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ORIGINAL ARTICLE

Molecular detection of genes encoding resistance to tetracycline and quinolones among *Shigella* strains isolated from children with acute diarrhea in southwest Iran

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ABSTRACT

Background and Objectives: An increase in the antibiotic resistance of *Shigella* isolates has caused major global challenges in antimicrobial therapy. Knowledge of local antibiotic resistance trends is essential for selecting appropriate antibiotic treatment regimens. This study aimed to evaluate the frequency of efflux-mediated tetracycline resistance (*tet*) and plasmid-mediated quinolone resistance (*qnr*) genes among *Shigella* isolates.

Materials and Methods: This survey investigated 91 *Shigella* isolates, obtained from children with acute diarrhea. The isolates were identified using standard biochemical tests and confirmed by polymerase chain reaction (PCR) assay. Besides, the susceptibility of isolates to six selected antibiotics was assessed by the disk diffusion method. All tetracycline-resistant and nalidixic acid and ciprofloxacin resistant strains were screened for *tet* and *qnr* genes by a multiplex PCR assay.

Results: According to the results of antibiotic susceptibility tests, the highest level of antibiotic resistance was related to tetracycline (80.2%) and doxycycline (78.1%), respectively. All isolates were sensitive to tigecycline. The PCR results showed that 40.6%, 3.1%, 21.8%, 61.6% and 28.7% of the isolates carried *qnrA*, *qnrB*, *qnrS*, *tetA*, and *tetB* genes, respectively. None of the isolates contained *tetC* and *tetD* genes.

Conclusion: The current findings revealed that *tetA* and *qnrA* genes might play a key role in conferring tetracycline and quinolone resistance.

Keywords: Diarrhea; *Shigella*; Tetracycline resistance; Quinolone resistance

INTRODUCTION

Shigella is the most common cause of bacillary dysentery in children younger than five years in underdeveloped and developing countries. It is responsible for about 700,000 deaths worldwide each year (1). Although shigellosis is often a self-limiting disease, antibiotic therapy is recommended to decrease

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the duration of diarrhea and prevent the transmission of infection (2). In recent years, there has been a marked increase in multidrug-resistant (MDR) *Shigella* species around the world (3). Quinolones are broad-spectrum that have excellent activity against *Shigella* infections. The plasmid-mediated quinolone resistance (PMQR) genes are members of the pentapeptide repeat family of proteins that protect DNA from quinolone by binding to DNA gyrase and topoisomerase IV, which cause resistance to quinolones (4). PMQR genes are classified into five classes which are *qnrA, qnrB, qnrC, qnrS,* and *qnrD.* PMQR are very important in medicine because these plasmids are transmissible and in addition to transferring resistance genes to quinolones, they also carry other resistance genes such as β-lactamase and integrons genes (5).

Tetracyclines, as broad-spectrum antibiotics disrupting protein synthesis, are used against various bacterial infections. The most well-known tetracyclines include tetracycline, minocycline, doxycycline, and tigecycline (6). The main mechanism of tetracycline resistance is attributed to the ribosomal protection system and antibiotic extrusion by active efflux pumps (2). Tetracycline efflux has been associated with the major facilitator superfamily (MFS) antibiotic efflux system, which is encoded by 12 tetracycline resistance (*tet*) genes in Gram-negative bacteria, such as *Shigella* species (7, 8). So far, five *tet* genes (*tetA, tetB, tetC, tetD,* and *tetG*) have been discovered in *Shigella* strains. The *tetA* and *tetB* genes are the most frequently identified *tet* genes, while others (*tetC, tetD,* and *tetG*) are seldom identified alone (7, 9).

Tetracyclines are not routinely used to treat shigellosis, but increasing resistance to first-line drugs has turned them into an alternative treatment for these infections (2). Since the resistance of *Shigella* to ampicillin and cotrimoxazole has spread, and also, the resistance to fluoroquinolones is increasing. Evaluation of the effectiveness of doxycycline and minocycline for the effective treatment of shigellosis is promising. To the best of our knowledge, this is the first study to evaluate the genetic diversity of tetracycline and quinolone resistance *Shigella* strains in this region. The present study aimed to assess the prevalence of efflux-mediated *tet* and quinolone resistance genes among *Shigella* strains isolated from diarrheal fecal samples of children in the southwest of Iran.

