The Frequency of Mutations in Quinolone Resistance-Determining Regions and Plasmid-Mediated Quinolone Resistance in *Shigella* Isolates Recovered from Pediatric Patients in Tehran, Iran: An Overlooked Problem

Sajad Yaghoubi,[1] Reza Ranjbar,[2] Mohammad Mehdi Soltan Dallal,[1,3] Mohammad Hasan Shirazi,[1] and Mohammad Kazem Sharifi-Yazdi[4,5]

Fluoroquinolone (FQ) resistance in clinical isolates of *Shigella* species has been increasing reported in recent years. This study was carried out to find the mutations within the quinolone resistance-determining regions (QRDRs) and the prevalence of plasmid-mediated quinolone resistance (PMQR) determinants among the clinical isolates of *Shigella* sp. in Tehran, Iran. A total of 50 *Shigella* isolates were collected from five teaching therapeutic centers in Tehran, Iran and analyzed for antibiotic susceptibility over a period of 20 months from July 2015 to January 2017. The PCR and direct nucleotide sequencing were used for genetic alterations in the QRDRs. The PMQR genes were detected using PCR. The results revealed four types of mutations in the QRDR of *gyrA*: 20 (40%) had a S83L mutation, 1 (2%) had a S83A mutation, 2 (4%) had a D87G mutation, and 1 (2%) isolate had a D87Y mutation. Mutations were also found at codon N57D, D200N, and E210K in three isolates. Seven hospitalized children had *qnrS* determinants, and one isolates had the mutation S83A, while two isolates had double mutations at S83L and/or D87G (Ser83Leu and Asp-87Gly). The PMQR gene-positive isolates had the single replacement of serine with leucine. In hospitalized children, two isolates had two types of PMQR determinants (*qnrS* and *qnrA*) and (*qnrS* and *qnrB*) at once. The results of this study indicate that the emergence of strains with mutations in the QRDR regions and the capture of PMQR determinants in strains may lead to failure in therapy with FQ and the widespread emergence of strains with high-level FQ resistance.

Keywords: shigellosis, DNA gyrase, topoisomerase IV, *qnr* gene

Introduction

*S*HIGELLA SPECIES COMMONLY cause foodborne diseases and community-acquired infectious gastroenteritis worldwide.[1,2] The supportive care of patients with shigellosis includes both oral rehydration therapy and treatment with antibiotics.[3,4] Unfortunately, in the last few years, the extensive and indiscriminate use of antibiotics in the treatment of shigellosis has created challenges for antimicrobial therapy and caused the emergence of *Shigella* strains with multidrug-resistant (MDR) phenotypes.[3]

Increase in antimicrobial resistance to commonly used antibiotics including tetracycline (TET), sulfonamides, am-

picillin (AMP), and trimethoprim/sulfamethoxazole among *Shigella* isolates has led to the conclusion that these agents are no longer recommended for empirical treatment.[3] For example, oral quinolone for proven or suspected *Shigella* infections is recommended by most authorities.[1,3] Nalidoxic acid (NAL) and ciprofloxacin (CIP) are alternative medicines that have been highly effective, although many authorities are hesitant about their use in children.[1,3] The quinolone-resistant *Shigella* isolates have emerged in various parts of the world, particularly in Africa and Asia.[5,6] Fluoroquinolones (FQs) act by inhibiting both gyrase and topoisomerase IV, which eventually leads to cell death.[7] FQ resistance is commonly caused by mutations in the *gyrA* and *parC* genes and in the

[1]Division of Microbiology, Department of Pathobiology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran.
[2]Molecular Biology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran.
[3]Food Microbiology Research Center, Tehran University of Medical Sciences, Tehran, Iran.
[4]Zoonosis Research Centre, Tehran University of Medical Sciences, Tehran, Iran.
[5]Department of Medical Laboratory Sciences, School of Para Medicine, Tehran University of Medical Sciences, Tehran, Iran.

highly conserved quinolone resistance-determining regions (QRDRs); these mutations alter the DNA gyrase-binding sites of quinolone.[3,7,8]

FQ resistance is also mediated by a novel mechanism of plasmid-mediated quinolone resistance (PMQR), as defined by a series of tandem five amino acid repeats, which protects topoisomerase IV and DNA gyrase from the quinolone. There are five Qnr proteins: QnrA, QnrB, QnrC, QnrS, and QnrD.[9] Aminoglycoside N-acetyltransferase acts as a second PMQR mechanism to diminish CIP activity by adding an acetyl group to the CIP, which decreases its effect.[10] The QepA protein is considered to be a third PMQR mechanism because it causes FQs to be extruded from the cell.[11] PMQR gene-positive isolates are susceptible to quinolones and FQs, although studies have revealed that these isolates can lead to higher levels of FQ resistance.[12]

Since PMQR determinants are transferable broad host range plasmids, they spread easily, between children in childcare facilities; they are also related to the expression of an extended-spectrum β-lactamase (ESBL) phenotype.[13] FQ treatment of Shigella infections from strains that carry quinolone resistance genes or have mutations in the QRDRs are more difficult to treat and may increase the risk of drug therapeutic failure.[14,15] Therefore, continuous monitoring of FQ resistant Shigella isolates and genetic testing on select strains with the previously mentioned characteristics are essential. This study was carried out to find the mutations within the QRDRs and the prevalence of PMQR determinants among the clinical isolates of Shigella sp. in Tehran, Iran.

