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Determination of Antibiotic Resistance Pattern and frequency of *CTX-M*, *TEM*, and *SHV* B-Lactamase Encoding Genes among *Shigella* Isolates from Inpatients in Tehran, Iran

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ABSTRACT

Background and Objectives: The emergence of extended-spectrum β-lactamase (ESBL)-producing *Shigella* spp. is becoming a health concern worldwide. This study aimed to investigate antibiotic resistance pattern and frequency of bla_{CTX-M}, bla_{SHV}, and bla_{TEM} genes among *Shigella* isolates from patients in hospitals of Tehran, Iran.

Methods: In this cross-sectional study, 52 non-repeated *Shigella* strains were isolated from hospitalized patients in Milad, Emam Khomeini and Shariati hospitals in Tehran (Iran) from November 2015 to December 2016. Bacterial identification, serotyping, and antimicrobial susceptibility testing were performed according to the standard guidelines. The bla_{CTX-M}, bla_{SHV}, and bla_{TEM} resistance genes were identified using multiplex polymerase chain reaction.

Results: Among 52 *Shigella* isolates, *S. sonnei* (44.2%) was the predominant species, followed by *S. flexneri* and *S. dysenteriae* (23%). Over 67% of the isolates were multidrug resistant. The highest rates of resistance were observed against cefalotin (67.3%), tetracycline (67.3%), amikacin (63.5%), trimethoprim-sulphamethoxazole (48.1), and ampicillin (42.3%). The lowest resistance rate was against ciprofloxacin (1.9%). We detected the bla_{TEM} and bla_{CTX-M} genes in 61.5% and 19.2% of the isolates, respectively. However, the bla_{SHV} gene was not detected in any of the isolates. In addition, 16.4% of the isolates harbored the bla_{TEM} and bla_{CTX-M} genes simultaneously. Ciprofloxacin was the most effective antibiotics according to the ESBL genes distribution.

Conclusion: Our findings indicate the high prevalence of multidrug resistance and ESBL genes in *Shigella* isolates, which elucidates the need for appropriate infection control measures for limiting the spread of resistant strains.

Keywords: *Shigella*, Multiplex Polymerase Chain Reaction, Drug Resistance.

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INTRODUCTION

Shigellosis is a global human health problem and the leading cause of diarrhea, which results in about 700,000 deaths per year worldwide (1). Despite the recent public health improvements, incidence of shigellosis is still being reported regularly (2, 3). The genus *Shigella* belongs to the *Enterobacteriaceae* family and consists of four subgroups according to their biochemical and serological properties: A (*Shigella dysenteriae*), B (*Shigella flexneri*), C (*Shigella boydii*), and D (*Shigella sonnei*). All *Shigella* species can cause shigellosis, but *S. flexneri* and *S. sonnei* are the predominant causative agents in developing and industrialized countries, respectively (4).

Antibiotic therapy in patients with shigellosis can help reduce the duration of illness, shedding of the organism in the feces, and the risk of person-to-person spread. A range of antibiotics is effective for the treatment of shigellosis. However, treatment options are limited due to the emergence of multidrug-resistant (MDR) strains. The World Health Organization (WHO) has recommended ciprofloxacin as the first-line treatment (5), but increased resistance to this antibiotic and other fluoroquinolones has been reported in many countries (2, 6). Azithromycin, mecillinam, and ceftriaxone are also considered to be effective for treatment of shigellosis (5).

The emergence of resistance to third-generation cephalosporins (3GC) in *Shigella* spp. is an important public health concern, mainly in developing countries (7). Resistance to 3GC is largely due to production of extended-spectrum β-lactamases (ESBLs), which is often plasmid-mediated (8). Most ESBLs are derivatives of the TEM and SHV β-lactamase families, but CTX-M β-lactamases have also been associated with ESBL-producing strains. These β-lactamases show greater activity against cefotaxime than ceftazidime, but increased activity against ceftazidime can occur due to point mutations (9).