MATERIALS AND METHODS

Study design and bacterial identification. This study was conducted on 91 *Shigella* isolates from patients with acute diarrhea, who were examined in our previous study (10). Samples were collected from children younger than 15 years, admitted to the teaching hospitals of Ahvaz and Abadan in southwest of Iran. The patients had not consumed any antibiotics in the last two weeks. Various standard microbiological and biochemical tests were performed for the identification of *Shigella* strains. In brief, all specimens were cultured on Hektoen enteric agar and xylose lysine deoxycholate agar (Merck, Germany) plates and incubated at 37°C overnight. The grown Gram-negative rods were identified by bacteriological and biochemical tests, such as citrate utilization, indole test, methyl red test, and urease test. All isolates that were confirmed as Shigella were preserved in Tryptic Soy Broth (TSB) (Merck, Germany), containing glycerol (30%) at -70°C.

Antimicrobial susceptibility tests. Antibiotic susceptibility toward tetracycline (30 μg), doxycycline (30 μg), tigecycline (15 μg), minocycline (30 μg), chloramphenicol (30 μg), nalidixic acid (30 μg), ciprofloxacin (5 μg), levofloxacin (5 μg), and erythromycin (15 μg) (Mast Ltd., UK.) was specified by standard disk diffusion (Kirby-Bauer) method on Muller-Hinton agar plates (Merck, Germany) according to the Clinical Laboratory Standard Institute (CLSI) 2020 recommendations (11). *E. coli* ATCC 25922 strain was used as a quality control in susceptibility testing. The minimum inhibitory concentrations (MICs) of ciprofloxacin for the resistant *Shigella* isolates were performed using broth microdilution method according to CLSI 2020.

PCR for the screening of *tet* genes. All tetracycline-resistant strains were investigated for the identification of tetracycline resistance genes include *tetA, tetB, tetC,* and *tetD.* The specific primers of *tetA, tetB, tetC* and *tetD* are shown in Table 1. The amplification reaction was carried out by thermal cycler (Eppendorf, Germany) with an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing 55°C for 30 s, extension 72°C for 1min and final extension 72°C for 5 min (12). PCR products were electrophoresed on ethidium bromide (0.5mg/mL) containing 2% of agarose gel prepared in

Table 1. The primers used for the detection of *tet* and *qnr* genes

Genes	Primer Sequence (5´-3´)	Product Size (bp)
qnrA	F-ATTTCTCACGCCAGGATTTG	516
	R-GATCGGCAAAGGTTAGGTCA	
qnrB	F-GATCGTGAAAGCCAGAAAGG	469
	R-ACGATGCCTGGTAGTTGTCC	
qnrS	F-ACGACATTCGTCAACT GCAA	417
	R-TAAATTGGCACCCTGTAGGC	
tetA	F-CGCCTTTCCTTTGGGTTCTCTATATC	182
	R- CAGCCCACCGAGCACAGG	
tetB	F- GCCAGTCTTGCCAACGTTAT	975
	R- ATAACACCGGTTGCATTGGT	
tetC	F- TTCAACCCAGTCAGCTCCTT	560
	R- GGGAGGCAGACAAGGTATAGG	
tetD	F- GAGCGTACCGCCTGGTTC	537
	R- TCTGATCAGCAGACAGATTGC	

1× TAE (Tris/Acetate/EDTA) buffer and visualized in the gel document system (Protein simple, USA). *Shigella sonnei* ATCC 9290 was used as a positive control strain and *E. coli* ATCC 25922 was used as the negative control.

PCR amplification of *qnr* genes. Multiplex PCR was done by modification of previously described PCR protocol (4) for PCR amplification of PMQR *qnrA*, *qnrB*, and *qnrS* genes. All isolates presenting resistance to nalidixic acid and ciprofloxacin were screened for these determinants. The specific primers of *qnrA*, *qnrB* and *qnrS* are listed in Table 1. The confirmed *Klebsiella pneumoniae* strain containing the *qnr* gene was used as a positive control for *qnr* genes.

Ethics. The study design was in line with the Helsinki Declaration and has previously received ethical permission from the Institutional Ethics Committee of the Abadan University of Medical Sciences, Abadan, Iran (Ethical code: IR.ABADANUMS.REC.1399.053).

