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Genotypic Diversity of Multidrug Resistant *Shigella* species from Iran

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Background: In many developing countries, shigellosis is endemic and also occurs in epidemics and treatment of multidrug-resistant (MDR) isolates are important. The aims of this study were to determine the antimicrobial susceptibility, prevalence of class 1 and 2 integrons and the clonal relatedness of isolates.

Materials and Methods: Antimicrobial susceptibility tests were performed by disc diffusion method. Polymerase chain reaction (PCR)-sequencing technique was employed for detection and characterization of integrons. The genetic relatedness was evaluated by using enterobacterial repetitive intergenic consensus (ERIC) PCR.

Results: There was a high percentage of resistance to trimethoprim-sulfamethoxazole (TMP/SMX) (93.7%), ampicillin (AMP) (87.3%), streptomycin (STR) (84.5%) and tetracycline (TET) (78.9%). MDR phenotype was seen in 95.1% of total isolates. Most common MDR profile was TMP/SMX/STR/AMP resistant pattern. Among the 142 *Shigella* spp. analyzed in this study, 28 isolates were positive for class 1 integron with two types of gene cassette arrays (*dfrA17/aadA5* = 31.7% and *dfrA7* = 3.8%). The class 2 integron was more frequently detected among the isolates (94.7%) with *dfrA1/sat1/aadA1* (69.4%) and *dfrA1/sat1* (30.6%) gene cassettes. ERIC-PCR results showed 6, 5, 4 and 3 main genotypes among *S. flexneri, S. sonnei, S. boydii* and *S. dysenteriae* isolates, respectively.

Conclusion: Our findings revealed that multidrug resistant *Shigella* species with high prevalence of class 2 integron were very common in Iran. In addition, ERIC-PCR patterns showed limited variety of clones are responsible for shigellosis in the region of the study.

Key Words: *Shigella*; Integron; Multidrug resistant; Enterobacterial repetitive intergenic consensus; Polymerase chain reaction

Introduction

Shigellosis, caused by *Shigella* species, is an acute enteric infection that is characterized by a watery and mucoid bloody diarrhea, also known as bacillary dysentery [1, 2]. Antibiotic therapy can reduce the duration and severity of the disease and has been effective in improving the dysenteric syndrome of shigellosis and reduce the risk of transmission [3]. However,

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resistance to commonly used antibiotics has been increasing among *Shigella* spp. worldwide and multidrug-resistant (MDR) *Shigella* infections are widespread [4, 5]. In *Shigella* species, resistance to ampicillin, trimethoprim, streptothricin and streptomycin is often associated with the presence of class 1 and class 2 integrons that contain resistance gene cassettes [3]. However, there are few data available to describe the prevalence of class 1 and class 2 integrons of *Shigella* species in Iran and there is no report on this subject in Northwest of Iran. Additionally, typing of bacterial strains helps us to understand transmission dynamics and control the spread of disease and accurately interpret epidemiological evolution of infectious diseases in the societies [6, 7].

The objective of this work was to determine the antimicrobial susceptibility pattern in *Shigella* species isolated from Iran. Also, we have investigated the prevalence of class 1 and 2 integrons and their cassette arrays among the isolates. Moreover, the clonal relatedness of all *Shigella* species was evaluated using enterobacterial repetitive intergenic consensus (ERIC) PCR genotyping method for the first time in Iran.

Materials and Methods

1. Bacteria isolates

Between May 2014 and May 2015, a total of 142 non-duplicate *Shigella* isolates were obtained from the feces of patients suffered from acute diarrhea and gastroenteritis in Iran (Table 1). Moreover, fresh stool samples were cultured on selective media including Xylose lysine desoxycholate (XLD) (Merck, Hamburg, Germany) agar and Hektoen Enteric agar plates (Merck) which were incubated at 37˚C for 18–24 h. All presumptive colonies (non-lactose-fermenting colonies) were more identified as *Shigella* spp. by standard *Enterobacteriaceae* differentiation biochemical tests [6]. Furthermore, specific polyvalent antisera (SIFIN, GmbH Berlin, Germany) were used for serogrouping of *Shigella* isolates by a slide agglutination test [7].