CTX-M, SHV, and TEM type ESBL producing *Shigella* species have been identified in different parts of the world (6, 8, 10). Studies on ESBL-producing *Shigella* isolates have revealed presence of insertion element upstream of the $bla_{\text{CTX-M}}$ gene, which may contribute to the mobility and dissemination of the genes t o other bacteria (8). The frequency of ESBL- producing *Shigella* species is increasing in Iran (11, 12). According to studies in Iran, the prevalence of ESBL-producing *Shigella* strains is higher than the rates observed in many other countries (11, 13). Studying the role of these isolates in infectivity and continuous monitoring of ESBL genes may be beneficial for determining appropriate treatment and control strategies in Iran. Therefore, the present study was conducted to evaluate antibiotic susceptibility profiles and to screen the presence of bla_{TEM}, $bla_{\text{CTX-M}}$, and bla_{SHV} genes among clinical isolates of *Shigella*.

MATERIALS AND METHODS

This cross-sectional study was conducted between November 2015 and December 2016 at three teaching hospitals in Tehran, Iran. Stool samples were collected from patients admitted with symptoms of diarrhea prior to administration of antibiotics. Samples were immediately transferred to laboratory and then inoculated on xylose lysine deoxycholate agar (Merck, Germany). The plates were incubated at 37 ºC for 24 hours and then *Shigella* suspected colonies were examined by conventional biochemical tests, including triple sugar iron agar, urea agar, SIM medium and IMVIC (indole, methyl red, Voges-Proskauer, and citrate) (14). Specific antisera (DIFCO, U.S.A) were used for serogrouping of *Shigella* isolates using a slide agglutination test (15). The study was approved by the Ethics Committee of Islamic Azad University of Tehran Medical Sciences (No: IR.IAU.TMU.REC.1396.275).

Antibiotic susceptibility testing was performed using the Kirby-Bauer disk diffusion method, according to CLSI guidelines (2017). The following antibiotic disks were used in the susceptibility testing: amikacin (30 µg), tetracycline (30 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg), furazolidone (100 µg), cefolatin (30 µg), doxycycline (30 µl), trimethoprim-sulfamethoxazole (25 µg), cefazolin (30 µg), gentamicin (120 µg), ceftriaxone (30 µg), and ceftazidime (30 µg) (Mast Diagnostics, UK). In addition, *E. coli* ATCC 25922 was used as the control strain (16). To confirm ESBL production, combination disk test using cefotaxime and ceftazidime disks (30 µg) with and without clavulanic acid (10 µg) was carried out on

Mueller Hinton agar (Merck, Germany) according to CLSI guidelines. Growth inhibition zone around cefotaxime or ceftazidime disks combined with clavulanic acid was compared with that around cefotaxime or ceftazidime disks alone. Growth inhibition zone diameter of >5mm with clavulanic acid than without confirmed ESBL production (16).

Boiling method was used for extraction of DNA from pure colonies after an overnight growth of *Shigella* isolates on Luria-Bertani agar (Oxoid, UK). Concentration of the extracted DNA was assessed by a spectrophotometer (Genway, England). Multiplex polymerase chain reaction (PCR) was performed using specific primers for simultaneous detection of the bla_{SHV}, bla_{CTX-M}, and bla_{TEM} (Table 1). PCR reactions were carried out in a final volume of 25 µl containing 2 µl DNA solution, Master Mix (CinnaGen Co., Iran), and 10 pmol of each primer. PCR amplification conditions were as follows: initial denaturation at 95 °C for 15 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 2 min, followed by final extension at 72 °C for 10 min (17). *Klebsiella pneumoniae* ATCC 700603 was used as positive control.

SPSS statistical software (version 20; SPSS Inc., Chicago, IL, USA) was used for data analysis. The chi-square test was used to evaluate significant differences in antimicrobial resistance and the correlation between resistance genes and antibiotics resistance. A P-value of less than 0.05 was considered statistically significant.