Materials and Methods

Bacterial strains

A total of 50 Shigella strains were isolated from 490 pediatric patients with diarrhea who were admitted to five teaching therapeutic centers in Tehran, Iran over a period of 20 months from July 2015 to January 2017. The samples were cultured on Shigella–Salmonella agar, xylose lysine deoxycholate, MacConkey agar, and Hektoen enteric agar (Difco Laboratories, Inc., Detroit). Individual isolates were surveyed using previously defined standard biochemical and serological tests.[16] All isolates were confirmed to be Shigella spp. using API 20E test strips (bioMérieux, France). Serogrouping of the Shigella isolates was performed using slide agglutination with specific antisera (Mast Group Ltd., Merseyside, United Kingdom). The confirmed Shigella isolates were kept in brain heart infusion broth with 40% glycerol at 70°C.

Antibiotic susceptibility testing

The FQs CIP and NAL were tested by Etest Method in accordance with the recommendations of the Clinical Laboratory Standards Institute.[17] The Kirby–Bauer disk diffusion method was used with the following antimicrobials: chloramphenicol 30 μg (CHL), ceftazidime 30 μg (CAZ)+ clavulanic acid 10 μg (CLA), cefotaxime 30 μg (CTX)+ CLA 10 μg, NAL 30 μg, CIP 5 μg, gentamicin 10 μg, TET 30 μg, AMP 20 μg, co-trimoxazole 5 μg (SXT), CTX 30 μg, CHL 10 μg, and CAZ 30 μg+ceftriaxone 30 μg (CRO) (Mast Group Ltd., Merseyside, United Kingdom).[17] The ESBL

phenotype of the Shigella isolates was detected using the double-disk synergy test. Briefly, the test was performed using both CTX 30 μg and CAZ 30 μg alone and in combination with CLA. All ESBL-producing isolates were tested using the minimum inhibitory concentration (MIC) test for CTX, CRO, and CAZ with the Etest (AB Biodisk, Solna, Sweden). Escherichia coli ATCC 25922 and ATCC 35218, and were used as control strains.

PCR amplification

The genomic DNA of the Shigella isolates was extracted and applied to a template. PCR assays were carried out that argeted the QRDRs of the gyrA, gyrB, parC, and parE genes.[18] PCR was also used to investigate the PMQR determinants qnrS, qnrA, qnrB, aac(6′)-Ib-cr, and QepA, using the described primers listed in Table 1. The protocol followed for the PCR by the qnr gene was based on the method described by Wang et al.[19] All Shigella isolates were selected for these determinants. PCR amplifications were carried out using the methods described by Wang et al.[19] and Chen et al.[20] The primers and PCR amplifications of CTX-M15, integron class 1 (int1) and integron class 2 (intII) were carried out based on previously described studies.[21–24]

DNA sequence analysis

Prominent amino acid substitutions in the QRDRs of gyrA and parC from NAL-resistant Shigella isolates were compared with the sequence of a susceptible strain of S. sonnei [ARS 04283.1] for gyrA. The S. flexneri reference sequence WP_001281839.1 was used for the DNA topoisomerase IV subunit A [parC].

Analysis of data and sequence

The database software program SPSS (version 16.0; Armonk, New York) was used for the statistical analyses. Differences in FQ resistance rates and mutation frequencies were surveyed using the χ test, where $p < 0.05$ was considered statistically significant.

Results

Bacteria isolation and serotypes

Fifty children with culture-confirmed shigellosis were studied. S. sonnei was the frequently isolated serogroup 40 (80%), followed by S. flexneri 9 (18%) and S. dysenteriae 1 (2%); S. boydii was not isolated ($p < 0.001$). Table 2, shows the resistance groups based on their FQ resistance profiles. Data analysis revealed that 11 (22%) of the children who tested positive for one of the Shigella isolates were hospitalized; thus, 7 (17.5%) of the 40 with S. sonnei, 3 (33%) of the 9 with S. flexneri, and 1 (100%) with S. dysenteriae. The predominant Shigella isolates in both hospitalized and nonhospitalized children was S. sonnei.