2. Antimicrobial susceptibility

Antibiotic susceptibility testing was performed by using Kirby-Bauer disc diffusion method according to Clinical and Laboratory Standards Institute recommendations (CLSI) [8]. The antimicrobial disks tested were ampicillin (AMP), cefoxitin (FOX), cefazolin (CFZ), ceftriaxone (CRO), cefotaxime (CTX), ceftazidime (CAZ), cefepime (FEP), amoxicillin-clavulanate (AMC), aztreonam (ATM), gentamicin (GEN), streptomycin (STR), amikacin (AMK), nalidixic acid (NAL), ofloxacin (OFX), ciprofloxacin (CIP), levofloxacin (LVX), chloramphenicol (CHL), trimethoprim-sulfamethoxazole (TMP/SMX), tetracycline (TET), azithromycin (AZM), imipenem (IPM) and meropenem (MEM) (Mast Co., Bootle, Merseyside, UK). *Escherichia coli* ATCC 25922 was used as the quality control strain. The MDR was defined as resistance to 3 or more unrelated antibiotics [9].

3. Detection of class 1 and 2 integrons

Total DNA of the isolates was prepared using the boiling method, as previously described [10]. The presence of class 1 and 2 integrons were investigated by PCR method of the conserved integrase gene with specific primers set of *int1* and *int2*. Also, to detect the gene cassettes of class 1 integron, PCR was performed with the primer pair CS-F/CS-R, which spans the entire cassette integrating site (3'-CS and 5'-CS) of integron (Table 2). Cassettes of class 2 integron was investigated by another PCR with the primer pair hep74 and hep51, specific to the conserved regions of class 2 integron, following previously described conditions and procedures [10-12]. Eventually, to determine the content of gene cassettes both DNA strands of the PCR products were sequenced by Macrogen Inc. (Seoul, South Korea).

4. ERIC-PCR

Genomic DNA was extracted from all isolates by DNA extraction kit (Bioneer, Daejeon, South Korea) following the manufacturer's instructions. Genotyping of the *Shigella* isolates was performed using primers (Table 2) and protocol of Dalla-Cos-

Table 1. *Shigella* species isolated from different parts of Iran used in this study

City	No. (%) isolates				
	Shigella dysenteriae	*Shigella flexneri*	*Shigella boydii*	*Shigella sonnei*	Total
Ardabil	0 (0)	10 (55.6)	4 (22.2)	4 (22.2)	18
Kerman	2 (4)	31 (62)	4 (8)	13 (26)	50
Urmia	1 (2.5)	10 (25)	5 (12.5)	24 (60)	40
Tabriz	3 (8.8)	22 (64.7)	3 (8.8)	6 (16.7)	34
Total	6 (4.2)	73 (51.4)	16 (11.3)	47 (33.1)	142

Table 2. Oligonucleotides used in this study

Primer	Oligonucleotide (5'-3')	Produced size (bp)	References
int1-F	CAGTGGACATAAGCCTGTTC	160	[10-12]
int1-R	CCCGAGGCATAGACTGTA		
int2-F	CACGGATATGCGACAAAAAGGT	789	[10-12]
int2-R	GTAGCAAACGAGTGACGAAATG		
CS-F	GGCATCCAAGCAGCAAG	Variable	[10-12]
CS-R	AAGCAGACTTGACCTGA		
hep74	CGGGATCCCGGACGGCATGCACGATTTGTA	Variable	[10-12]
hep51	GATGCCATCGCAAGTACGAG		
ERIC-1	ATGTAAGCTCCTGGGGATTCAC	Variable	[13]
ERIC-2	AAGTAAGTGACTGGGGTGAGCG		

ta et al [13]. ERIC-PCR patterns were evaluated with GelClust software [14]. Similarity for a potential clonal relatedness was determined with Dice Unweighted Pair Group Method with Arithmetic Mean (UPGENA) method [13].

Results

1. Patient data

The age distribution data revealed that *Shigella* species were isolated from 59 cases (41.5%) in the <5-years, 44 (31%) in the 6–12-years and 39 (27.5%) in >13-years old age groups. Considering the gender of patients, 81 (57%) were male and 61 (43%) were female patients.

2. Antimicrobial profiles

There was a high percentage of resistance to TMP/SMX, AMP, STR and TET. A lower percentage of resistance was also against AZM, FOX, OFX, CIP and LVX while no resistance was found to AMK and carbapenems including IPM and MEM (Table 3). MDR phenotype was seen in 95.1% of the total isolates.