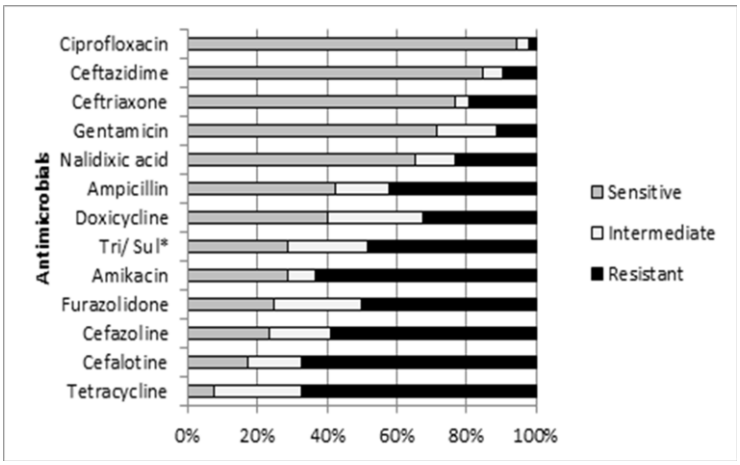
RESULTS

Of 945 samples collected during the study period, 52 *Shigella* strains were isolated. Among these isolates, *S. sonnei* was the predominant species (n=23, 44.2%), followed by *S. flexneri* (n=12, 23%), *S. dysenteriae* (n=12, 23%), and *S. boydii* (n=5, 9.6%). Figure 1 shows the antimicrobial resistance patterns of *Shigella* isolates against the tested antibiotics. Highest rate of resistance was against cefalotin (67.3%), tetracycline (67.3%), amikacin (63.47%), trimethoprim-sulphamethoxazole (48.1), ampicillin (42.3%), and doxycycline (32.7%). Moderate to low rate of resistance was observed against nalidixic acid (23.1%), ceftriaxone (19.2%), azithromycin (11.5), ceftazidime (9.6%), gentamicin (11.5%), and ciprofloxacin (1.9%). The isolates showed intermediate resistance to doxycycline (26.9%), trimethoprim-sulphamethoxazole (23.1%), cefazoline (17.3%), and gentamicin (17.3%). All *Shigella* isolates were resistant to nalidixic acid, but all *S. flexneri*, *S. dysenteriae*, and *S. boydii* isolates were sensitive to ciprofloxacin.

Table 1- Sequence of the primers used in multiplex PCR

Genes	Oligonucleotide sequence (5' to 3')	Amplicon size (bp)	References
bla_{SHV}	ATGCGTTATATTCGCCTGTG TGCTTTGTTATTCGGGCCAA	747	(18)
bla_{TEM}	TCGCCGCATACACTATTCTCAGAATGA ACGCTCACCGGCTCCAGATTTAT	445	(17)
bla_{CTX-M}	ATGTGCAGCACCAGTAAAGTGATGGC TGGGTAAAGTAAGTGACCAGAATCAGCGG	593	(19)

Figure 1-Antibiotic susceptibility pattern of *Shigella* isolates to various antibiotics. Tri/Sul*: trimethoprim/sulfamethoxazole



Strains resistant to three or more classes of antibiotics were identified as MDR (20). Multidrug resistance was detected in 15 (65.2%) *S. sonnei*, 10 (83.3%) *S. dysenteriae*, 8 (66.6%) *S. flexneri*, and 1 (20%) *S. boydii* isolates.

Multidrug resistance profiles of *Shigella* isolates are reported in Table 3. Phenotypic evidence of ESBL production was observed in 19 (36.5%) *Shigella* isolates, among which *S. dysenteriae* (7/12, 58.3%) was the predominant ESBL producer, followed by *S. flexneri* (4/12, 33.3%), *S. sonnei* (7/23, 30.4%), and *S. boydii* (1/5, 20%).

All 52 *Shigella* isolates were also tested for the presence of the bla_{TEM}, bla_{SHV}, and bla_{CTX-M} genes. All isolates were negative for bla_{SHV}, but 32 (61.54%) isolates harbored bla_{TEM}. Ten (19.23%) isolates contained bla_{CTX-M} and seven isolates (13.46%) carried both bla_{TEM} and bla_{CTX-M}. Figure 2 displays the PCR gel electrophoresis of the bla_{TEM}, bla_{CTX-M}, and bla_{SHV} genes. The frequency of bla_{TEM} was highest in *S. dysenteriae* (83.3%), followed by *S. sonnei* (60.87%). However, the frequency of simultaneous harboring of bla_{TEM} and bla_{CTX-M} was highest among *S. sonnei* isolates (Figure 3).

Figure 2- Multiplex PCR assay for detection of bla_{TEM}, bla_{CTX-M}, and bla_{SHV} on 1.5% agarose gel. Lane 1: negative control, lane 2-4: positive for bla_{TEM} and bla_{CTX-M}, lane 5-7: positive for bla_{TEM}, lane 8-10: positive for bla_{CTX-M}, and lane 11: positive control (*K. pneumoniae* ATCC 700603).