FQ susceptibility

Of the Shigella isolates, 22 (55%) S. sonnei, 7 (77.8%) S. flexneri, and 1 (100%) of the S. dysenteriae were resistant to NAL. For the S. flexneri isolates, 6 (85.7%) serotype 2a

TABLE 1. OLIGONUCLEOTIDE USED IN THIS STUDY

Primers	Primer sequence (5′ → 3′)	Target	Amplicon size (bp)
gyrA	F: TACACCGGTCAACATTGAGG	Specific	647 (18)
	R: TTAATGATTGCCGCCGTCGG		
gyrB	F: GTCTGAACTGGGCCTGAATGC	Specific	309 (18)
	R: AGCAGCTCGGAATATTTCGACAA		
parC	F: GTCTGAACTGGGCCTGAATGC	Specific	248 (18)
	R: AGC AGC TCG GAATATTTC GACAA		
parE	F: ATG CGT GCG GCT AAA AAA GTG	Specific	289 (18)
	R: TCGTCGCTGTCAGGATCGATAC		
CTX_{M15}	F: CACACGTGGAATTTAGGGACT	Specific	995 (21)
	R: GCCGTCTAAGGCGATAAACA		
intII	F: GCAAATGAAGTGCAACGC	Specific	466 (23)
	R: ACACGCTTGCTAACGATG		
intI	F: GGGTCAAGGATCTGGATTTCG	Specific	483 (24)
	R: ACAGGGTGTAAATCATCGTC		
qnrA	F: ATTTCTCACGCCAGGATTTG	Specific	516 (19)
	R: GATCGGCAAAGGTTAGGTCA		
qnrB	F: GATCGTGAAAGCCAGAAAGG	Specific	469 (19)
	R: ACGATGCCTGGTAGTTGTCC		
qnrS	F: ACGACATTCGTCAACTGCAA	Specific	417 (19)
	R: TAAATTGGCACCCTGTAGGC		
aac(6′)-Ib-cr	F: TTGCGATGCTCTATGAGTGGCTA	Specific b	482 (38)
	R: CTCGAATGCCTGGCGTGTTT		
qepA	F: CTGCAGGTACTGCGTCATG	Specific c	403 (40)
	R: CGTGTTGCTGGAGTTCTTC		

and 1 (14.3%) serotype 1a were resistant to NAL. Statistical analysis showed that the incidence of resistance differed considerably between NAL and CIP (Table 2). The overall rate of resistance to CIP was 10%, and most of the CIP-positive isolates belonged to *S. sonnei*. Out of 30 *Shigella* isolates that were resistant to NAL, 5 isolates showed CIP resistance (10%) at MIC >32 µg/ml and 5 isolates showed CIP resistance at $1 \le$ MIC ≤ 0.25 µg/ml, while the rest of the isolates showed CIP resistance at <0.25 µg/ml. Of the five CIP-positive isolates, two were associated with hospitalization, while all five were resistant to NAL at MIC >8 µg/ml. isolates numbers (INs) 250 and 302 from the five CIP-positive strains were resistant to extended-spectrum cephalosporins and had the *CTX-M-15* gene. All CIP-resistant isolates were also resistant to SXT, NAL, AMP, and TET, and harbored the class 2 integron.

Mutations in gyrA, gyrB, parC, and parE

Mutations in the *gyrA* gene were identified in 20 (40%) of the isolates. There were three common types of mutations detected in the QRDR of gyrA: 20 isolates (40%) had S83L, while 3 (6%), INs 274, 302, and 250, had D87Y and D87G mutations. Mutation at codon 83 of *gyrA*, which leads to a serine substitution of the leucine residue (transition C to T), was the most common substitution in all of the *Shigella* isolates. In the *Shigella* isolates without mutations and in the single isolate with a Ser83 to leucine substitution, the CIP MICs were ≤ 1 µg/ml. The MIC in isolates with a single *gyrA* mutation (S83L) was lower than the MIC in isolates with a double *gyrA* mutation (S83L and D87G) (Fig. 1). None of the *Shigella* strains had any mutations in the QRDR regions of the *gyrB* or *parE* genes. All hospitalized patients who were infected with either *S. sonnei* or *S. flexneri* had a single mutation in the region of S83L. Single mutations in *parC*

(S80I) were seen with the simultaneous presence of a *gyrA* mutation. All CIP-resistant isolates (MIC >32 µg/ml) had a detectable mutation in the QRDR of *gyrA* at positions 83 or 87 (S83L and/or D87G) and a single mutation in *parC* at position 80 except for one isolate (Fig. 2). One common mutation in IN 303 [GCG (S83A)], and common mutations [D87G] in INs 302 and 250 were found (Table 2). In the amino acid region of 87 GAC (Asp), amino acid substitutions D87G, D87Y were detected in 302 and 250, 274, respectively. Mutations were also found at codon N57D, D 200N, and E210K in INs 271, 251, and 306. The mutations at codon 91 [His (Glu)], and [Leu (Arg)] appear outside the QRDR region of *parC* in IN 265, and one common mutation [Pro (Ser)] in three isolates were detected. The presence of a number of mutations should be noted in positions N57D, and D210 N in IN 306, D87Y in IN 274 in *gyrA*, and IN 303 S83A, in outside and inside region of the QRDR domain in the *gyrA* gene, which is reported for the first time in Iran.

