M}}$, bla_{TEM}, and bla_{DHA} were detected in 93 (44%), 84 (40%), and 3 (1.4%) of the isolates, respectively. Ten distinct plasmid replicon types, including I1-Iγ, K, W, FIB, Y, P, FIC, FIA, HI1, and B/O were identified among the isolates. The study sheds light on the persistent challenges posed by multidrug-resistant (MDR) shigellosis to public health in different regions of Iran. Despite advancements in hygiene practices, the prevalence and population composition of *Shigella* species have remained largely unchanged. Also, the spread of beta-lactamase genes and various plasmid replicon types is increasing among the *Shigella* spp across Iran, which poses challenges for their treatment. More efficient strategies and monitoring efforts should be considered to prevent their further spread.

Corresponding Author:
d.kalantar@kmu.ac.ir
parvin.mohseni.sisakht@gmail.com

https://orcid.org/0000-0002-8694-2888

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1. Introduction

Foodborne bacterial pathogens such as *Shigella* species (spp.) are among the most critical public health concerns around the world (1). The *Shigella* spp., including *Shigella dysenteriae*, *Shigella flexneri*, *Shigella sonnei*, and *Shigella boydii* are members of the *Enterobacteriaceae* family and cause acute gastroenteritis with high morbidity and mortality, especially in children in developing countries (2). Shigellosis, caused by *Shigella* spp., is an acute enteric infection that is usually characterized by watery and bloody mucoid diarrhea. It is highly contagious due to its low infectious dose. Antibiotic therapy, usually used for infants, the elderly, and immunocompromised patients, can reduce the duration and severity of shigellosis, as well as and the risk of transmission.

Ciprofloxacin, pivmecillinam, ceftriaxone, and azithromycin are antibiotic agents used for the treatment of shigellosis, especially in patients with bloody diarrhea (3). However, antibiotic resistance is increasing among *Shigella* spp. due to the misuse or overuse of antibiotic agents in the treatment of shigellosis, and multidrug resistant (MDR) *Shigella* isolates have been reported in some countries (4). The ability of this organism to acquire different antibiotic resistance genes through mobile genetic elements such as integrons, transposons, and plasmids is a major factor in the spread and emergence of MDR isolates.

Most MDR *Shigella* isolates are resistant to cephalosporins, ciprofloxacin, and azithromycin through acquired plasmid-mediated resistance mechanisms (4). Detection of ESBL- and AmpC- positive *Shigella* spp. is important because they are usually MDR and therapeutic options for them are limited. Hence, updating our data on the rate and mechanisms of resistance to antibiotic agents in *Shigella* spp. is essential for using effective and justified therapy to decrease the morbidity and mortality rates associated with shigellosis. Conjugative plasmids play an important role in the evolution and dissemination of antibiotic resistance and virulence factors among bacteria through facilitation of horizontal gene transfer (3). Due to the importance of *Shigella* spp. in causing gastrointestinal infections and the significant challenges in the treatment of infections resulting from MDR isolates, this study was conducted to investigate the presence of ESBLs, pAmpC beta-lactamase genes, and plasmid replicon types among *Shigella* isolates in different cities in Iran.

2. Materials and Methods

2.1. Sampling, Culture, and Shigella spp. isolates

This study was performed on 210 clinical isolates of *Shigella* spp. that were collected from patients with diarrhea (A single specimen each) and kept at -80 °C in Tryptic Soy Broth (TSB) supplemented with 30% glycerol from 2019 to 2021. The bacterial isolates were obtained from six cities in Iran--Ahvaz, Kerman, Shahr-e Kord, Tabriz, Urmia, and Ardabil-in Iran, and were confirmed using standard microbiological tests and polymerase chain reaction (PCR) with specific primers. The sequences and annealing temperatures of the primers are shown in Table 1.

2.2. Antimicrobial susceptibility of isolates and detection of ESBL-producing isolates

Disk diffusion method was used to evaluate the antibiotic susceptibility pattern of the isolates to different antibiotic agents (Padtan Teb Laboratory Instruments Co., Iran), including ceftazidime (CAZ, 30 µg), ampicillin (AMP, 10 µg), cefotaxime (CTX, 30 µg), cefoxitin (FOX, 30 µg), ceftriaxone (CRO, 30 µg), gentamicin (GEN, 10 µg), levofloxacin (LEV, 5 µg), amikacin (AMK, 30 µg), streptomycin (STR, 10 µg), ciprofloxacin (CIP, 5 µg), chloramphenicol (CHL, 30 µg), nalidixic acid (NAL, 30 µg), azithromycin (AZM, 15 µg), tetracycline (TET, 30µg), and trimethoprim-sulfamethoxazole (SXT, 1.25/23.75 µg), according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (5). *Shigella* isolates that were resistant to one of the third-generation cephalosporins-including ceftazidime, cefotaxime, and ceftriaxone-were selected for confirmation of ESBL production according to CLSI guidelines, using the combination disc test with clavulanic acid (5).

2.3. DNA extraction

The DNA of the isolates was extracted using the boiling method. Briefly, a single colony from each isolate was suspended in 400 µL of DNase- and RNase-free water and heated at 100°C for 10 minutes. The lysates were then centrifuged at 12,000 × g for 10 minutes and the supernatants were used as the DNA template for PCR and multiplex PCR assays.