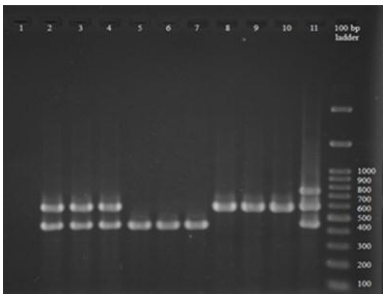
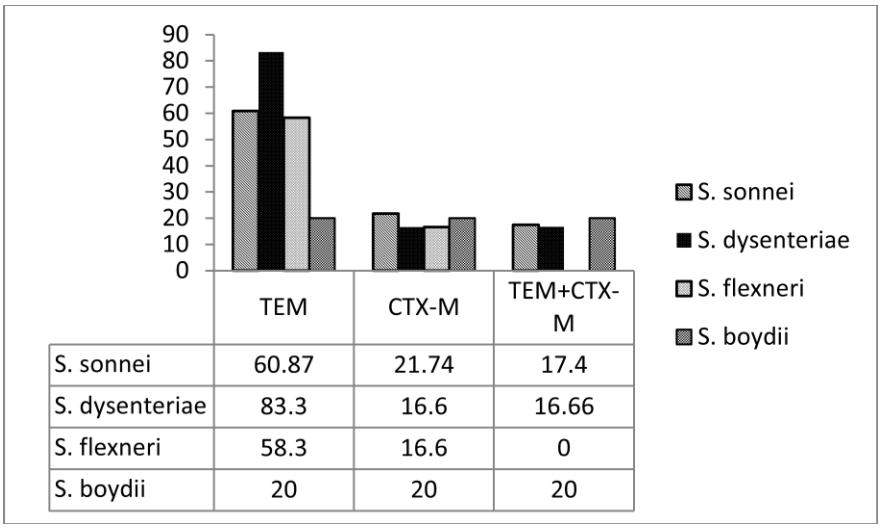


Figure 3- Frequency of the bla_{TEM}, bla_{CTX-M} and bla_{SHV} genes in *Shigella* isolates



	TEM	CTX-M	TEM+CTX-M
S. sonnei	60.87	21.74	17.4
S. dysenteriae	83.3	16.6	16.66
S. flexneri	58.3	16.6	0
S. boydii	20	20	20

All MDR *Shigella* strains carried at least one *bla* gene (Table 3). The results showed that all bla_{TEM}-containing *Shigella* strains were resistant to cefalotin, 75% were resistant to tetracycline, amikacin, and cefazolin, 72% were resistant to trimethoprim-sulfamethoxazole, and 55.5% were resistant to ampicillin and furazolidone. All *Shigella* strains that simultaneously carried bla_{TEM} and bla_{CTX-M} were resistant to ampicillin, amikacin, tetracycline, nalidixic acid, furazolidone, cefalotin, doxycycline, trimethoprim-sulfamethoxazole, cefazolin, and ceftriaxone. Furthermore, only one strain harboring both bla_{TEM} and bla_{CTX-M} was resistant to ciprofloxacin (Table 2).

Table 2- Multidrug resistance profile of *Shigella* isolates (n=35) based on the distribution of *bla* genes

