Molecular epidemiology and genetic characterization of *Shigella* in pediatric patients in Iran

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SUMMARY

Infection with *Shigella* is considered a major cause of morbidity and mortality in children with diarrhea in developing countries, especially in Iran. Due to the importance of country-level epidemiological data, molecular characterization of genetic determinants of *Shigella* spp. is a necessity.

The aim of the present study was to investigate the prevalence of integron types, bla_{CTX-M}, bla_{SHV} and bla_{TEM} β-lactamase genes of *Shigella* isolates in pediatric patients in Tehran, Iran. In a time period of 18 months from May 2015 to August 2017, 75 *Shigella* spp. were isolated from non-duplicative diarrheal stool specimens in six different hospitals in Tehran. The isolates from patients were further analyzed for their antibiotic susceptibility and extended-spectrum beta-lactamase (ESBL) production. Polymerase chain reaction was performed for amplification of the integrons (I, II, III), *TEM*, *SHV*, *CTX-M15*. The prevalence of *S. sonnei*, *S. flexneri*, *S. dysenteriae* and *S. boydii* were 40 (53.3%), 33 (44%), 1 (1.3%) and 1 (1.3%), respectively. The results of an antimicrobial resistance test showed that the high percentage of resistance to nalidixic acid (NA), ampicillin (AMP) and trimethoprim/sulfamethoxazole (SXT) included 38 (50.6%), 59 (81.3%) and 64 (88%) isolates, respectively. Further results revealed that 52% and 76% of *Shigella* isolates carried intI and intII genes, respectively. In this study, the rates of CTX-M (10.7%), SHV (28%) and TEM (21.3%) were determined, all of which were positive for blaCTX-M15. This study showed the high prevalence of multidrug resistant *S. sonnei* and *S. flexneri.* Furthermore, it highlighted the increasing integrons (*intI* and *intII*) and ESBL genes, especially $bla_{CTX-M15}$, in *Shigella* isolates.

Keywords: *Shigella*, anti-bacterial agents, diarrhea, ESBLs, integrons.

INTRODUCTION

Shigellosis is a global health problem with signs of gastroenteritis and bacillary dysentery, especially in children less than 5 years old [1, 2]. Approximately 165 million cases and 1.1 million deaths happen annually, with the distribution of these adverse outcomes weighted heavily towards developing countries [2]. Shigellosis in humans are caused by four *Shigella* spp., *S. sonnei*, *S. flexneri*, *S. dysenteriae*, and *S. boydii*. The frequency of different serogroups varies depending on geographic location [2]. Besides the self-limiting period of the infection, antibiotic therapy has been effective in alleviating bacillary dysentery for the past several decades. *Shigella* strains have increasingly acquired resistance to various antimicrobials, such as tetracycline/streptomycin (*i.e.*, in combination),

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ampicillin, and trimethoprim/sulfamethoxazole. Elements of antimicrobial resistance in *Shigella* strains are commonly borne within mobile genetic elements (MGEs) [3]. MGEs may mediate the distribution of resistance factors among species, even genera. The gene-capture systems, or integrons, are conserved sequences (3′-CS and 5′-CS) of bacterial genome that are able to obtain gene cassettes, which can carry antimicrobial resistance elements, by site-specific recombination [4]. The most common classes of integrons are the transferable class I integron followed by class II and class III integrons, respectively [3]. Class I integrons harbor many antimicrobial resistance-determinants cassettes that encode extended spectrum β-lactamase (ESBLs), dihydroflavonol-4-reductase/trimethoprim (dfr), disinfectants and aminoglycoside-modifying enzymes (AMEs), and sulfonamide (sul1) [5, 6]. Class II integrons are frequently present in *S. sonnei* isolates and their gene cassette arrays are commonly constant, consisting of dfrA1 and aadA1 [6]. These genes confer resistance to trimethoprim and streptomycin, respectively [5]. Class III integrons are located in transposable elements (TEs) and have been described, but the 3′-CS is still not well defined [5].