Characterization of qnr genes

Of the 50 *Shigella* isolates, 13 (26%) carried the PMQR determinants: 11 (22%) carried *qnrS*, 5 (10%) harbored only *qnrB*, and 3 (6%) carried *qnrA* determinants. All the 11 qnrS positive were resistant to NAL, and 5 were also resistant to CIP (Table 2). We found that the MDR isolates carried the *qnrS*, *qnrA*, *qnrB*, *intII*, *intI*, and *bla*$_{CTX-M-15}$ genes. None of the isolates were positive for the *aac(6′)-Ib-cr* and *QepA* genes. All isolates, except for four, that were carrying the PMQR gene had a single replacement of serine with leucine. For the five CIP-positive strains, the isolates harboring the *qnrS* gene were resistant to NAL. In the hospitalized children, INs 306 and 303 had two types of PMQR determinants

TABLE 2. CHARACTERISTICS OF THE SIXTEEN MULTIDRUG-RESISTANT ISOLATES

Number	species	MIC µg/ml		Nucleotide and amino change in gyrA		ParC position	PMQR determinant(s)			Integron genes		b-Lactamases	Resistance profile
		NA	Cip	83 [TCG (Ser)]	87 [GAC (Asp)]	80 [AGC (Ser)]	qnrS	qnrA	qnrB	intI	IntII		
287	S	8	0.25	TTG (Leu)	GAC (Asp)	AGC (Ser)	P	N	N	intI	IntII	N	SXT, NA, AMP, CTX, GM, TET
274	S	>32	1	TCG (Ser)	TAC (Tyr)	AGC (Ser)	N	N	N	N	N	N	SXT, NA, AMP, GM
265	S	>32	>32	TCG (Ser)	[GAC (Asp)]	AGC (Ser)	P	N	N	N	IntII	N	SXT, NA, CIP, AMP, CTX
275	S	16	>32	TTG (Leu)	GAC (Asp)	ATC (Ile)	P	N	N	N	IntII	N	SXT, NA, CIP, AMP, TET
250	S	>32	>32	TTG (Leu)	GGC (Gly)	ATC (Ile)	P	P	N	N	IntII	CTX-M-15	SXT, NA, CIP, AMP, TET
302	S		>32	TTG (Leu)	GGC (Gly)	ATC (Ile)	P	N	P	intI	IntII	CTX-M-15	SXT, NA, CIP, AMP, TET, CHL
256	S	>32	>32	TTG (Leu)	GAC (Asp)	ATC (Ile)	P	N	N	intI	IntII	N	SXT, NA, CIP, AMP, CHL
253	F	32	0.5	TCG (Ser)	GAC (Asp)	AGC (Ser)	P	N	N	N	N	N	SXT, NA, AMP, TET
222	S	32	0.032	TTG (Leu)	GAC (Asp)	AGC (Ser)	N	N	N	N	N	N	SXT, NA, AMP, CHL
306	F	32	0.016	TTG (Leu)	GAC (Asp)	AGC (Ser)	P	N	P	N	IntII	N	SXT, NA, AMP, GM, TET
305	S	32	0.032	TTG (Leu)	GAC (Asp)	AGC (Ser)	N	P	P	N	N	N	SXT, NA, AMP, TET
392	F	32	1	TTG (Leu)	GAC (Asp)	AGC (Ser)	N	N	P	N	N	N	SXT, NA, AMP, TET
303	F	32	0.75	GCG(Ala)	GAC (Asp)	AGC (Ser)	P	N	P	N	IntII	N	SXT, NA, AMP, TET
312	S	32	0.125	TCG (Ser)	GAC (Asp)	AGC (Ser)	P	N	N	intI	N	CTX-M-15	SXT, NA, AMP, TET, CHL
386	S	32	0.5	TCG (Ser)	GAC (Asp)	AGC (Ser)	P	N	N	N	IntII	CTX-M-15	SXT, NA, AMP, TET
234	S	1	0.032	TCG (Ser)	GAC (Asp)	AGC (Ser)	N	N	N	IntI	IntII	N	SXT, AMP, TET, CHL

PMQR, plasmid-mediated quinolone resistance; F, *flexneri*, S, *sonnei*; *intI*, integrin; QRDR, quinolone resistance-determining region; AMP, ampicillin; CTX, cefotaxime; CIP, ciprofloxacin; CAZ, ceftazidime; NA, nalidixic acid; CIP, ciprofloxacin; GM, gentamicin; CHL, chloramphenicol; SXT, co-trimoxazole; MIC, minimum inhibitory concentration; TET, tetracycline; N, none; P, positive.