3. Distribution of class 1 and 2 integrons

Among the total of 142 isolates, 79 (55.6%) carried class 1 integron (*int*+) and 114 (80.3%) carried class 2 integron (*int*+). PCR amplification of internal variable region of 79 class 1 integron positive strains using CS-F/CS-R primers produced two different products of approximately 750 and 1600 bp in 3 (3.8%) and 25 (31.7%) isolates, respectively. Fifty-one (64.5%) isolates produced no products, which is indicative of an empty integron with no resistance gene cassette. Sequence analysis of variable region indicated the presence of *dfrA7*, and *dfrA17-aadA5* resistance gene cassettes among the isolates, which correspond to 750 and 1600 bp PCR products, respectively (Fig. 1). Amplification of internal variable region for 114 class 2 integron positive isolates using hep74 and hep51 primers showed, 108 (94.7%) isolates harbored resistance gene cassettes; however, no gene cassette was obtained for another six (5.3%) integron-positive isolates. In addition, two types gene cassette were identified in class 2 integrons. Seventy-five (69.4%) strains had a 2,300 bp fragment and 33 (30.6%) strains had a 1,500 bp fragment. Sequence analysis of the gene cassette indicated the presence *dfrA1-sat1-aadA1* and *dfrA1-sat1* resistance gene cassettes that are related to 2,300 and 1,500 bp fragments, respectively (Fig.1).

4. Genotyping

The 73 *S. flexneri* isolates were classified into 6 major clusters (A to F) and 41 subcluster. Moreover, five different genotypes (A to E) and 28 subtypes were found among the 47 *S. sonnei* isolates. All of 16 *S. boydii* and 6 *S. dysenteriae* isolates were also classified into 4 major clusters (A to D) and 13 subcluster as well as 3 cluster (A to C) and 4 sub cluster, respectively (Fig. 2-4).

Discussion

The emergence and dissemination of MDR *Shigella* strains have become an important concern worldwide which can complicate the selection of empirical agents for the treatment of shigellosis [15]. Our findings revealed that 95.1% of *Shigella* spp. isolated from patients showed a MDR phenotype. Most common MDR profiles observed in this study were TMP/SMX/STR/AMP (76.1%), TMP/SMX/STR/TET (73.2%) and TMP/SMX/STR/AMP/TET (62.7%). Previous studies have also shown the dominance of resistance to TMP/SMX and STR and foremost

Table 3. Antimicrobial resistance among *Shigella* isolates collected from Iran

Antibiotics	No. (%) isolates				
	Shigella flexneri (N = 73)	*Shigella sonnei* (N = 47)	*Shigella boydii* (N = 16)	*Shigella dysenteriae* (N = 6)	Total (N = 142)
Trimethoprim-sulfamethoxazole	66 (90.4)	47 (100)	14 (87.5)	6 (100)	133 (93.7)
Streptomycin	67 (91.8)	38 (80.9)	10 (62.5)	5 (83.3)	120 (84.5)
Ampicillin	68 (93.2)	36 (76.6)	14 (87.5)	6 (100)	124 (87.3)
Tetracycline	62 (84.9)	34 (72.3)	11 (68.7)	5 (83.3)	112 (78.9)
Cefazolin	46 (63)	34 (72.3)	10 (62.5)	3 (50.0)	93 (65.5)
Amoxicillin-clavulanate	52 (71.2)	22 (48.6)	7 (43.8)	2 (33.3)	83 (58.5)
Ceftriaxone	38 (52.0)	30 (63.8)	7 (43.8)	3 (50.0)	78 (54.9)
Cefotaxime	39 (53.4)	30 (63.8)	6 (37.5)	3 (50.0)	78 (54.9)
Aztreonam	25 (34.2)	16 (34.0)	5 (31.2)	3 (50.0)	49 (34.5)
Nalidixic acid	19 (26.0)	17 (36.2)	8 (50.0)	0 (0)	44 (31.0)
Ceftazidime	25 (34.2)	12 (25.5)	5 (31.2)	2 (33.3)	44 (31.0)
Cefepime	21 (28.8)	12 (25.5)	5 (31.2)	3 (50.0)	41 (28.9)
Chloramphenicol	28 (38.4)	1 (2.1)	4 (25.0)	0 (0)	33 (23.2)
Gentamicin	6 (8.2)	16 (34.0)	3 (18.8)	1 (16.7)	26 (18.3)
Azithromycin	6 (8.2)	6 (12.8)	4 (25.0)	0 (0)	16 (11.3)
Cefoxitin	7 (9.5)	3 (6.4)	0 (0)	0 (0)	10 (7.0)
Ciprofloxacin	1 (1.4)	0 (0)	5 (31.2)	0 (0)	6 (4.2)
Ofloxacin	1 (1.4)	0 (0)	5 (31.2)	0 (0)	6 (4.2)
Levofloxacin	0 (0)	0 (0)	4 (25.0)	0 (0)	4 (2.8)
Amikacin	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Meropenem	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Imipenem	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