2.4. Detection of ESBLs and pAmpC among *Shigella* spp

ESBL resistance genes, including bla_{TEM}, bla_{SHV}, and $bla_{CTX\text{-}M}$, were screened using PCR among ESBL-positive isolates. Cefoxitin-resistant isolates were also selected for identification of plasmid-mediated AmpC beta-lactamase genes, including bla_{MOX}, bla_{ACC}, bla_{FOX}, bla_{CMY}, bla_{EBC}, and bla_{DHA}, using a multiplex-PCR amplification

technique previously described by Pérez-Pérez and Hanson (6). The PCR products were electrophoresed on 1.5% agarose gel for 45 minutes at 100 V, and the gels were analyzed usung a gel documentation imaging system. The sequence and annealing temperatures of the primers used for the detection of beta-lactamase genes are presented in Table 1.

2.5. Plasmid replicon type profiling

PCR-based plasmid replicon typing (PBRT) was performed based on the Carattoli *et al.*, method, using 18 pairs of specific primers to identify replicons FIA, FIB, FIC, HI1, HI2, I1-Iγ, N, L/M, P, W, A/C, T, K, B/O, X, Y, FIIAs, and Frep in five multiplex-PCR and three simplex-PCR (7). The sequence of primers used in PBRT techniques is presented in Table 2.

2.6. Statistical analysis

The results were statistically evaluated using SPSS software (version 24) and P-values $\leq$ 0.05 were considered statistically significant, based on the chi-square test and Fisher's exact test.

3. Results

3.1. *Shigella spp.* isolates

In the present study, a total of 210 *Shigella* spp. isolates were collected from 6 different cities, including the cities Kerman [n= 64 (30.4%)], Ahvaz [n=67 (33.5%)], Ardabil [n=18 (8.5%)], Urmia [n=19 (9%)], Tabriz [n=20 (9.5%)], and Shahr-e Kord [n=22 (10.4%)], where *S. dysenteriae* [n=5 (2%)], *S. flexneri* [n=102 (49%)], *S. boydii* [n=28 (13%)], and *S. sonnei* [n=75 (36%)] were reported. The prevalence of *Shigella* spp. in different cities in Iran is shown in Table 3.

3.1. Result of antimicrobial susceptibility

In this study, the resistance rate to antibiotic agents was as follows: trimethoprim-sulfamethoxazole (97%), streptomycin (94%), ampicillin (80%), tetracycline (77%), chloramphenicol (62%) and cefotaxime (57%), ceftriaxone (49%), nalidixic acid (31%), ceftazidime (30%), gentamicin (13%), azithromycin (12%), levofloxacin (6%), amikacin (6%), ciprofloxacin (6%), and cefoxitin (5%). Interestingly, all isolates were resistant to at least three classes of antibiotics and were considered MDR isolates. The rate of antibiotic resistance in different cities is presented in Table 4.

3.2. Prevalence of ESBLs and pAmpC beta-lactamase genes (*bla* genes)

Based on the results, 114 isolates (54%) of the isolates were ESBL-positive, and bla_{TEM} and bla_{CTX-M} were detected in 84 (40%), and 93 (44%) of ESBL-positive isolates, respectively. bla_{DHA} as one of the pAmpC-associated genes, was detected only in three isolates (1.4%): two isolates belonged to *S. flexneri* and one to *S. sonnei*. The prevalence of β-lactamase genes among *Shigella* spp. in different cities in Iran is shown in Table 5.

3.3. Prevalence of plasmid replicon types

In this study, the replicon types I1-Iγ (72.8%), K (54.2%), W (47.1%), FIB (30.9%), Y (21.9%), P (13.3%), FIC (6.6%), FIA (4.2%), HI1 (1.9%), and B/O (1.4%) were reported. The Distribution of *bla* genes and plasmid replicon types among *Shigella* spp. in Kerman, Ardabil, Shahr-e Kord, Tabriz, Ahvaz, and Urmia, Iran, is presented in Tables 6 and 7.

4. Discussion

Shigellosis poses a considerable threat to global public health, with a particularly high prevalence in developing countries. Administering antibiotics has been shown to reduce both the severity and duration of the infection, as well as the excretion of the organism in feces, thereby aiding in the prevention of its continued transmission. This study offers insights into the molecular epidemiology of antibiotic resistance profiles, ESBLs, AmpC, and plasmid replicon types among *Shigella* spp. isolated from diarrheal samples in Iran. In the current study, *S. flexneri* (49%) and *S. sonne*i (36%) were identified as the most common species, and our results were largely consistent with the results of recent studies in Iran and various countries (8-10). Between 2001-2019, several studies identified *S. flexneri* and *S. sonnei* as the predominant species of *Shigella* in Ahvaz and Tehran, Iran (8, 9). Given that *S. flexneri* and *S. sonnei* are typically predominant in developing and developed countries, respectively, the findings of aforementioned studies are consistent with those of our own. The results of the current study showed that despite the high standard of hygiene in our region in recent years, we observed no noticeable change in the prevalence and population composition of isolated *Shigella* spp.

In recent years, an epidemiological transition in the prevalence of *Shigella* serogroups has been observed. Specifically, there has been a notable emergence of *S. sonnei* in regions where *S. flexneri* previously prevailed. This shift has been documented across various areas in Asia, Latin America, and the Middle East (10).