Resistance to the new cephalosporins is facilitated by production of ESBLs [5]. ESBL production is often related to resistance to other classes of antibiotics, leading to considerable limitations when attempting to treat individuals with ESBL-producing strains of *Shigella* (or other bacteria) [7]. ESBL-producing *Shigella* isolates have been related to numerous endemic and epidemic outbreaks throughout Europe [8]. In the United States, such outbreaks have newly been reported in Asia, including in Iran [9-11]. In recent years, the increasing use third-generation cephalosporins (3GC) for treatment of shigellosis can be responsible for emergence of ESBLs producing *Shigella* sp. [11]. Therefore, understanding about molecular aspect of antimicrobial resistance-conferring genetic elements of *Shigella* species is important because of both epidemiological and clinical indications in developing countries and Iran [8]. The aim of the present study was to investigate the prevalence of integron types, *blaCTX-M*, and *blaSHV* and *blaTEM* β-lactamase genes of *Shigella* isolates in pediatric patients in Tehran, Iran.

MATERIALS AND METHODS

In this cross-sectional study, 75 out of the 946 samples analyzed were obtained from children with sporadic diarrhea admitted to six teaching/therapeutic centers (Children's Medical Center and Bahman, Shariati, Valiasr, Imam Khomeini and Mofid Hospitals) during a period of 18 months (from May 2015 to October 2016) in Tehran, Iran. Among these isolates, 11 (14.66%) and 64 (85.33%) were related to hospitalized and non-hospitalized patients, respectively. This study assessed the use of exclusion criteria in sampling after the initial one day of the onset of symptoms, consumption of antibiotics before sampling, samples without a label and questionnaire, and receiving the sample more than three days after collection. The specimens were immediately transferred to Department of Microbiology in Tehran University. For the isolation of *Shigella* spp., samples were streaked onto Salmonella-Shigella and MacConkey agar plates and incubated at 37 °C for 24 hours. Biochemical identification was performed by standard methods. In order to identify species, serological reactions were done by the slide agglutination test with specific antisera (Denka Saiken, Tokyo, Japan). *S. boydii* ATCC 9207, *S. dysenteriae* ATCC 13313, *S. sonnei* ATCC 1202, and *S. flexneri* ATCC 9290 were used as quality controls in each test. Antimicrobial susceptibility testing (AST) was done for all *Shigella* isolates, irrespective of the serotype. Furthermore, 84.4% of the children in this study who resulted positive for one of the four *Shigella* isolates were non-hospitalized.

Antimicrobial susceptibility testing

In agreement with Clinical and Laboratory Standards Institute (CLSI document M100-S14) guidelines, antimicrobial susceptibility was carried out on the Mueller-Hinton agar plates (Merck Co., Germany) using the Kirby-Bauer (KB) method disc diffusion to the following antimicrobials: gentamicin (GM,10 µg), chloramphenicol (CHL, 30 µg), nalidixic acid (NA, 30 µg), ciprofloxacin (CP, 5 µg), tetracycline (TET, 30 µg), ampicillin (AMP, 20 µg), imipenem (IPM 10 µg), co-trimoxazole (SXT, 5 µg), cefotaxime (CTX, 30µg), ceftazidime (CAZ, 30 µg), ceftriaxone (CRO, 30µg) and azithromycin (AZM, 15 µg) (MAST Diagnostics, Merseyside, UK). ESBL phenotype of *Shigella* isolates was identified by double disc diffusion synergy test (DDST) method. Briefly, the test was performed using both