4

```
                        50         60         70         80
                ....|....| ....|....| ....|....| ....|....|
QRDR region    1  ---------- ---------- ---------- ----------  1
ARS04283.1 DNA 41 LKPVHRRVLY AMNVLGNDWN KAYKKSARVV GDVIGKYHPH 80
271            12 LKPVHRRVLY AMNVLGDDWN KAYKKSARVV GDVIGKYHPH 51
251            12 LKPVHRRVLY AMNVLGNDWN KAYKKSARVV GDVIGKYHPH 51
256            12 LKPVHRRVLY AMNVLGNDWN KAYKKSARVV GDVIGKYHPH 51
274-1          12 LKPVHRRVLY AMNVLGNDWN KAYKKSARVV GDVIGKYHPH 51
302            12 LKPVHRRVLY AMNVLGNDWN KAYKKSARVV GDVIGKYHPH 51
249            12 LKPVHRRVLY AMNVLGNDWN KAYKKSARVV GDVIGKYHPH 51
273            12 LKPVHRRVLY AMNVLGNDWN KAYKKSARVV GDVIGKYHPH 51
303            12 LKPVHRRVLY AMNVLGNDWN KAYKKAARVV GDVIGKYHPH 51
287            12 LKPVHRRVLY AMNVLGNDWN KAYKKSARVV GDVIGKYHPH 51
306            12 LKPVHRRVLY AMNVLGNDWN KAYKKSARVV GDVIGKYHPH 51
234            12 LKPVHRRVLY AMNVLGNDWN KAYKKSARVV GDVIGKYHPH 51
250            12 LKPVHRRVLY AMNVLGNDWN KAYKKSARVV GDVIGKYHPH 51
Clustal Consens

                        90        100        110        120
                ....|....| ....|....| ....|....| ....|....|
QRDR region    1  GDSAVYDTIV RMAQ------ ---------- ----------  14
ARS04283.1 DNA 81 .......... ....PFSLRY MLVDGQGNFG SIDGDSAAAM 120
271            52 ..L....... ....PFSLRY MLVDGQGNFG SIDGDSAAAM 91
251            52 ..L....... ....PFSLRY MLVDGQGNFG SIDGDSAAAM 91
256            52 ..L....... ....PFSLRY MLVDGQGNFG SIDGDSAAAM 91
274-1          52 ......Y... ....PFSLRY MLVDGQGNFG SIDGDSAAAM 91
302            52 ..L...G... ....PFSLRY MLVDGQGNFG SIDGDSAAAM 91
249            52 ..L....... ....PFSLRY MLVDGQGNFG SIDGDSAAAM 91
273            52 ..L....... ....PFSLRY MLVDGQGNFG SIDGDSAAAM 91
303            52 ..A....... ....PFSLRY MLVDGQGNFG SIDGDSAAAM 91
287            52 .......... ....PFSLRY MLVDGQGNFG SIDGDSAAAM 91
306            52 ..L....... ....PFSLRY MLVDGQGNFG SIDGDSAAAM 91
234            52 .......... ....PFSLRY MLVDGQGNFG SIDGDSAAAM 91
250            52 ..L...G... ....PFSLRY MLVDGQGNFG SIDGDSAAAM 91
Clustal Consens 1 ** *** *** ****                              12
```

FIG. 1. Nucleotide sequence of a *gyrA* region of the *Shigella* isolates, ARS04283.1: DNA gyrase subunit A [*Shigella sonnei*].

```
                        60         70         80         90        100
                ....|....| ....|....| ....|....| ....|....| ....|....|
QRDR region    1  ---------- ---------- -------GDS ACYEAMVLMA ----------  13
WP 001281839.1 51 ELGLNARAKF KKSARTVGDV LGKYHPH... .......... QPFSYRYPLV 100
307            1  ---------- -KSAGTVGDV LGKYHPH... .......... QPFSYRYPLV 39
304            1  ---------- -KSARTVGDV LGKYHPH... .......... QPFSYRYPLV 39
257            1  ---------- -KSARTVGDV LGKYHPH... .......... QPFSYRYPLV 39
100            1  ---------- -KSAGTVGDV LGKYHPH... .......... QPFSYRYPLV 39
250            1  ---------- -KSAGTVGDV LGKYHPH..I .......... QPFSYRYPLV 39
291-2          1  ---------- -KSASTVGDV LGKYHPH... .......... QPFSYRYPLV 39
265            1  ---------- -KSARTVGDV LGKYHPH... .......... HPFSYRYPLV 39
Clustal Consens                                 ** **********

                       110        120        130        140        150
                ....|....| ....|....| ....|....| ....|....| ....|....|
QRDR region    13 ---------- ---------- ---------- ---------- ----------  13
WP 001281839.1 101 DGQGNWGAPD DPKSFAAMRY TESRLSKYSE LLLSELGQGT ADWVPNFDGT 150
307            40 DGQGNWGAPD DPKSFAAMRY TESRLSKYPE ---------- ----------  69
304            40 DGQGNWGAPD DPKSFAAMRY TESRLSKYSR ---------- ----------  69
257            40 DGQGNWGAPD DPKSFAAMRY TESRLSKYSR ---------- ----------  69
100            40 DGQGNWGAPD DPKSFAAMRY TESRLSKYPE ---------- ----------  69
250            40 DGQGNWGAPD DPKSFAAMRY TESRLSKYSE ---------- ----------  69
291-2          40 DGQGNWGAPD DPKSFAAMRY TESRLSKYSE ---------- ----------  69
265            40 DGQGNWGAPD DPKSFAAMLY TESRLSKYPN ---------- ----------  69
Clustal Consens
```

FIG. 2. Nucleotide sequence of a *parC* region of the *Shigella* isolates, Reference Sequence Strain; WP_001281839.1 DNA topoisomerase IV subunit A [*Shigella flexneri*].