Table 1. The primer sequences are used for conformation of different species of *Shigella*.

Gene	Primer sequence (5′-3′)	Product size (bp)	Annealing (°C)	Use
Sboy	F-TCTGATGTCACTCTTTGCGA R-GAATCCGGTACCCGTAAGGT	248	59	**For confirmation *S. boydii***
Sflex	F-TTTATGGCTTCTTTGTCGGC R-CTGCGTGATCCGACCATG	537	56	**For confirmation *S. flexneri***
Sson	F-AATGCCGTAAGGAATGCAAG R-CTTGAAGGAGATTCGCTGCT	503	58	**For confirmation *S. sonnei***
Sdys	F-TCTCAATAATAGGGAACACAG R-CATAAATCACCAGCAAGGTT	211	56	**For confirmation *S. dysenteriae***
***bla*$_{SHV}$**	F-TCAGCGAAAAACACCTTG R-TCCCGCAGATAAATCACC	471	50	**For detection of ESBL genes**
***bla*$_{TEM}$**	F- GAGTATTCAACATTTCGTGTC R- TAATCAGTGAGGCACTATCTC	861	60	
***bla*$_{CTX-M}$**	F-CGCTTTGCGATGTGCAG R-ACCGCGATATCGTTGGT	550	60	
***bla*$_{MOX}$**	F-GCTGCTCAAGGAGCACAGGAT R-CACATTGACATAGGTGTGGTGC	520		
***bla*$_{CIT}$**	F-TGGCCAGAACTGACAGGCAAA R-TTTCTCCTGAACGTGGCTGGC	462		
***bla*$_{DHA}$**	F-AACTTTCACAGGTGTGCTGGGT R-CCGTACGCATACTGGCTTTGC	405	57	**For detection of pAmpC genes using multiplex-PCR**
***bla*$_{ACC}$**	F-AACAGCCTCAGCAGCCGGTTA R-TTCGCCGCAATCATCCCTAGC	346		
***bla*$_{EBC}$**	F-TCGGTAAAGCCGATGTTGCGG R-CTTCCACTGCGGCTGCCAGTT	302		
***bla*$_{FOX}$**	F-AACATGGGGTATCAGGGAGAT R-GCAAAGCGCGTAACCGGATTGG	190		

Table 2. The list of primers for plasmid replicon typing of isolates.

Replicon type	Primer sequence (5′-3′)	Target site	Amplicon size (bp)
HI1	F-GGAGCGATGGATTACTTCAGTAC R-TGCCGTTTCACCTCGTGAGTA	parA-parB	**471**
HI2	F-TTTCTCCTGAGTCACCTGTTAACAC R-GGCTCACTACCGTTGTCATCCT	iterons	**644**
I1	F-CGAAAGCCGGACGGCAGAA R-TCGTCGTTCCGCCAAGTTCGT	RNAI	**139**
X	F-AACCTTAGAGGCTATTTAAGTTGCTGAT R-TGAGAGTCAATTTTTATCTCATGTTTTAGC	ori g	**376**
L/M	F-GGATGAAAACTATCAGCATCTGAAG R-CTGCAGGGGCGATTCTTTAGG	repA,B,C	**785**
N	F-GTCTAACGAGCTTACCGAAG R-GTTTCAACTCTGCCAAGTTC	repA	**559**
FIA	F-CCATGCTGGTTCTAGAGAAGGTG R-GTATATCCTTACTGGCTTCCGCAG	iterons	**462**
FIB	F-GGAGTTCTGACACACGATTTTCTG R-CTCCCGTCGCTTCAGGGCATT	repA	**702**
W	F-CCTAAGAACAACAAAGCCCCCG R-GGTGCGCGGCATAGAACCGT	repA	**242**
Y	F-AATTCAAACAACACTGTGCAGCCTG R-GCGAGAATGGACGATTACAAAACTTT	repA	**765**
P	F-CTATGGCCCTGCAAACGCGCCAGAAA R-TCACGCGCCAGGGCGCAGCC	iterons	**534**
FIC	F-GTGAACTGGCAGATGAGGAAGG R-TTCTCCTCGTCGCCAAACTAGAT	repA2	**262**
A/C	F-GAGAACCAAAGACAAAGACCTGGA R-ACGACAAACCTGAATTGCCTCCTT	repA	**465**
T	F-TTGGCCTGTTTGTGCCTAAACCAT R-CGTTGATTACACTTAGCTTTGGAC	repA	**750**
FIIAS	F-CTGTCGTAAGCTGATGGC R-CTCTGCCACAAACTTCAGC	repA	**270**
FrepB	F-TGATCGTTTAAGGAATTTTG R-GAAGATCAGTCACACCATCC	RNAI/repA	**270**
K/B	F-GCGGTCCGGAAAGCCAGAAAAC R-TCTTTCACGAGCCCGCCAAA	RNAI	**160**
B/O	F-TCTGCGTTCCGCCAAGTTCGA	RNAI	**159**

Table 3. Prevalence of *Shigella* spp. in different cities in Iran.