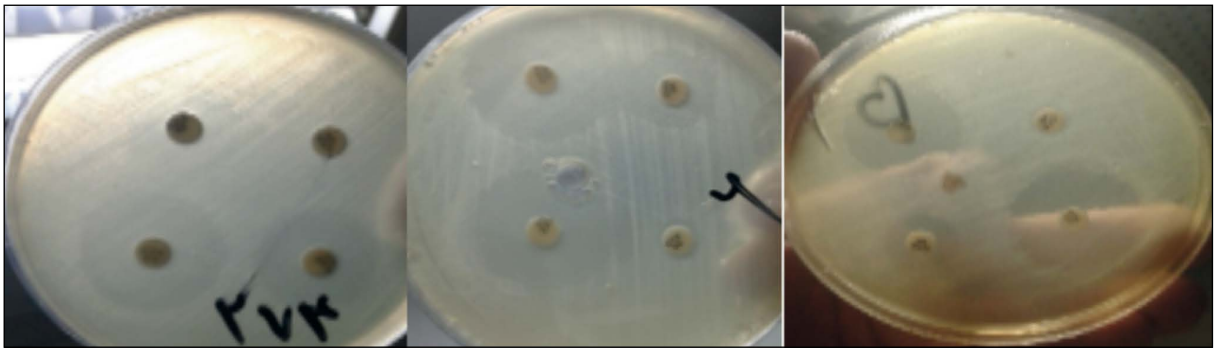

Figure 1 - Double disc diffusion synergy test (DDST) method. Disc synergy test for extended-spectrum beta-lactamases producing strains.

cefotaxime (CTX, 30µg) and ceftazidime (CAZ, 30µg) alone and in combination with clavulanic acid. An increase of 5 mm in the zone diameter for either CAZ and/or CTX in combination with CLA in contrast to its zone when used alone was considered as ESBL-producing strains (Figure 1) [13]. All ESBL-producer isolates were tested by the Etest (AB Biodisk, Solna, Sweden) to determine the MICs for CAZ and CTX according to CLSI document M100-S14 (12). All ESBLs-producing *Shigella* isolates were tested for CRO, CAZ and CTX susceptibility by E-test according to the manufacturer's guidelines (Liofilchem, Italy).

PCR and Multiplex-PCR method

Multiplex-PCR and PCR (*int*, *TEM*, *SHV*, *CTXM* and Amp-C genes) were performed by the PCR instrument mastercycler gradient (Eppendorf

Table 1 - Oligonucleotide primer sequences used for the amplification of *TEM*, *SHV* and *CTX-M* integrons and resistance genes.

Gene	*Primer Sequence (5′→3′)*	*Product size (bp)*	*Annealing Temperature °C*	*Reference*
bla*TEM*	F-ATGAGTATTCAACATTTCCG	868	55	(20)
	R-CAATGCTTAATCAGTGAGG			
bla*SHV*	F-AAGATCCACTATCGCCAGCAG	230	55	(21)
	R-ATTCAGTTCCGTTTCCCAGCGG			
bla*CTX-M1*	F- GAC GAT GTC ACT GGC TGA GC	499	55	(18)
	R- AGC CGCCGA CGCTAATAC A			
bla*CTX-M2*	F- GCG ACC TGG TTA ACT ACA ATC C	351	55	(18)
	R- CGGTAGTATTGCCCT TAA GCC			
bla*CTX-M4*	F- GCT GGA GAA AAG CAG CGG AG	307	55	(18)
	R- GTA AGC TGA CGC AAC GTC TG			
bla*CTX-M15*	F: CACACGTGGAATTTAGGGACT	995	55	(19)
	R: GCCGTCTAAGGCGATAAACA			
CTXM4	F: GCT GGA GAA AAG CAG CGG AG	474	60	(18)
	R: GTA AGC TGA CGC AAC GTC TG			
intI1	F- GGGTCAAGGATCTGGATTTCG	483	50	(15)
	R- ACAGGGTGTAAATCATCGTC			
intI2F	F- GCAAATGAAGTGCAACGC	466	50	(16)
	R- ACACGCTTGCTAACGATG			
intI3F	F- GCCTCCGGCAGCGACTTTCAG	980	55	(17)
	R- ACGGATCTGCCAAACCTGACT			