resistance phenotype were TMP/SMX/TET/TMP among *Shigella* spp. in Iran and other countries [5, 15-17]. Therefore, these antibiotics should no longer be considered as appropriate empirical therapy. Additionally, cephalosporins are considered as alternative drugs for shigellosis treatment. Unfortunately, this study has demonstrated emerging resistance to these drugs with 65.5%, 54.9%, 54.9% and 31% resistance rate to cefazolin, cefotaxime, ceftriaxone and ceftazidime, respectively. A previous study in China had shown low cephalosporin resistance among *Shigella* spp [15]. It is also noteworthy that the result of present study showed a high dissemination of cephalosporins resistance among *Shigella* spp. in Iran compared with two previous studies [2, 18]. Importantly, 28.9% of our collected *Shigella* spp. were also resistant to cefepime. The emergence of co-resistance to older antibiotics and 3rd- or 4th-generation cephalosporin's in *Shigella* is an excessive challenge to the public health for the effective treatment of shigellosis. The main mechanism of resistance to cephalosporin's in our isolates was production of extended spectrum beta-lactamase enzymes

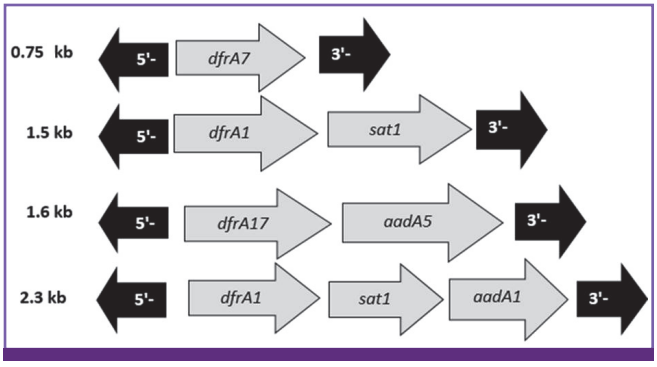

Figure 1. Structures of class 1, 2 integrons variable regions found in this study.

(unpublished data). However, in the present study, we observed high in vitro activity of azithromycin (88.7%), fluoroquinolone (95.8-97.2%), amikacin and carbapenems (100%), making them the recommended drugs of choice for treatment of shigellosis.