***Shigella* spp.**	**City; n (%)**					
	Ardabil; 18 (%)	**Ahvaz; 67 (%)**	**Kerman; 64 (%)**	**Urmia; 19 (%)**	**Shahre-kord; 22 (%)**	**Tabriz; 20 (%)**
S. dysenteriae	0	0	2 (3.1)	1 (5.3)	0	2 (10)
S. flexneri	10 (55.6)	27 (40.3)	34 (53.2)	8 (42.1)	10 (45.5)	13 (65)
S. boydii	4 (22.2)	10 (14.9)	9 (14.06)	1 (5.3)	3 (13.6)	1 (5)
S. sonnei	4 (22.2)	30 (44.8)	19 (29.6)	9 (47.3)	9 (40.9)	4 (20)
Total	18 (100)	67 (100)	64 (100)	19 (100)	22 (100)	20 (100)

Table 4. The frequency of resistance to different antibiotic agents in different cities in Iran among *Shigella* spp.

Antibiotic agents	**City; n (%)**					
	Ardabil	**Ahvaz**	**Kerman**	**Urmia**	**Shahre-kord**	**Tabriz**
Cefoxitin	6 (33.3)	-	4 (6.2)	-	-	2 (10)
Ciprofloxacin	3 (16.66)	1 (1.4)	5 (7.8)	1 (5.2)	1 (4.5)	2 (10)
Ampicillin	17 (94.44)	42 (62.6)	57(89)	14 (73.6)	20 (90.9)	19 (95)
Gentamicin	8 (44.44)	2 (2.9)	8 (12.5)	4 (21)	4 (18.1)	2 (10)
Nalidixic acid	9 (50)	24 (35.8)	16 (25)	8 (42.1)	5 (22.7)	4 (20)
Levofloxacin	3 (16.6)	3 (4.4)	4 (6.2)	-	3 (13.6)	1 (5)
Amikacin	-	4 (5.9)	7 (10.9)	-	2 (9)	-
Streptomycin	13 (72.2)	64 (95.5)	64 (100)	18 (94.7)	22 (100)	18 (90)
Azithromycin	4 (22.2)	3 (4.4)	5 (7.8)	5 (26.3)	2 (9)	7 (35)
Tetracycline	9 (50)	63(94)	44 (68.7)	16 (84.2)	16 (72.7)	15 (75)
Trimethoprim-sulfamethoxazole	17 (94.44)	67 (100)	64 (100)	17 (89.4)	21 (95.4)	19 (95)
Chloramphenicol	2 (11.1)	14 (20.8)	26 (40.6)	6 (31.5)	4 (18.1)	4 (20)
Ceftriaxone	11 (61.1)	27 (40.2)	29 (45.3)	10 (52.6)	10 (45.4)	17 (85)
Cefotaxime	14 (77.7)	31 (46.2)	34 (53.1)	10 (52.6)	14 (63.6)	18 (90)
Ceftazidime	8 (44.4)	26 (38.8)	4 (6.2)	3 (15.7)	12 (54.5)	11 (55)

Table 5. Prevalence of β-lactamase genes among *Shigella* spp in different cities in Iran.

City	**β-lactamase genes, n (%)**			
	***bla*$_{CTX-M}$**	***bla*$_{SHV}$**	***bla*$_{TEM}$**	***bla*$_{DHA}$**
Ardabil (18)	11(61.1)	0	7 (38.8)	1 (5.5)
Ahvaz (67)	16 (23.8)	0	25 (37.3)	0
Kerman (64)	29 (45.3)	0	19 (29.6)	2 (3.1)
Urmia (19)	10 (52.6)	0	7 (36.8)	0
Shahr-e Kord (22)	10 (45.45)	0	14 (63.6)	0
Total (190)	76 (40)	0	72 (37.8)	3 (1.57)

Table 6. Prevalence of plasmid replicon types among *Shigella* spp in different cities in Iran.

City (n)	**Plasmid replicon types, n (%)**									
	HI1	**I1-Iγ**	**FIA**	**FIB**	**W**	**Y**	**P**	**FIC**	**K**	**B/O**
Ardabil (18)	0	15 (83.3)	0	7 (38.8)	12 (66.6)	1 (5.5)	1 (5.5)	0	10 (55.5)	0
Ahvaz (67)	1 (1.4)	43(64.1)	5 (7.4)	18 (26.8)	12 (17.9)	1 (1.49)	0	0	33 (49.2)	2 (2.9)
Kerman (64)	0	48(75)	2 (3.1)	10 (5.6)	41	29 (45.3)	18 (28.1)	11 (17.1)	40 (62.5)	3 (4.6)
Urmia (19)	2 (10.5)	16 (84.2)	0	6 (31.5)	8 (42.1)	5 (26.3)	1 (5.2)	1 (5.2)	7 (36.8)	0
Shahre-kord (22)	1 (1.4)	15 (68.1)	0	14 (63.6)	14 (63.6)	5 (22.7)	4 (18.1)	4 (18.1)	11 (50)	0
Total (190)	4 (2.1)	137 (72.1)	7 (3.68)	55 (28.9)	87 (45.7)	41 (21.57)	24 (12.63)	16 (8.42)	101 (53.1)	5 (2.63)

Table 7. Distribution of *bla* genes and plasmid replicon types among *Shigella* spp. in Kerman, Ardabil, Shahr-e Kord, Tabriz, Ahvaz, and Urmia in Iran.