Co., Germany) for detection of *intI, intII, intIII*, bla_{TEM}, bla_{SHV}, bla_{CTX-M} and *AMP-C* genes. The overnight grown colonies on Xylose lysine deoxycholate (XLD) agar plates were selected for template genomic DNA extraction by boiling method. The primer sequences used in this study are listed in Table 1 [15-21]. The total volume of MPCR (int genes) and PCR (*blaTEM*, *blaSHV*, and *blaCTX-M*) reaction mixture was 20 µl, containing a 1.0 µl of extracted template DNA (variable in Multiplex-PCR), 2.0 µl of 10× PCR buffer, 0.6 µl $MgCl_2$ (50 mM), 0.6 µl dNTPs (10 mM), 1 µl of each primer, 0.7 µl of Taq DNA polymerase (5 U/µl) (Amplicon Co., Denmark) and 12.1 µl ddH_2O (variable in MPCR and PCR). The MPCR conditions for amplification of *int* genes were as follows: one cycle represents an initial denaturation at 95°C for 60s, 30 cycles of denaturation for 30 s at 94°C, annealing for 30s at 50˚C (Tm detail mentioned in Table1) and extension for 60s at 72°C, and a final extension for 6 min at 72°C and PCR conditions for amplification of ESBL genes were an initial denaturation at 95°C for 60s, 30 cycles of denaturation for 30 s at 94°C, annealing for 30s at 55°C (Tm detail mentioned in Table 1) and extension for 60s at 72°C, and a final extension for 7 min. Therefore, the reaction mixture was completed in a thermal gradient cycler (Eppendorf Co., Germany). PCR products were subjected to electrophoresis in a 1.0% agarose gel, stained with ethidium bromide (EtBr) and imagined under UV light. The PCR products were purified from the agarose gel using a QIAquick Gel Extraction kit (Qiagen, Germany). Both DNA strands of the PCR product were sequenced using an ABI automatic DNA sequencer (model 3730xl; Perkin-Elmer). The isolates positive for bla_{CTXM1} group, due to high prevalence $bla_{CTX-M15}$ in *shigella* isolates were further analyzed by PCR with $bla_{CTX-M15}$ specific primers (Table 1).

Data analysis

IBM SPSS Statistics program SPSS version 19.0 was used to the statistical analyses. Differences at a statistical level of $P<0.05$ were considered statistically significant.

RESULTS

Bacterial isolation

Over an 18-month period (from May 2015 to October 2016), 75 (7.9%), no repetitive isolates of *Shigella* spp. were collected at the six teaching Hospital (Tehran, Iran). The prevalence of *S. sonnei*, *S. flexneri*, *S. dysenteriae* and *S. boydii* were 40 (53.33%), 33 (44.0%), 1 (1.33%) and 1 (1.33%) respectively. Data analysis revealed that 14.66% of the children were hospitalized: seven (17.5%) were related to *S. sonnei*, 3 (33%) to *S. flexneri*, and a single case to *S. dysenteriae*. *Shigella sonnei* was detected in 12 (30%) patients in the ≤5 years age group and in 28 patients (70%) in the >5 years of age group, while *S. flexneri* was identified in 17 (51.50%) children in the ≤5 year age group, but this difference was not statistically significant (P=0.16). Twenty-three (57.5%) and ten (30.30%) of males were infected respectively with *S. sonnei* and *S. flexneri*.

Antibiotic resistance profile

All strains were screened for resistance to 12 antimicrobials and the resistance percentage to GM, IPM, Chl, NA, CP, TET, AMP, SXT, CTX, CAZ, CRO and AZM were 7%, 0%, 52%, 50.6%, 4%, 38.6%, 81.3%, 88%, 19.6%, 20%, 24% and 28% respectively, as shown in Figure 2. All isolates were susceptible to IPM. *S. flexneri* isolates showed high levels of resistance against AMP (81.3%), SXT (88%) and NA (50.6%) while low-level resistance showed to CIP (4%), IPM (0%) and CTX (19.6%). Moreover, 88%, 55% and 81.3% of *S. sonnei* isolates were resistant to SXT, NA and AMP, respectively (Figure 2). Therefore, all isolates were resistant to three different antibiotics (SXT, AMP, and NA) and the prevalence of multi-drug resistance (MDR) was 91%. DDST using CAZ, CTX in combination with CLA as an inhibitor revealed that, out of 75 *Shigella* isolates, 10.6% (n=8 [4 *S. flexneri* and 4 *S. sonnei*]) isolates were ESBL-producers (Figure 1).