Noteworthy this is the first comprehensive report about the

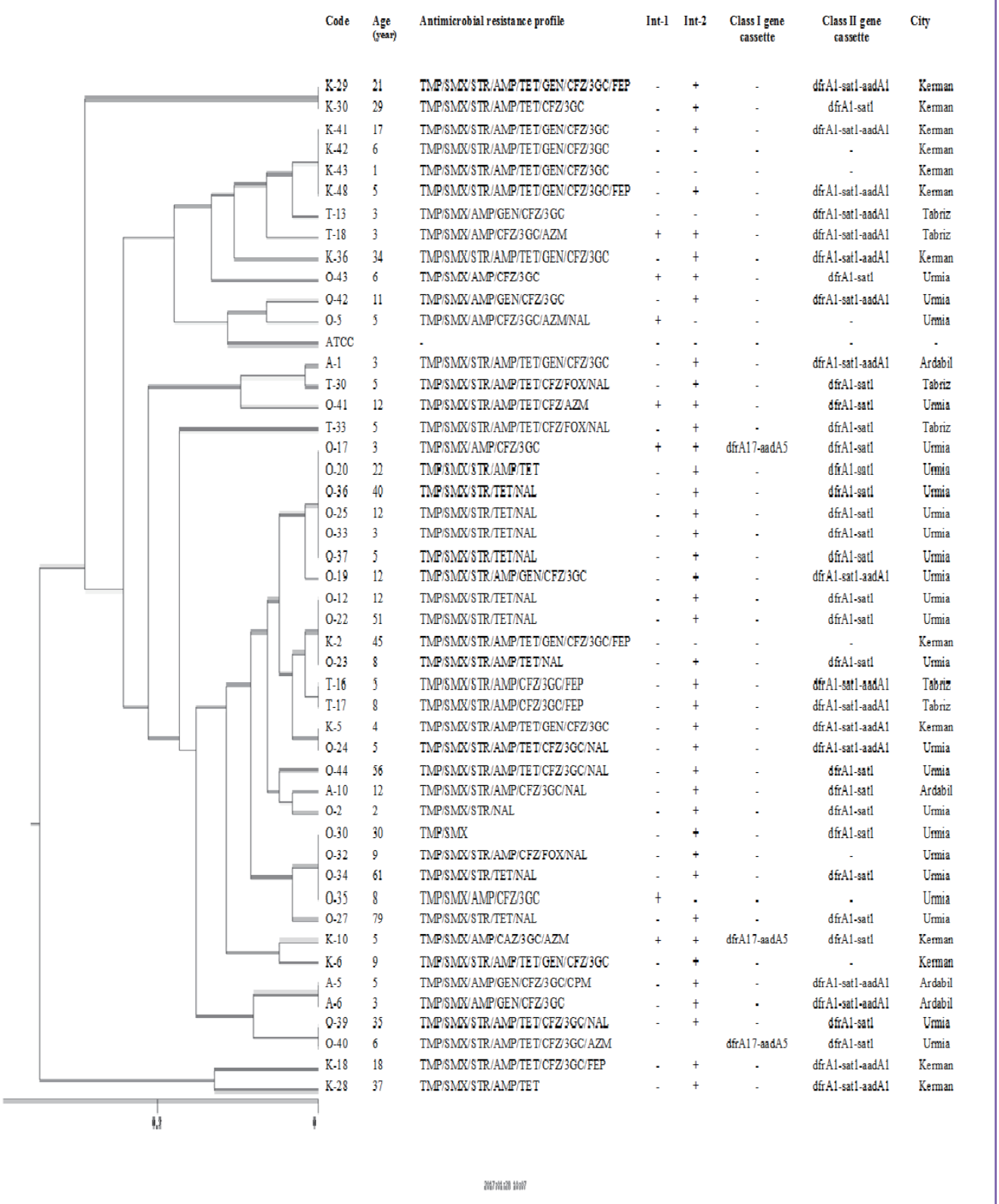

Figure 2. Dendrogram based on ERIC-PCR of 73 *Shigella flexneri* in relation to the resistance profile and integron resistance gene content.

ERIC, enterobacterial repetitive intergenic consensus; PCR, polymerase chain reaction; AMP, ampicillin; FOX, cefoxitin; CFZ, cefazolin; 3GC, 3rd generation of cephalosporins; FEP, cefepime; GEN, gentamicin; STR, streptomycin; NAL, nalidixic acid, OFX, ofloxacin; CIP; ciprofloxacin; LVX, levofloxacin; CHL, chloramphenicol; TMP/SMX, trimethoprim-sulfamethoxazole; TET, tetracycline; AZM, azithromycin; Int 1, integrase 1; Int 2, integrase 2.