(*qnrS* and *qnrB*), but were sensitive to CIP (Table 2). Seven of the hospitalized children had *qnrS* determinants, and IN 303 had any mutation S83L. All four ESBL-producing isolates carried *qnrS*, although a single clinical isolate harbored *qnrS* and *qnrA* simultaneously. However, isolates numbers 250 and 302 had double mutations at S83L and/or D87G. It should be emphasized that clinical isolate harbored qnr genes, (*qnrS* and *qnrA*) and (*qnrS* and *qnrB*), simultaneously first reported in FQ-R *Shigella* strains in Iran.

Discussion

Diarrhea caused by *Shigella* species is a major public health problem in developing countries, especially for children <5 years old.[4] FQ is the primary choice of treatment for MDR *Shigella* infections. Previous studies demonstrated that a high rate of FQ resistance is mostly due to QRDR alterations and PMQR determinant mechanisms.[4] This is the first study in Iran to investigate the genetic characterization of novel mutations in the QRDR region and outside it and the presence of two types of PMQR determinants. Our results indicated the seriousness of the problem associated with NAL-resistant *Shigella* spp. Thirty isolates showed resistance to NAL and most of the isolates had a mutation at codon 83 [TCG (Ser)]. In contrast, a low percentage (<5%) of the *Shigella* isolates demonstrated resistance to NAL in other countries, including Turkey (4.7%)[4] and the United States.[25] This difference in rate of resistance may be due to the strict rules for antimicrobial therapy prescription in different countries. Bhattacharya *et al.* reported that resistance to NAL by almost all isolates existed in India by 2006–2009.[26] Our findings are in contrast with previous studies in India in which *Shigella* resistance to CIP gradually increased up to 48% over a 5-year period between 2002 and 2007.[16] In this study, high number of NAL-resistant strains displayed the most common mutation in *gyrA* (Ser83Leu), with another mutation that was only observed in the CIP-resistant isolates at codon 87 of the *gyrA* region. There is satisfactory agreement between our results and the research findings by Ruiz *et al.*[27] who reported that a mutation in codon 83 of the *gyrA* played an important role in the acquisition of high-level NAL resistance in clinical isolates of *Shigella*. In this study, some of the CIP-resistant isolates showed double mutations in the QRDR of a subunit of *gyrA* (Ser83Leu and Asp87Gly). In contrast to our results, another report noted that mutations in gyrA and parC were essential to achieve a high level of FQ resistance.[18]

Results of this study showed that the CIP MICs for all isolates with just a single *gyrA* mutation resulting in the substitution of serine (TCG) by leucine (TTG) were ≤1 μg/ml. Our findings is in agreement with previous reports that decreased susceptibility to CIP (MIC: 0.125–2 μg/ml) in *S. sonnei* strains is related to an alteration of Ser83 in *gyrA* [16]. Our results in contradiction with previous research[5] on the prevalence of *qnrS*. However, *qnrS* was the most prevalent PMQR determinant, and had a higher prevalence than was shown in previous studies.

Pu *et al.*[28] reported that 5 of 15 (34%) FQ-resistant isolates harbored the *qnrS* determinant; their serotypes were *S. flexneri 1a*, *2a*, and *4c*, which is in contrast with our results. Das *et al.* reported that *qnrB* was the most prevalent gene (93.7%), followed by *qnrC* (18.7%).[29] Cui *et al.*[30]

performed a study in which 22% (*n* = 58) of the isolates contained the aac(6′)-Ib-cr gene and two isolates contained the *qnrS* gene.[29] Cui *et al.*[30] performed a study in which 58 (22%) of isolates contained the aac(6′)-Ib-cr gene, and 3% isolates contained the *qnrS* gene. In Iran, Ranjbar *et al.*[3] found that, of the 23 quinolone-resistant isolates, 4 (17.4%) contained the *qnrS* gene. In the present study, all PMQR-positive *Shigella* isolates were positive for class 2 integrons. The five *Shigella* isolates that were CIP-resistant were also resistant to at least four other antibiotic agents, and were positive for *qnrS* and for class 2 integrons. This raises the possibility that these genes had a significant correlation among the *Shigella* isolates. Liu *et al.*[31] showed that 58.3% of the PMQR-positive isolates were also positive for the *bla*_{CTX-M} gene, and that 50% of the aac(6′)-Ib-cr-positive isolates harbored class 1 or class 2 integrons, which agreed with our data. Four ESBL-producing *S. sonnei* isolates possessed the CTX-M-15 gene; two of the isolates were CIP-resistant and harbored the *qnrS* gene. Our results agree with several studies from different countries, such as India,[32] Germany,[33] Spain,[34] The Netherlands,[35] and Denmark,[36] all of which mentioned or elaborated on the relationship of the *bla*_{CTX-M-15} with qnr determinants and other ESBLs.[40] In a study by Xiong *et al.*,[37] more than half of the PMQR determinants were associated with *bla* genes, especially *bla*_{CTX-M-14}. Remarkably, in our research, we found that INs 303 and 302 harbored both the *qnrS* and *qnrB* genes and IN 392 harbored both the *qnrA* and *qnrB* determinants. Similar studies on *Shigella* isolates[28] and *E. coli* isolates[38] reported that the isolates carried the aac(6′)-Ib-cr and qnrA genes.[39]