Antimicrobial resistance profiles	Number (%) of resistant isolates					ESBL genes
	Total (n=34)	*S. sonnei* (n=15)	*S. dysenteriae* (n=10)	*S. flexneri* (n=8)	*S. boydii* (n=1)	
AK/T/KF	1 (2.9)	0	1 (10)	0	0	bla_{TEM}
AK/T/FZ/KF	1 (2.9)	1 (6.7)	0	0	0	bla_{TEM}
AK/T/KF/TS/CZ	3 (8.8)	0	1 (10)	2 (25)	0	bla_{TEM}
AP/AK/T/KF/CZ	1 (2.9)	0	1 (10)	0	0	bla_{TEM}
AK/T/FZ/KF/CZ	2 (5.9)	2 (13.3)	0	0	0	bla_{TEM}
AK/T/FZ/KF/TS	1 (2.9)	0	1 (10)	0	0	bla_{TEM}
AP/AK/T/NA/KF	1 (2.9)	1 (6.7)	0	0	0	bla_{TEM}
FZ/KF/DX/TS/CZ	1 (2.9)	1 (6.7)	0	0	0	bla_{TEM}
AK/T/KF/CZ/CRO	1 (2.9)	1 (6.7)	0	0	0	bla_{TEM}
FZ/KF/DX/CZ/CRO	1 (2.9)	1 (6.7)	0	0	0	bla_{CTX-M}
AP/AK/T/FZ/KF/CZ	1 (2.9)	1 (6.7)	0	0	0	bla_{TEM}
AP/AK/T//KF/TS/CZ	1 (2.9)	0	0	1 (12.5)	0	bla_{TEM}
AK/T/FZ/KF/TS/CZ	1 (2.9)	0	0	1 (12.5)	0	bla_{TEM}
AK/T/KF/DX/TS/CZ	1 (2.9)	0	0	1 (12.5)	0	bla_{TEM}
AP/AK/T/FZ/KF/TS/CZ	4 (11.8)	2 (13.3)	1 (10)	1 (12.5)	0	bla_{TEM}
AP/AK/T/FZ/KF/DX/TS/CZ	2 (5.9)	0	2 (20)	0	0	bla_{TEM}
AP/AK/T/NA/FZ/KF/TS/CZ/	1 (2.9)	0	1 (10)	0	0	bla_{TEM}
AP/AK/T/NA/FZ/KF/DX/TS/CZ	1 (2.9)	1 (6.7)	0	0	0	bla_{TEM}
AP/AK/T/NA/FZ/KF/DX/TS/CZ/CRO	1 (2.9)	0	1 (10)	0	0	bla_{TEM}; bla_{CTX-M}
AP/AK/T/NA/FZ/KF/DX/TS/CZ/GM/CRO	2 (5.9)	0	0	2 (25)	0	bla_{CTX-M}
AP/AK/T/NA/FZ/KF/DX/TS/CZ/CRO/CZA	1 (2.9)	1 (6.7)	0	0	0	bla_{TEM}; bla_{CTX-M}
AP/AK/T/NA/FZ/KF/DX/TS/GM/CRO	1 (2.9)	0	1 (10)	0	0	bla_{TEM}; bla_{CTX-M}
AP/AK/T/NA/FZ/KF/DX/TS/CZ/CRO/CZA	1 (2.9)	1 (6.7)	0	0	0	bla_{TEM}; bla_{CTX-M}
AP/AK/T/NA/FZ/KF/DX/TS/CZ/GM/ CRO/CZA	1 (2.9)	0	0	0	1 (100)	bla_{TEM}; bla_{CTX-M}
AP/AK/T/NA/CIP/FZ/KF/DX/TS/CZ/GM/CRO/CZA	2 (5.9)	2 (13.3)	0	0	0	bla_{TEM}; bla_{CTX-M}

AK: amikacin, T: tetracycline, NA: nalidixic acid, C: ciprofloxacin, FZ: furazolidone, KF: cefolatin, DXT: doxycycline, TS: trimethoprim-sulfamethoxazole, CZ: cefazolin, GM: gentamicin, CRO: ceftriaxone, and CZA: ceftazidime.

DISCUSSION

Shigellosis is a major cause of morbidity and mortality among children in the developing countries (21). MDR *Shigella* isolates narrow the choice of effective antimicrobials and treatment options for shigellosis (22). Besides, with the advent of ESBL-producing *Shigella* strains, 3GC is no longer effective against MDR *Shigella*. Hence, knowledge about the contribution of the MDR strains to infectivity in hospitalized patients and continuous monitoring of the involved genes in different regions can be beneficial for development and implementation of preventive and control measures.

S. sonnei and *S. flexneri* are the most commonly isolated species in industrialized (23) and developing countries (24), respectively. In our study, *S. sonnei* was the most common cause of shigellosis, which is consistent with findings of other studies in Iran (25-27). Reasons behind the dominance of *S. sonnei* in industrialized countries remain unclear (28), but an increasing incidence of shigellosis caused by *S. sonnei* generally correlates with improving economic prosperity (29).