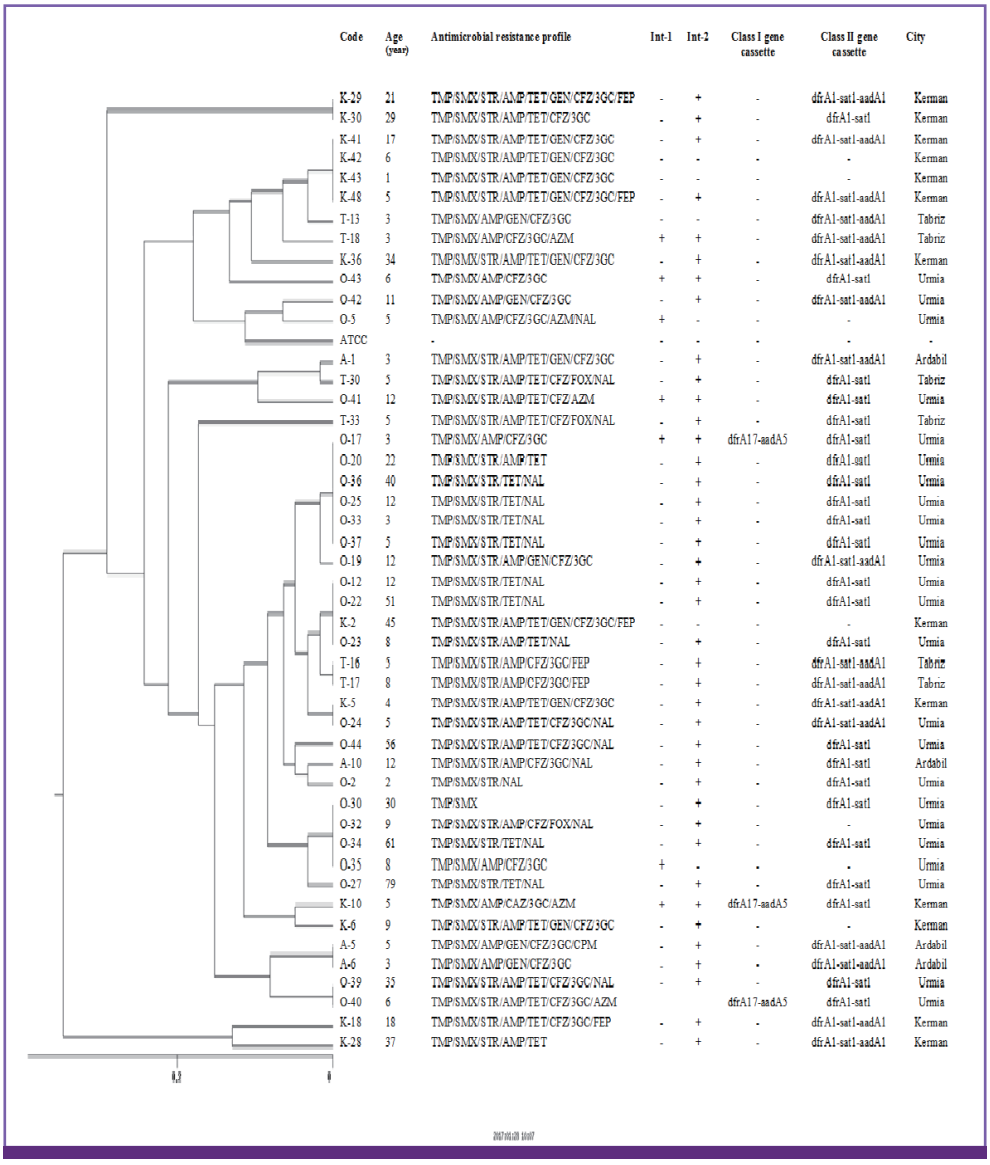

Figure 3. Dendrogram based on ERIC-PCR of 47 *Shigella sonnei* in relation to the resistance profile and integron resistance gene content.
ERIC, enterobacterial repetitive intergenic consensus; PCR, polymerase chain reaction; AMP, ampicillin; FOX, cefoxitin; CFZ, cefazolin; 3GC, 3rd generation of cephalosporins; FEP, cefepime; GEN, gentamicin; STR, streptomycin; NAL, nalidixic acid; OFX, ofloxacin; CIP, ciprofloxacin; LVX, levofloxacin; CHL, chloramphenicol; TMP/SMX, trimethoprim-sulfamethoxazole; TET, tetracycline; AZM, azithromycin; Int 1, integrase 1; Int 2, integrase 2.

integrons cassettes in all four *Shigella* species from different parts of Iran. Previous studies have also shown the similar array of gene cassettes of class 1 integrons among enteric bacteria including *Proteus*, *E. coli*, *Klebsiella pneumoniae* and *Shigella* spp. in Iran and other countries, which provide evidence for transfer of resistance genes through integrons between different species [16, 17, 19, 20]. The class 2 integron was more frequently detected among the isolates (94.7%). Similar observations in other countries have shown the high prevalence of class 2 integron among *Shigella* spp. [3, 21, 22].

Furthermore, two different gene cassettes detected in class 2 integrons among our isolates (1,500 and 2,300 bp in size). These two arrays were defined as *dfrA1/sat1* (30.6%) and *dfrA1/sat1/aadA1* (69.4%). Interestingly, this high prevalence of class 2 integron with dominant of *dfrA1/sat1/aadA1* arrays found in *Shigella* spp. in the present study is in conformity with previous studies conducted [23, 24]. The occurrence of gene cassettes *dfrA1/sat1/aadA1* in *Shigella* flexneri (73.9%) and gene cassettes *dfrA1/sat1* in *Shigella sonnei* (51%) were more than the other *Shigella* species.