City	*bla* genes	Species	Replicon plasmid type profile
Ardabil	bla_{TEM}	*S. flexneri*	I1-Iγ, K
	bla_{TEM}	*S. flexneri*	I1-Iγ, FIB, K
	bla_{CTX-M}	*S. flexneri*	I1-Iγ, FIB, W, K
	bla_{CTX-M}	*S. flexneri*	I1-Iγ, FIB, W, Y, P
	bla_{CTX-M}	*S. flexneri*	I1-Iγ, FIB, W, K
	bla_{CTX-M}, bla_{TEM}	*S. flexneri*	I1-Iγ, FIB, W, K
	bla_{CTX-M}, bla_{TEM}	*S. flexneri*	I1-Iγ, K
	bla_{CTX-M}, bla_{TEM}	*S. flexneri*	I1-Iγ, FIB, W
	bla_{CTX-M}	*S. sonnei*	I1-Iγ, FIB, W
	bla_{CTX-M}	*S. sonnei*	I1-Iγ, W, K
	bla_{CTX-M},bla_{TEM}	*S. sonnei*	I1-Iγ, W
	bla_{CTX-M}, bla_{TEM}	*S. sonnei*	I1-Iγ, W
	bla_{CTX-M}	*S. sonnei*	I1-Iγ, FIB, W
	bla_{CTX-M}	*S. sonnei*	I1-Iγ, W, K
Urmia	bla_{CTX-M}, bla_{TEM}	*S. boydii*	I1-Iγ, K
	bla_{CTX-M}, bla_{TEM}	*S. boydii*	I1-Iγ, W
	bla_{CTX-M}, bla_{TEM}	*S. flexneri*	I1-Iγ, FIB, W, FIC, K
	bla_{CTX-M}, bla_{SHV}	*S. flexneri*	I1-Iγ, FIB, W
	bla_{CTX-M}, bla_{TEM}	*S. sonnei*	I1-Iγ, FIB, W
	$bla_{CTX-M,}$ bla_{TEM}	*S. boydii*	I1-Iγ, W, Y
	bla_{CTX-M}	*S. sonnei*	I1-Iγ, FIB, W
	$bla_{CTX-M,}$ bla_{TEM}	*S. dysenteriae*	I1-Iγ, K
	bla_{CTX-M}	*S. flexneri*	I1-Iγ, FIB, W
	bla_{CTX-M}	*S. flexneri*	I1-Iγ Y, K
	bla_{CTX-M}, bla_{TEM}	*S. flexneri*	I1-Iγ, FIB, W
	bla_{TEM}	*S. flexneri*	I1-Iγ, W
	bla_{TEM}	*S. flexneri*	I1-Iγ, Y, FIC, K
	bla_{CTX-M}, bla_{TEM}	*S. flexneri*	I1-Iγ
	bla_{CTX-M}, bla_{TEM}	*S. flexneri*	I1-Iγ
	bla_{CTX-M}, bla_{TEM}	*S. flexneri*	I1-Iγ, W, Y, P
	bla_{CTX-M}, bla_{TEM}	*S. flexneri*	I1-Iγ, K, B/O
	bla_{CTX-M}, bla_{TEM}	*S. flexneri*	I1-Iγ, W, K
	bla_{CTX-M}, bla_{TEM}	*S. flexneri*	I1-Iγ, W, Y, K
	bla_{CTX-M}, bla_{TEM}	*S. flexneri*	I1-Iγ, W, Y, K
	bla_{CTX-M}, bla_{TEM}	*S. flexneri*	I1-Iγ, K, B/O
	bla_{CTX-M}	*S. sonnei*	I1-Iγ, W, K
	bla_{CTX-M}	*S. sonnei*	I1-Iγ, FIB, W, Y, P, K
	bla_{CTX-M}	*S. sonnei*	I1-Iγ, W, K
	bla_{CTX-M}	*S. sonnei*	I1-Iγ, FIB, K
	bla_{CTX-M}	*S. sonnei*	I1-Iγ, FIB, Y, K
	bla_{CTX-M}	*S. sonnei*	I1-Iγ, FIA, FIB, W, K
	bla_{CTX-M}	*S. sonnei*	I1-Iγ, K, FIC, P, Y, W, FIB, FIA
Kerman	bla_{CTX-M}	*S. sonnei*	W, Y, P
	bla_{CTX-M}	*S. sonnei*	I1-Iγ, W, K
	bla_{CTX-M}	*S. sonnei*	I1-Iγ, B/O
	bla_{CTX-M}	*S. sonnei*	I1-Iγ, W
	bla_{CTX-M}	*S. sonnei*	I1-Iγ, W, Y, P, K
	bla_{CTX-M}	*S. sonnei*	K, FIC, Y
	bla_{CTX-M}	*S. sonnei*	I1-Iγ, W
	bla_{CTX-M}	*S. sonnei*	I1-Iγ, W, Y, P, K
	bla_{CTX-M}	*S. boydii*	I1-Iγ, FIC, K
	bla_{CTX-M}	*S. boydii*	I1-Iγ, W, P, K
	bla_{CTX-M}, bla_{TEM}	*S. boydii*	I1-Iγ, W, Y, P, K
	bla_{CTX-M}, bla_{TEM}	*S. boydii*	I1-Iγ, FIB, W
	bla_{TEM}	*S. sonnei*	I1-Iγ, K, FIC, P, Y, W, FIB, FIA
	bla_{CTX-M}	*S. sonnei*	K, FIC, Y
	bla_{TEM}	*S. sonnei*	I1-Iγ, W
	bla_{TEM}	*S. sonnei*	I1-Iγ, W, Y, P, K
	bla_{CTX-M}, bla_{TEM}	*S. sonnei*	I1-Iγ, W, Y, P, FIC, K
	bla_{CTX-M}, bla_{TEM}	*S. sonnei*	W, Y, P
	bla_{CTX-M}, bla_{TEM}	*S. flexneri*	W, K