RESULTS

Bacterial isolation and antimicrobial susceptibility test. Of 91 *Shigella* spp., 51.6% (n=47), 39.6% (n=36) and 8.8% (n=8) samples were identified as *S. flexneri*, *S. sonnei*, and *S. boydii* respectively. All 91 *Shigella* isolates were susceptible to tigecycline and levofloxacin. The highest rates of resistance were to tetracycline 80.2% (n=73), followed by doxycycline resistance 78.1% (n=71). The analysis of antimicrobial resistance patterns of the isolates to six antibiotics is summarized in Table 2. The MIC values of ciprofloxacin ranged 1-256 µg/L. The analysis of antimicrobial resistance patterns of the isolates to nine antibiotics is summarized in Table 2.

Detection of tetracycline and quinolone resistance genes. The percentage of *qnrA*, *qnrB* and *qnrS* genes in *Shigella* spp. were 40.6% (n=13), 3.1% (n=1) and 21.8% (n=7) respectively. The distribution of *qnr* genes in *Shigella* spp. is shown in Table 3.

The *tetA* and *tetB* genes were detected in 61.6% (n=45) and 28.7% (n=21) of tetracycline-resistant strains, respectively. Among tetracycline-resistant isolates, 9.6% (n=7) simultaneously harbored *tetA* and *tetB* genes. However, *tetC* and *tetD* genes were not found in any of the isolates (Table 4).

DISCUSSION

Antimicrobial resistance in *Shigella* strains has become an emerging and increasing threat to human health, especially in pediatrics. Since shigellosis is a highly infectious disease, physicians should become increasingly aware of the regional antimicrobial resistance patterns to prevent the spread of pathogens and ensure efficient clinical care (13). According to the World Health Organization (WHO) guidelines, ciprofloxacin is now a good drug of choice for the treatment of shigellosis. However, increase in the use of ciprofloxacin has led to the development of resistance to this antibiotic in *Shigella* strains (14). In areas where there is high resistance in *Shigella* strains, newer fluoroquinolones such as norfloxacin and levofloxacin are recommended for treatment (15). The results of our study showed that ciprofloxacin and levofloxacin were the best antibiotics against *Shigella* strains. In the present study, 35.1% of *Shigella* isolates were resistant to nalidixic acid. The results of this research consistent with the previous studies (15, 16). Resistance to quinolones is mainly attributed to mutations in the quinolone resistance-determining region (QRDR) of DNA gyrase and protection of DNA gyrase by the Qnr protein produced from

Table 2. Antibiotic resistance patterns of the *Shigella* spp.

Antibiotic Species	TET	DTX	MN	TGC	NA	CIP	LVX	ERY	CHL
S. flexneri	37 (78.7%)	37 (78.7%)	2 (4.2%)	0 (0%)	17 (36.1%)	5 (10.6%)	0 (0%)	32 (68.1%)	29 (61.7%)
S. sonnei	31 (86.1%)	31 (86.1%)	1 (2.7%)	0 (0%)	14 (38.8)	1 (2.7%)	0 (0%)	27 (75%)	23 (63.8%)
S. boydii	5 (62.5%)	3 (37.5%)	0 (0%)	0 (0%)	1 (12.5%)	0 (0%)	0 (0%)	2 (25%)	4 (50%)
Total	73 (80.2%)	71 (78.1%)	3 (3.2%)	0 (0%)	32 (35.1%)	6 (6.5%)	0 (0%)	61 (67.1%)	56 (61.5%)

TET: Tetracycline, DTX: Doxycycline, MN: Minocycline, TGC: Tigecycline, ERY: Erythromycin, CHL: Chloramphenicol, NA: Nalidixic acid, CIP: Ciprofloxacin, LVX: Levofloxacin

Table 3. The distribution *qnrA,qnrB* and *qnrs* among quinolones-resistant *Shigella* spp.