Uncommonly, nineteen isolates with empty class 1 and class 2 integron were resistance to TMP/SMX This can be attributed

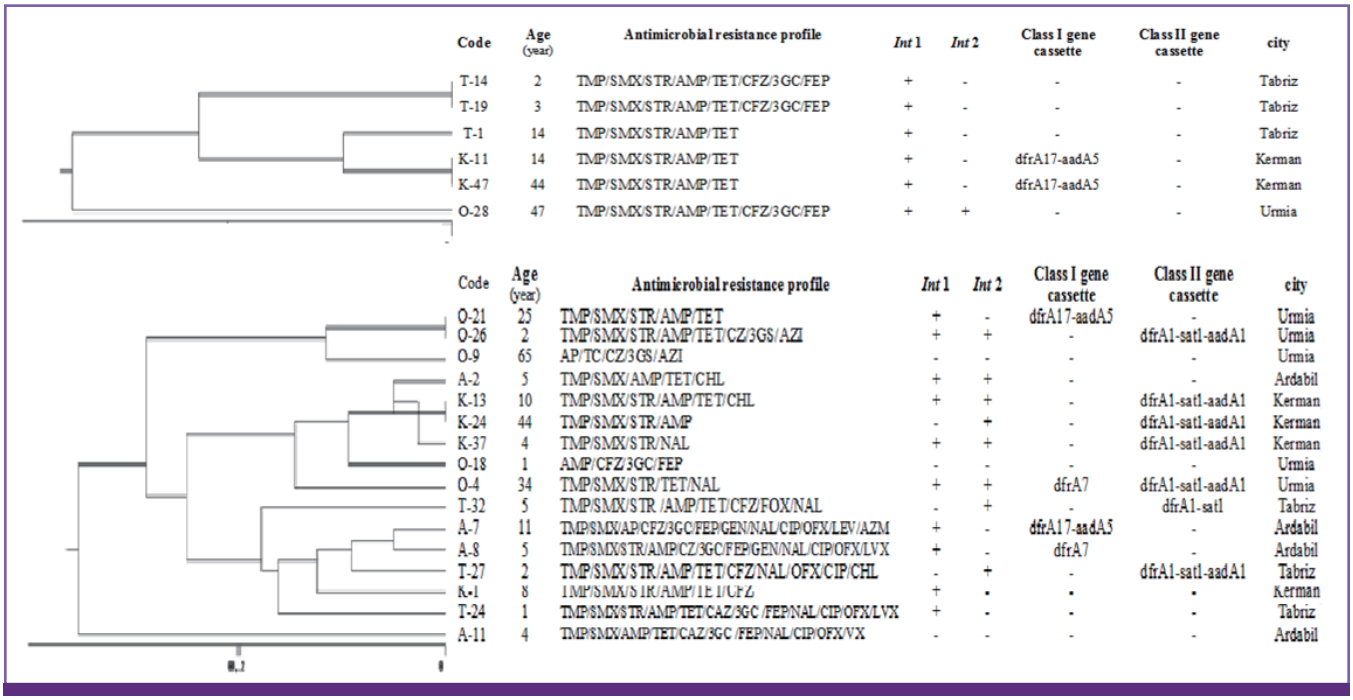

Figure 4. Dendrogram based on ERIC-PCR of 16 *Shigella boydii* and 6 *Shigella dysenteriae* in relation to the resistance profile and integron resistance gene content.

ERIC, enterobacterial repetitive intergenic consensus; PCR, polymerase chain reaction; AMP, ampicillin; FOX; cefoxitin; CFZ, cefazolin; 3GC, 3rd generation of cephalosporins; FEP, cefepime; GEN, gentamicin; STR, streptomycin; NAL, nalidixic acid; OFX, ofloxacin; CIP, ciprofloxacin; LVX, levofloxacin; CHL, chloramphenicol; TMP/SMX, trimethoprim-sulfamethoxazole; TET, tetracycline; AZM, azithromycin; Int 1, integrase 1; Int 2, integrase 2.

to sulfanilamide resistance gene (*sul1*) offered as part of the 3 conserved segment of all class 1 integrons [25]. Furthermore, taking into account 16 (11.2%) isolates resistant to STR that did not carry a class 1 or 2 integron, indicated that mechanisms other than integrons mediate STR resistance in this isolates of *Shigella*.