In our study, most *Shigella* isolates were resistant and intermediately resistant to trimethoprim-sulfamethoxazole and ampicillin, the first-line drugs suggested by the CLSI for shigellosis (30). These results are in line with the results of other studies (31, 32). Quinolones are a good choice for the treatment of shigellosis (30). However, more than 34% of *Shigella* isolates in our study were resistant and intermediately resistant to nalidixic acid. In a study by Ghavam et al. in Iran, a similar frequency for resistance to nalidixic acid was observed (26), but in some countries higher resistance rates have been reported (7, 31).

Multi-drug resistance to the antimicrobial agents used for the treatment of shigellosis has been reported in many parts of the world (31, 33, 34). The results of our study suggest that multidrug resistance is common amongst all *Shigella* species, and this property was more notable in the case of *S. dysentrie*. Inappropriate prescription and the easy access to antibiotics among outpatients could facilitate the spread of MDR *Shigella* strains in the community (34).

In the case of infections caused by MDR strains, 3GC and fluoroquinolones are used for treatment of children and adults, respectively (35). We detected very little resistance to ciprofloxacin (1.92%), which is comparable with the results of other studies in Iran (12, 27, 36). However, a higher prevalence for ciprofloxacin-resistant *Shigella* isolates has been documented in other countries (31, 33, 34). In our study, the rate of resistance to ceftriaxone was 19.23%, which is higher than the rates reported by studies in Iran (11, 27, 37) and other countries (31, 38). However, even higher prevalence rates for ceftriaxone resistance among *Shigella* isolates has been reported in China (39). We also observed simultaneous resistance to ciprofloxacin and ceftriaxone, which further limits therapeutic options.

In a study by Pourakbari and colleagues, *S. sonnei* was more sensitive to cephalosporines (ceftizoxime, ceftriaxone, ceftazidime, and cephalothin) than *S. flexneri*, and *S. flexneri* was more sensitive to nalidixic acid than *S. sonnei* (40). Contrary to these findings, we found no significant difference in antibiotic susceptibility among *S. sonnei* and *S. flexneri* isolates.

In recent years, a high frequency of *TEM* gene has been identified in *Shigella* and other *Enterobacteriaceae* (22). This is of great clinical importance because the gene confers resistance to ampicillin, which was the principal antibiotic used for treatment of shigellosis (41). In our study, the bla_{TEM} gene was detected in 61.5% of the *Shigella* isolates and in all MDR strains. Ampicillin resistance was found to be significantly associated with *TEM* β-lactamase genes in all isolates, which is in agreement with the results of other studies (10, 42). Ampicillin resistance in *S. flexneri* and *S. sonnei* is mainly due to the presence of bla_{OXA} and bla_{TEM}, respectively (42). Likewise, we found a significant correlation between ampicillin resistance and presence of bla_{TEM} in *S. sonnei* isolates. However, no significant relationship was found between resistance to ampicillin and presence of bla_{TEM} in *S. flexneri* isolates.

In the present study, the frequency of $bla_{\text{CTX-M}}$ was relatively high among *Shigella* isolates. Zahedi Bialvaei et al. reported an even higher frequency for the $bla_{\text{CTX-M}}$ gene (12). Among the *Shigella* isolates, $bla_{\text{CTX-M}}$ was more frequently found in *S. sonnei* (21.74%), which is higher (33, 43) and in one case lower (23)

than the frequencies reported by studies in other countries. This could be because the transfer of the *bla*$_{CTX-M}$ gene is plasmid-mediated.

To the best of our knowledge, this is the first report on the emergence of simultaneous resistance to ceftriaxone and ciprofloxacin among *bla*$_{CTX-M}$-positive *Shigella* isolates in Iran. With the increase in globalization, the potential widespread dissemination of these strains could be a public health threat, not only in Iran but also in the rest of the world. Therefore, effective infection control policies are needed for preventing the dissemination of resistant strains. Phage- based control of shigellosis can be considered as an alternative strategy for the treatment of MDR *Shigella* strains (44).

CONCLUSION

We revealed the high prevalence of *bla*$_{TEM}$ and *bla*$_{CTX-M}$ in *Shigella* strains isolated from patients in Iran. Antimicrobial susceptibility testing also demonstrated the high level of multi-drug resistance among these isolates. These findings raise serious concerns about the dissemination of ESBL-producing *Shigella* strains, and emphasize the need for controlled use of antibiotics, especially 3GC, to avoid further antibiotic resistance.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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