Distribution of integrons in the Shigella strains

Identification of class I, II and III integrons in all *Shigella* isolates was performed using MPCR assay (Figure 3). The results revealed that 52% (39/75), 76% (57/75), 33.3% (25/75) of *Shigella* isolates carried *intI*, *intII* and both *intI*/*intII* genes. No class III integrons were detected. Class 1 integrons were found in *S. sonnei* 21 (52.5%) and *S. flexneri* 18 (54.5%), whereas class 2 integrons were found in a total of 57 (76%) strains: *S. sonnei* 34 (85%) and *S. flexneri* 23 (69.7%). Thirty-five (35%) of *S. sonnei* isolates carrying a class 1 integron showed an additional class 2 integron. Both class 1 and 2 integrons are present in 11 (33.3%) *S. flexneri*.

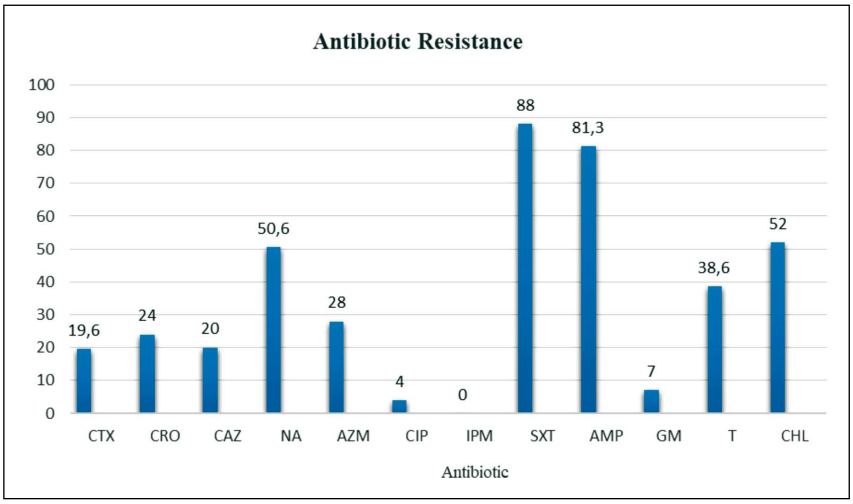

Figure 2 - Percentage of resistance in *Shigella* species. CTX, cefotaxime; CRO, ceftriaxone; CAZ, ceftazidime; NA, nalidixic acid; ATM, aztreonam; CIP, ciprofloxacin; IPM, imipenem; SXT, trimethoprim-sulfamethoxazole; AMP, ampicillin; GM, gentamicin; T, tetracycline; CHL, chloramphenicol.

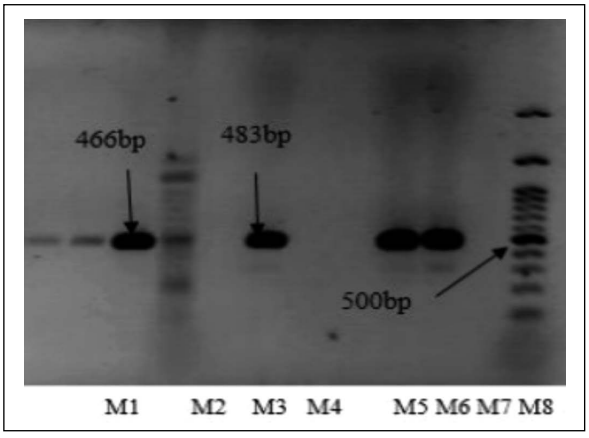

Figure 3 - Gel-Electrophoresis of the PCR products of integrons control samples (positive control: M1, M3, negative control: M2).

Detection of β*-lactamase genes*

PCR detection results gave rise to different *bla* genes encoding major ESBLs such as *TEM*, *SHV*, and *CTX-M* (Figure 4). All ESBL producers were positive for $bla_{CTX\text{-}M15}$ ESBLs genes amplification test showed that the prevalence of bla_{SHV} and bla_{TEM} were 37.5% (n; 3) and 25% (n; 2), respectively. The prevalence of *SHV* and *TEM* genes in *S. flexneri* were 1 (12.5%) and 1 (12.5%), respectively (Figure 5). Two (25%) and 1 (12.5%) of *S. sonnei* isolates carrying *SHV* and *TEM*, genes, respectively.