Conclusion

Treatment with FQ in patients contaminated with *Shigella* strains that carry quinolone resistance genes or that have chromosomal mutations that confer quinolone resistance may increase the risk of secondary diseases, long-course treatment (hospitalization), and drug therapeutic failure; it can also cause an increase in the quantity of organisms shed in the feces. To rectify this situation, in addition to limiting the administration of FQ in Iran to control the spread of resistance, it is essential to perform continuous monitoring and genetic testing to detect FQ-resistant *Shigella* strains. Therefore, FQ should not be the first-line treatment for these children patients since high failure rates are to be expected.

Disclosure Statement

No competing financial interests exist.

References

1. Mamishi, S., N. Mashoori, N. Mahboobi, and B. Pour Akbari. 2009. Increasing resistance to nalidixic acid in

Shigella subgroups in a comparative study between 2001–2003 and 2004–2006. Singapore Med. J. 50:791–793.

2. Yaghoubi, S., R. Ranjbar, M.M.S. Dallal, S.Y. Fard, M.H. Shirazi, and M. Mahmoudi. 2017. Profiling of virulence-associated factors in *Shigella* species isolated from acute pediatric diarrheal samples in Tehran, Iran. Osong Public Health Res. Perspect. 8:220–226.

3. Ranjbar, R., V. Behnood, H. Memariani, A. Najafi, M. Moghbeli, and C. Mammina. 2016. Molecular characterisation of quinolone-resistant *Shigella* strains isolated in Tehran, Iran. J. Global Antimicrob. Resist. 5:26–30.

4. Özmert, E.N., O.T. İnce, E. Örün, S. Yalçın, K. Yurdakök, and D. Gür. 2011. Clinical characteristics and antibiotic resistance of Shigella gastroenteritis in Ankara, Turkey between 2003 and 2009, and comparison with previous reports. Int. J. Infect. Dis. 15:e849–e853.

5. Yang, H., G. Duan, J. Zhu, W. Zhang, Y. Xi, and Q. Fan. 2013. Prevalence and characterisation of plasmid-mediated quinolone resistance and mutations in the gyrase and topoisomerase IV genes among *Shigella* isolates from Henan, China, between 2001 and 2008. Int. J. Antimicrob. Agents 42:173–177.

6. Hu, L.-F., J.-B. Li, Y. Ye, and X. Li. 2007. Mutations in the GyrA subunit of DNA gyrase and the ParC subunit of topoisomerase IV in clinical strains of fluoroquinolone-resistant *Shigella* in Anhui, China. J. Microbiol. 45:168–170.

7. van Eijk, E., B. Wittekoek, E.J. Kuijper, and W.K. Smits. 2017. DNA replication proteins as potential targets for antimicrobials in drug-resistant bacterial pathogens. J. Antimicrob. Chemother. 72:1275–1284.

8. Paula, C.M.D.d., M.P. Geimba, P.H.d. Amaral, and E.C. Tondo. 2010. Antimicrobial resistance and PCR-ribotyping of *Shigella* responsible for foodborne outbreaks occurred in southern Brazil. Braz. J. Microbiol. 41:966–977.

9. Cattoir, V., L. Poirel, V. Rotimi, C.-J. Soussy, and P. Nordmann. 2007. Multiplex PCR for detection of plasmid-mediated quinolone resistance qnr genes in ESBL-producing enterobacterial isolates. J. Antimicrob. Chemother. 60:394–397.

10. Iabadene, H., Y. Messai, H. Ammari, N. Ramdani-Bouguessa, S. Lounes, R. Bakour, and G. Arlet. 2008. Dissemination of ESBL and Qnr determinants in *Enterobacter cloacae* in Algeria. J. Antimicrob. Chemother. 62:133–136.

11. Poirel, L., V. Cattoir, and P. Nordmann. 2012. Plasmid-mediated quinolone resistance; interactions between human, animal, and environmental ecologies. Front. Microbiol. 3:24.