	bla$_{CTX-M}$, *bla*$_{TEM}$	*S. flexneri*	I1-Iγ, K
	bla$_{CTX-M}$, *bla*$_{TEM}$	*S. flexneri*	I1-Iγ
	bla$_{CTX-M}$, *bla*$_{TEM}$	*S. flexneri*	I1-Iγ, K
	bla$_{CTX-M}$, *bla*$_{TEM}$	*S. flexneri*	I1-Iγ, FIA
	bla$_{CTX-M}$, *bla*$_{TEM}$	*S. flexneri*	I1-Iγ
	bla$_{CTX-M}$, *bla*$_{TEM}$	*S. flexneri*	I1-Iγ, FIB
	bla$_{CTX-M}$, *bla*$_{TEM}$	*S. flexneri*	W, FIA
	bla$_{TEM}$	*S. flexneri*	FIA
	bla$_{TEM}$	*S. sonnei*	I1-Iγ, FIB, K
	bla$_{TEM}$	*S. sonnei*	I1-Iγ, K
	bla$_{CTX-M}$, *bla*$_{TEM}$	*S. sonnei*	I1-Iγ
	bla$_{CTX-M}$, *bla*$_{TEM}$	*S. sonnei*	I1-Iγ, FIB
Ahvaz	*bla*$_{CTX-M}$, *bla*$_{TEM}$	*S. sonnei*	I1-Iγ, FIB
	bla$_{CTX-M}$	*S. sonnei*	I1-Iγ, K
	bla$_{CTX-M}$, *bla*$_{TEM}$	*S. sonnei*	I1-Iγ, FIB
	bla$_{CTX-M}$, *bla*$_{TEM}$	*S. sonnei*	I1-Iγ, W
	bla$_{TEM}$	*S. boydii*	I1-Iγ, W, K
	bla$_{TEM}$	*S. boydii*	I1IY, W, K
	bla$_{TEM}$	*S. boydii*	I1-Iγ
	bla$_{TEM}$	*S. flexneri*	I1-Iγ, FIA, FIB, W
	bla$_{CTX-M}$	*S. sonnei*	I1-Iγ, FIB, K
	bla$_{CTX-M}$	*S. sonnei*	I1-Iγ
	bla$_{CTX-M}$	*S. sonnei*	I1-Iγ, K
	bla$_{CTX-M}$, *bla*$_{TEM}$	*S. flexneri*	I1-Iγ, FIB
	bla$_{CTX-M}$	*S. flexneri*	I1-Iγ, K
	bla$_{TEM}$	*S. flexneri*	I1-Iγ, FIB, W
	bla$_{CTX-M}$	*S. sonnei*	I1-Iγ, FIB, W, K
	bla$_{CTX-M}$	*S. sonnei*	I1-Iγ, FIB, W, Y
	bla$_{CTX-M}$	*S. sonnei*	I1-Iγ, FIB, W, P, K
	bla$_{CTX-M}$, *bla*$_{TEM}$	*S. boydii*	I1-Iγ, FIA
	bla$_{CTX-M}$, *bla*$_{TEM}$	*S. dysenteriae*	I1-Iγ, K
	bla$_{CTX-M}$	*S. flexneri*	I1-Iγ, Y, K
	bla$_{CTX-M}$	*S. flexneri*	I1-Iγ, K
	bla$_{CTX-M}$	*S. flexneri*	I1-Iγ, FIB, W, K
Tabriz	*bla*$_{CTX-M}$, *bla*$_{TEM}$	*S. flexneri*	I1-Iγ, FIB, W
	bla$_{CTX-M}$, *bla*$_{TEM}$	*S. flexneri*	I1-Iγ, W, Y, P, K
	bla$_{CTX-M}$, *bla*$_{TEM}$	*S. flexneri*	I1-Iγ, FIB, W
	bla$_{CTX-M}$, *bla*$_{TEM}$	*S. flexneri*	I1-Iγ, K, B/O
	bla$_{CTX-M}$, *bla*$_{TEM}$	*S. flexneri*	I1-Iγ, Y
	bla$_{CTX-M}$, *bla*$_{TEM}$	*S. flexneri*	I1-Iγ
	bla$_{CTX-M}$, *bla*$_{TEM}$	*S. flexneri*	I1IY, FIB, W, P, K
	bla$_{CTX-M}$	*S. flexneri*	W, Y, K
	bla$_{CTX-M}$, *bla*$_{TEM}$	*S. sonnei*	I1-Iγ, FIB, W, K
	bla$_{CTX-M}$, *bla*$_{TEM}$	*S. sonnei*	I1-Iγ, FIB, W, Y
	bla$_{CTX-M}$, *bla*$_{TEM}$	*S. flexneri*	I1-Iγ, FIB, W, K
	bla$_{CTX-M}$, *bla*$_{TEM}$	*S. flexneri*	FIB, W, Y, P
	bla$_{TEM}$	*S. flexneri*	FIB, W
	bla$_{CTX-M}$, *bla*$_{TEM}$	*S. sonnei*	I1-Iγ, FIB, W, K
	bla$_{CTX-M}$, *bla*$_{TEM}$	*S. sonnei*	I1-Iγ, K
Shahre-kord	*bla*$_{TEM}$	*S. sonnei*	I1-Iγ, K
	bla$_{TEM}$	*S. flexneri*	I1-Iγ, FIB, W
	bla$_{CTX-M}$	*S. sonnei*	I1-Iγ, FIB, W, K
	bla$_{CTX-M}$	*S. sonnei*	I1-Iγ, K
	bla$_{CTX-M}$	*S. sonnei*	I1-Iγ, K
	bla$_{CTX-M}$, *bla*$_{TEM}$	*S. boydii*	I1-Iγ, FIB, W
	bla$_{CTX-M}$, *bla*$_{TEM}$	*S. boydii*	I1-Iγ, FIB, W, Y, P