Genes Species	qnrA	qnrB	qnrs	*qnrA+qnrB*	*qnrA+qnrS*	*qnrB+qnrS*	*qnrA+qnrB+qnrS*
S. flexneri (n=17)	7 (41.1%)	1 (5.8%)	4 (23.5%)	1 (5.8%)	1 (5.8%)	0 (0%)	3 (17.6%)
S. sonnei (n=14)	5 (35.7%)	0 (0%)	3 (21.4%)	3 (21.4%)	1 (7.1%)	0 (0%)	2 (14.2%)
S. boydii (n=1)	1 (100%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Total (n=32)	13 (40.6%)	1 (3.1%)	7 (21.8%)	4 (12.5%)	2 (6.25%)	0 (0%)	5 (15.6%)

Table 4. The distribution of *tetA* and *tetB* among tetracycline-resistant *Shigella* spp.

Genes Species	*tetA*	*tetB*	*tetA + tet B*
S. flexneri (n=37)	24 (64.8%)	11 (29.7%)	2 (5.4%)
S. sonnei (n=31)	19 (61.3%)	7 (22.5%)	5 (16.1%)
S. boydii (n=5)	2 (40%)	3 (60%)	0 (0%)
Total (n=73)	45 (61.6%)	21 (28.7%)	7 (9.6%)

PMQR genes and active efflux pump (15). In the current study, 40.6% of *Shigella* isolates carried the *qnrA* gene which was more prevalent in *S. flexneri* isolates. This result was nearly similar to that of Thanaa et al. (14). The results of the current study regarding other PMQR genes (*qnrB* and *qnrS*) are different from the previous studies (17, 18). The cause of the difference in frequency of these genes can be attributed to other factors such, as mutations in regions Quinolone resistance- determining regions (QRDR), changes in expression of efflux pumps, or even new mechanisms that may exist.

Tetracyclines are broad-spectrum antibiotics that can be used to treat various bacterial infections (7). Since tetracyclines, as bacteriostatic agents, have been used for a long time, there are some concerns about the increasing resistance of bacterial strains (19).

In the present study, the resistance of *Shigella* strains to tetracycline and doxycycline was significantly high, which discouraged their empirical use in our region. These results are consistent with previous reports (2, 20). The extent of *Shigella* resistance to first-line drugs such as tetracycline in our study may be based on the misuse and overuse of these antibiotics for the treatment of diarrhea in our region. In this study, all isolates were sensitive to tigecycline. Only 3.2% of the isolates were resistant to minocycline. The present results are consistent with the findings of studies conducted by Sheykhsaran et al. and Shahsavan et al. (2, 6). Low resistance to minocycline and tigecycline may be due to less use of these antibiotics. It seems that resistance to tetracycline can be related to efflux pump, ribosomal protection or chemical modification (21). In this study, 71.2% and 38.3% of tetracycline-resistant isolates contained *tetA* and *tetB* genes, respectively. However, none of the strains harbored *tetC* and *tetD* genes. In a similar study conducted by Shahsavan et al. in Tehran, the prevalence rates of *tetA* in *Shigella* isolates were 66% (2), which was approximately consistent with our findings. Both *tetA* and *tetB* genes are the most prevalent among tetracycline-resistant Enterobacteriaceae (22, 23). Studies show that *tetA* confers resistance to tetracycline, while *tetB* confers resistance to tetracycline and doxycycline (23, 24). In the current study, *tetA* gene was

more prevalent in *S. sonnei* and *S. flexneri* isolates. Two independent studies reported that *tetA* gene was more common among *S. sonnei* and *S. flexneri* isolates (2, 25), which is inconsistent with our findings.

It can be concluded that resistance to tetracycline in *S. sonnei* and *S. flexneri* may be related to expression of *tetA*. As shown in Table 4, based on the number of isolates, *tetB* gene was more prevalent in *S. boydii* strains. Our results are not consistent with previous study (2). The most reason for this contrast with other studies may be due to differences in the number of samples and geographical areas. The results of this study indicated that *tetA* and *tetB* genes are the most important resistant factors to tetracycline in *Shigella* isolates in our region.

CONCLUSION

In conclusion, the present findings indicated that tigecycline and levofloxacin exhibited considerable antimicrobial activities and could be useful in the treatment of *Shigella* infections. Also, this study found that active efflux pumps promoted tetracycline resistance in the investigated isolates. Because tetracyclines are not commonly prescribed for the treatment of shigellosis, tetracycline resistance may be due to clone dissemination in the strains. In addition, the high abundance of the *qnrA* gene in *Shigella* strains is suggestive of the potential horizontal transfer of the resistance genes between these strains.

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