DISCUSSION

Shigella was recovered from 7.9% of stool during the study. However, other studies conducted in Iran reported the prevalence rates of 3-21.7%

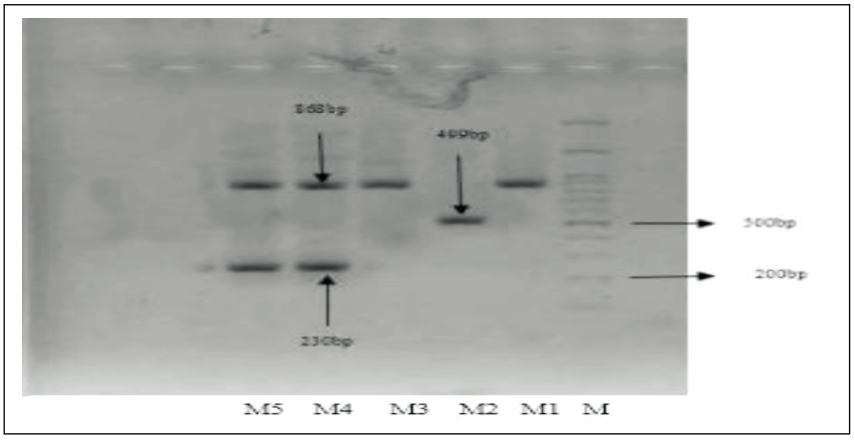

Figure 4 - PCR Products of Positive Controls for β-Lactamase gene; bla_{TEM} (Lanes M3, M4, M5), bla*CTXM1* (M2) and *blaSHV* (M4).

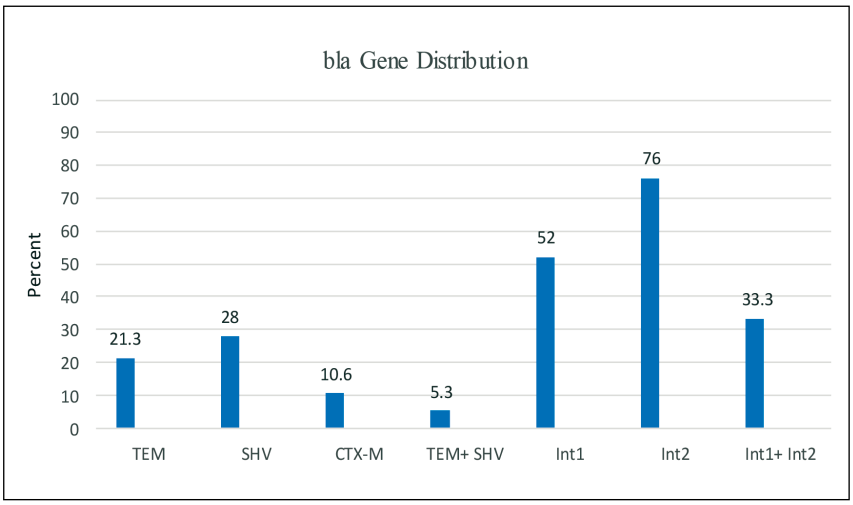

Figure 5 - Distribution of *bla* gene types and integron genes in 75 *Shigella* isolates.