Notably, in the United States, *S. flexneri* was the predominant serotype in the early 1960s, but it was supplanted by *S. sonnei* between 1964 and 1968, the cause of which remains unknown (10).

The increasing prevalence of *S. sonnei* in developing countries could potentially be attributed to improvements in water quality and sanitation practices. These improvements might limit the passive immunization typically conferred by *P. shigelloides*, which is commonly found in contaminated water sources. Furthermore, the amoeba *Acanthamoeba castellani* serves as a reservoir for *S. sonnei*, enabling its persistence even in highly chlorinated environments where *S. flexneri* struggles to thrive. Additionally, *S. sonnei* demonstrates a greater propensity for acquiring resistance compared to *S. flexneri*, giving it a competitive edge, particularly in regions with limited antimicrobial usage (11).

Shigella spp. can easily acquire and spread antimicrobial resistance genes, as in recent years, MDR-positive *Shigella* spp. have been reported abundantly throughout the world. In this study, all isolates were resistant to at least three classes of antibiotics and were considered MDR. Also, 54% of isolates produced ESBL, which could be a serious threat to public health. The frequency of ESBL-producing *Shigella* isolates was reported to be 7.5% by Ranjbar et al., in 2013 (12) in Tehran, Iran, and the results of this study, in comparison to their findings, represented a significant increase in ESBL-producing *Shigella* isolates in other regions of Iran. In a cross-sectional study in Ardabil, Iran, from 2019 to 2020, 10.2% of *Shigella* species were ESBL positive, and various beta-lactamase genes including $bla_{\text{CTX-M}}$ and bla_{TEM} were found among them (13). In another study in Tehran, Iran from 2015 to 2017 $bla_{\text{CTX-M-15}}$ (10.7 %), bla_{SHV} (28 %), and bla_{TEM} (21.3 %) were reported among *Shigella* isolates (14). In the present study, bla_{TEM} and $bla_{\text{CTX-M}}$ were the common ESBL genes among the isolates, with frequencies of 84 (40%) and 93 (44%), respectively, which is similar to findings in Argentina, Turkey, Lebanon, China, Korea, and Japan, where bla_{TEM} and bla_{CTX} were as the predominant ESBL genes in *Shigella* isolates (15-17).

This study revealed that more than 50% of the *Shigella* isolates exhibited resistance to ampicillin, tetracycline, trimethoprim-sulfamethoxazole, streptomycin, chloramphenicol, cefotaxime, and ceftriaxone. Many reports have highlighted a significant prevalence of resistance to ampicillin and trimethoprim-sulfamethoxazole among *Shigella* isolates, and based on these reports, trimethoprim-sulfamethoxazole and ampicillin are not appropriate choices for the treatment of shigellosis (18,19).

A high prevalence of resistance to nalidixic acid, trimethoprim/sulfamethoxazole, and ampicillin was reported in *Shigella* isolates among pediatric patients in different regions of Iran (14). Therefore, findings in different regions in Iran, similar to our results, showed that the rate of resistance to ampicillin, nalidixic acid, and trimethoprim/sulfamethoxazole is high.

In contrast to the findings by Xing et al. in China (18), in this study, resistance to nalidixic acid (31%) was low. This difference may be to the limited or nonexistent use of this drug in the treatment of shigellosis and other gastrointestinal tract infections in Iran. Gu et al., (20) demonstrated a significant increase in ciprofloxacin resistance in Asia and African countries over 12 years, whereas resistance to this antibiotic and third-generation cephalosporins was reported to be below 1% in the United States and some European countries. Ceftriaxone is presently the primary treatment for shigellosis in hospitalized patients. However, the irregular use of antibiotics has contributed to the development of resistant strains. Resistance to third-generation cephalosporins, particularly ceftriaxone, has been notably high in countries like Vietnam, China, and Iran. Despite reports of increasing antibiotic resistance in *Shigella* spp, the high resistance rate (49%) to ceftriaxone in this study is particularly noteworthy.