All *dfrA* carrying isolates were resistant to TMP/SMX, except three *S. flexneri* (T2, K32, K45) that this isolates harboring *dfrA1/sat1/aadA1* gene cassette of class 2 integron and were sensitive to TMP/SMX. Also all *aadA* carrying isolates were resistant to streptomycin, except four *Shigella* isolates (T8, T18, A7, K10) harboring resistance gene cassette with sensitive phenotype, maybe due to the different promoter strength or even inactive promoters. Some previous studies have reported similar results in *Shigella* species [17, 24]. Except two, all isolates that carried *dfrA1/sat1/aadA1* gene cassette array, were resistant to TMP/SMX and STR. The results indicate the importance of this gene cassette array in high resistance to TMP/SMX and STR.

A little information on the integron content of *S. boydii* and *S. dysenteriae* strains is available. In our study among 16 isolates of *S. boydii*, five isolates carried class 1 integron with two different gene arrays (*dfrA17-aadA1* in 2, *dfrA7* in one isolate) and six isolates contained class 2 integron with only *dfrA1/sat1/aadA1* gene cassette array. All of the six *S. dysenteriae* in the present study were resistance to streptomycin and trimethoprim-sulfamethoxazole. However, only two isolates (K11, K47) harbored *dfrA17-aadA5* gene cassette that related to a 1,600 bp product of class 1 integron and 4 other isolates did not carry any gene cassette of class 1 or 2 integron. So the cause of resistance to these antibiotics in these isolates could be due to other mechanisms of resistance that need to further researches.

Notably, our results showed that 21 *Shigella* isolates (14.7%) harbored both class 1 and 2 integrons simultaneously: 13 *S. flexneri* (with *dfrA1/sat1/aadA1* + *dfrA17/aadA5* gene arrays), six *S. sonnei* (with *dfrA1/sat1* + *dfrA17-aadA5* gene arrays) and two *S. boydii* (one with *dfrA1/sat1/aadA1* + *dfrA17-aadA5* and one with *dfrA1/sat1* + *dfrA7* gene cassettes arrays). This dual occurrence has been reported earlier only in 1, 7 and 10 isolates from Brazil [26] and Korea [27].

The results of ERIC-PCR analysis of 73 *S. flexneri* showed a major cluster (cluster E) with 22 isolate constituting 30.13% of total isolates specially distributes in Kerman and Tabriz province, all of which uniformly showed identical *dfrA1/sat1/aadA1* resistance gene cassette array of class 2 integron content. ERIC-PCR patterns of *S. sonnei* in this study has shown the main cluster with 30 isolates (cluster D) constituted 63.8% of the total

S. sonnei, all of which (except seven isolates) uniformly showed *dfrA1/sat1* resistance gene cassette of class 2 integron and absence of class 1 integron in these isolates (except three isolates). Considering the ERIC-PCR results an impressive feature is the high genetic homogeneity of the studied isolates. This result strongly suggests the distribution of a limited *S. sonnei* clone among Iranian population. Similar limited diversity in strains of *Shigella* spp. has been reported previously in India [28] and Japan [29]. Of the 16 *S. boydii* isolates, 4 different genotypes were obtained: 3 isolates in type A, 6 isolates each in type B and C and one isolate in type D. From six fluoroquinolone resistance isolates in this study, four isolates belonging to major cluster C. In *S. boydii*, strains belonging to the same ERIC cluster but having different antimicrobial resistance profile were observed, this is probably due to an integron or other mobile elements carrying the resistance gene integrated in the chromosome [30]. Eventually, in *S. dysenteriae*, isolates with the similar antimicrobial resistance profile and integron content placed in unique cluster and were found to be closely related.

In conclusion, we have identified prevalence of high resistance to widely used antimicrobial agents for shigellosis and demonstrated that MDR phenotype is common among *Shigella* species in Iran. To the best of our knowledge, this study was the first comprehensive and largest analysis of the genetic characteristics of integrons, their association with antibiotic resistance and ERIC-PCR typing of the clinical isolates of *Shigella* species from Iran. Significantly, the ERIC patterns in this study were associated with similar resistance phenotypes and the predominance of class 2 integrons in most strains of *Shigella* spp. Finally, the results suggest a few specific clones are responsible of shigellosis in these regions of Iran.

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Conflicts of interest

No conflict of interest.

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