[22-25]. This isolation rate is comparable with studies conducted from Argentina, and Ethiopia that documented rates of 9.7% and 7.5% respectively, but differed from 58% (Bangladesh), 4% (Nepal) and 3.3% (Indonesia) [26-28]. These contrasts may be due, in part, to continuing educational programs at elementary schools, aggressive infection-control measures, and possibly under-reporting of shigellosis cases by general practitioners. In the present study, 68.5%, 31.5% and 2.6% of non-duplicative *S. sonnei*, *S. flexneri* and *S. dysenteriae* serogroups were obtained from the all testes samples, respectively. *S. boydii* was not found in any of the stool samples. These data are consistent with Orrett et al., Khan et al., and Ranjbar et al. [29-31]. Our results showed that all isolates were susceptible to GM and IPM. 88% and 81.3% of *Shigella* isolates were resistant to SXT and AMP, respectively. 87.9%, 87.9% and 45.5% of *S. flexneri* were resistant to AMP, SXT and NA, respectively [31]. In the comparable study by Jafari et al., most *Shigella* isolates were resistant to AMP (95%) and SXT (91.7%) with greatest antibiotic resistance observed among *S. sonnei* (60.2% isolates) [32]. Shen and his colleagues showed that 88.0%, 89.2%, 85.5% and 79.5% of *S. flexneri* were resistant to AMP, NA, TET and SXT, respectively [33]. This conflict may be due to geographical distribution, source of samples and level of hygiene [34]. In different geographic regions of the world, *Shigella* isolates have become resistant to AMP and SXT, and quinolones such as NA [34]. In the present study, 50.6% of isolates were resistant to NA. It can be considered as a warning factor in drug therapy regimen in our country. From 75 strains in this study, 57 (34 (85%) *S. sonnei* and 23 (69.7%) S. *flexneri*) were positive for class 2 integron (int2+) and was the predominant class of integrons. These data are consistent with those of Bakhshi et al. and Ranjbar et al [6, 35]. In accordance with our results and on the basis of Jin et al., and Sow et al., Class 2 integron was the most predominant integron in *S. sonnei* [36, 37]. In contrast to our study, of 58 *S. flexneri* isolated from China in the study by Yuan Zhu et al., 91% isolates were MDR and 94.8%, 100% and 94.8% carrying class 1, 2 or both types of integrons, respectively [38]. Distribution of class 1 integrons in our study was 52% (n = 39/75) [52.5% *S. sonnei* and 54.5% *S. flexneri*]. In the current study, 8 (10.6%) of ESBL-producing *Shigella* were obtained in DDST, and all of them were positive for *blaCTX-M*. The prevalence of *blaSHV* and *blaTEM* were 37.5% (n=3 [1 (12.5%) *S. flexneri* and 2 (25%) *S. sonnei*) and 25% (n=2, [1 (12.5%) *S. flexneri* and 1 (12.5%) *S. sonnei*), respectively in ESBL isolates. Previous studies in Iran showed that the *blaCTX-M15* was the prevalent ESBL gene among clinical isolates of *Shigella* [39]. These data are similar to other studies conducted in Turkey and Saudi Arabia [40-42]. The outcome of a high prevalence of ESBL-producing genes in *Shigella* isolates will have disastrous consequences. Therefore, spread of resistance to 3rd generation cephalosporins to other areas must be managed and reemphasizes the necessity for the application of robust infection-control function

and careful use of antibiotics by clinicians in community and in hospitals. In agreement with Ranjbar et al., our results increase the level of concern about the distribution of ESBL among *S. sonnei* all over the country, because *S. sonnei* is now the commonest isolated *Shigella* species in Iran [39]. Shigellosis attributable to *S. sonnei* can pose a significant concern to patients and presents a challenge for illness management. The high prevalence of ESBL-producing *Shigella* species imposes a considerable concern to public health in Tehran, Iran [39]. Rigid surveillance policies as well as limits in drug prescription and following antimicrobial drug resistance patterns seems to be needed as the first step along with a judicious use of drugs to minimize the distribution of ESBL-producing *Shigella* [42]. Therefore, continuous monitoring should be made to prevent further spreading of resistant *Shigella* species. Finally, a number of potential limitations in this study need to be considered. First, gene cassettes inserted between the conserved segments of the integrons were not studied. In addition, presence of small sample size in our research was another limitation. Therefore, further investigations should be made to assess nucleotide sequences of the gene cassettes with a larger sample size. Taken together, these findings suggest significant prevalence of integrons and resistance gene markers in mobile genetic elements in *Shigella* spp., circulating in Tehran, Iran. In diarrhea-endemic areas, epidemiological data about the incidence and transmission of resistance genes through bacterial populations play a crucial role in monitoring antimicrobial resistance trends.

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Conflicts of interest

All authors: no conflicts.

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