In a study conducted by Jafari et al., in 2009 (21) in Tehran, Iran, more than 90% of *Shigella* isolates were found to be susceptible to ceftriaxone, ceftazidime, cefepime, and ciprofloxacin. Conversely, Mostafavi et al., (22) reported a very high level of resistance among *Shigella* serotypes to trimethoprim-sulfamethoxazole, ampicillin, and third-generation cephalosporins. Furthermore , studies conducted in Iran over the past 20 years have consistently revealed a high level of resistance to trimethoprim-sulfamethoxazole and ampicillin (22-25).

In this study, a significant relationship was observed between all the *S. sonnei* strains and resistance to trimethoprim-sulfamethoxazole ($P \leq 0.5$). Additionally, in the current study, *S. boydii*, *S. flexneri*, and *S. dysenteriae* strains exhibited a significant relationship with resistance to trimethoprim-sulfamethoxazole, ampicillin, and streptomycin ($P \leq 0.05$). This may indicate a clonal spread of these strains due to the horizontal transfer of plasmids carrying multiple resistance genes in Iran. This highlights the key role of horizontal transfer of multidrug resistance genes associated with endemic plasmids. Overall, the low resistance to ciprofloxacin (6%) in this study suggests that fluoroquinolones remain effective for treating shigellosis in different regions of Iran. However, due to the limitations of fluoroquinolone prescription in children because of their side effects, some cephalosporins are often used as an alternative treatment for shigellosis.

The PBRT method is a practical tool for evaluating the genetic relatedness of bacterial isolate in epidemiological studies. In this study, plasmid profiling showed that 72.8% of isolates harbored I1-Iγ replicon type as the most abundant replicon type. I1-Iγ replicons are limited to Enterobacteria hosts carrying the bla_{CTX-M}, bla_{SHV}, bla_{CMY}, bla_{TEM} genes, integrons and resistance genes to arsenic, tetracycline and streptomycin.

In the study by Ruekit *et al.*,(26) conducted in India on 29 *S. sonnei* isolates, only B/O (34.4%) and I1-Iγ (13.7%) replicon types were reported, while in this study, I1-Iγ and B/O in were reported in 72.8% and 1.4% of isolates, respectively. Due to the geographical distribution and species diversity of isolates in this study, clonal dissemination of *Shigella* isolates carrying the I1-Iγ replicon is unlikely. Also, in the present study, most ESBL isolates carried the I1-Iγ replicon, so there may be a co-relationship between these two factors. The results of this study suggests that the I1-Iγ replicon is likely more compatible with ESBL-positive *Shigella* isolates than other plasmid replicon types.

In the present study, isolates harboring multi-replicon types were observed abundantly, such that among plasmid-carrying isolates, 59.3% harbored at least three replicons simultaneously. Also, two isolates, both belonging to *S. soneii* from Kerman, carried eight replicon types. The presence of multiple replicons among the isolates is one of the main factors in facilitating genetic exchanges, therefore, while increasing the chance of horizontal gene transfer among isolates (a broad host range), it also increases the possibility of genetic recombination between different plasmids within each isolate, which can drive the evolution and diversity of these bacteria, potentially leading to new pathogenicity and resistance profile.

In conclusion, the study sheds light on the persistent challenges posed by shigellosis to global public health. Despite advancements in hygiene practices, the prevalence and population composition of *Shigella* species remain largely unchanged. The epidemiological transition from *S. flexneri* to *S. sonnei* observed in various regions, underscores the dynamic nature of this infectious disease. Antimicrobial resistance among *Shigella* spp. poses a significant threat, with multi-drug resistance and ESBL-producing isolates increasing worldwide, which may reduce treatment options.

Notably, the high resistance rate to ceftriaxone in Iran underscores the urgent need for prudent antibiotic stewardship. Additionally, this study highlights the possible role of plasmid replicon types in the development and dissemination of resistance genes among *Shigella* isolates, and the spread of the I1-Iγ replicon, which is usually associated with ESBL genes, underscores the importance of understanding plasmid dynamics in combating antimicrobial resistance. However, despite the challenges posed by antimicrobial resistance, some antibiotics like ciprofloxacin remain effective for treating shigellosis, although its use is limited, especially in pediatric patients. Ongoing surveillance and appropriate use of antibiotic agents are essential for managing shigellosis caused by drug-resistant isolates.

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Authors' Contribution

Study concept and design: SA. AM, D. K-N.
Acquisition of data: M. M, H. HN.
Analysis and interpretation of data: D. K-N, P. M, SA. AM.
Drafting of the manuscript: D. K-N, P. M, SA. AM.
Critical revision of the manuscript for important intellectual content: D. K-N, P. M.
Statistical analysis: D. K-N, P. M.
Administrative, technical, and material support: D. K-N.

Ethics

The present study with Reg. No. 97000972 was approved by the ethical committee of Kerman University of Medical Sciences, Kerman, Iran. The ethics approval code is IR.KMU.REC.1398.199.

Conflict of Interest

All authors of this manuscript have no conflicts of interest to disclose.

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Data Availability

The datasets used or analyzed during the study are available on reasonable requests from the corresponding author (**Email:** d.kalantar@kmu.ac.ir).

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