12. Guillard, T., P. Cholley, A. Limelette, D. Hocquet, L. Matton, C. Guyeux, A.-L. Lebreil, O. Bajolet, L. Brasme, J. Madoux, V. Vernet-Garnier, C. Barbe, X. Bertrand, and Christophe de Champs on behalf of CarbaFrEst Group. 2015. Fluoroquinolone resistance mechanisms and population structure of *Enterobacter cloacae* non-susceptible to ertapenem in North-Eastern France. Front. Microbiol. 6:1186.

13. Kao, C.-Y., H.-M. Wu, W.-H. Lin, C.-C. Tseng, J.-J. Yan, M.-C. Wang, C.H. Teng, and J.J. Wu. 2016. Plasmid-mediated quinolone resistance determinants in quinolone-resistant *Escherichia coli* isolated from patients with bacteremia in a university hospital in Taiwan, 2001–2015. Sci. Rep. 6:32281.

14. Qin, T., R. Bi, W. Fan, H. Kang, P. Ma, and B. Gu. 2016. Novel mutations in quinolone resistance-determining regions of gyrA, gyrB, parC and parE in *Shigella flexneri* clinical isolates from eastern Chinese populations between 2001 and 2011. Eur. J. Clin. Microbiol. Infect. Dis. 35:2037–2045.

15. Centers for Disease Control and Prevention. CDC Recommendations for Diagnosing and Managing *Shigella* Strains with Possible Reduced Susceptibility to Ciprofloxacin. April 18, 2017. Available at https://emergency.cdc.gov/han/han00401.asp (accessed Nov. 3, 2017).

16. Srinivasa, H., M. Baijayanti, and Y. Raksha. 2009. Magnitude of drug resistant Shigellosis: a report from Bangalore. Indian J. Med. Microbiol. 27:358.

17. Clinical and Laboratory Standards Institute. 2017. Performance Standards for Antimicrobial Susceptibility Testing. 27th ed. CLSI Supplement M100. Wayne, PA: Clinical and Laboratory Standards Institute.

18. Dutta, S., Y. Kawamura, T. Ezaki, G.B. Nair, K.-I. Iida, and S.-I. Yoshida. 2005. Alteration in the GyrA subunit of DNA gyrase and the ParC subunit of topoisomerase IV in quinolone-resistant *Shigella dysenteriae* serotype 1 clinical isolates from Kolkata, India. Antimicrob. Agents Chemother. 49:1660–1661.

19. Wang, A., Y. Yang, Q. Lu, Y. Wang, Y. Chen, L. Deng, H. Ding, Q. Deng, H. Zhang, C. Wang, L. Liu, X. Xu, L. Wang, and X. Shen. 2008. Presence of qnr gene in *Escherichia coli* and *Klebsiella pneumoniae* resistant to ciprofloxacin isolated from pediatric patients in China. BMC Infect. Dis. 8:68.

20. Chen, X., W. Zhang, W. Pan, J. Yin, Z. Pan, S. Gao, and X. Jiao. 2012. Prevalence of qnr, aac (6′)-Ib-cr, qepA, and oqxAB in *Escherichia coli* isolates from humans, animals, and environment. Antimicrob. Agents Chemother. 56:3423–3427.

21. Taneja, N., A. Mewara, A. Kumar, G. Verma, and M. Sharma. 2012. Cephalosporin-resistant *Shigella flexneri* over 9 years (2001–2009) in India. J. Antimicrob. Chemother. 67:1347–1353.

22. Sidjabat, H.E., D.L. Paterson, J.M. Adams-Haduch, L. Ewan, A.W. Pasculle, C.A. Muto, G.B. Tian, and Y. Doi. 2009. Molecular epidemiology of CTX-M-producing *Escherichia coli* isolates at a tertiary medical center in western Pennsylvania. Antimicrob. Agents Chemother. 53:4733–4739.

23. Sunde, M. 2005. Prevalence and characterization of class 1 and class 2 integrons in *Escherichia coli* isolated from meat and meat products of Norwegian origin. J. Antimicrob. Chemother. 56:1019–1024.

24. Hsiao, K.-Y., M.-F. Lee, and C.-F. Peng. 2014. Detection and characterization of class 1 integron-associated gene cassettes from *Pseudomonas aeruginosa* isolates in southern Taiwan. Biomark. Genom. Med. 6:74–78.

25. Shiferaw, B., S. Solghan, A. Palmer, K. Joyce, E.J. Barzilay, A. Krueger, and P. Cieslak. 2012. Antimicrobial susceptibility patterns of *Shigella* isolates in foodborne diseases active surveillance network (foodnet) sites, 2000–2010. Clin. Infect. Dis. 54(suppl 5):S458–S463.

26. Bhattacharya, D., A. Sugunan, H. Bhattacharjee, R. Thamizhmani, D. Sayi, K. Thanasekaran, S.P. Manimunda, A.R. Ghosh, A.P. Bharadwaj, M. Singhania, and S. Roy. 2012. Antimicrobial resistance in *Shigella*-rapid increase and widening of spectrum in Andaman Islands, India. Indian J. Med. Res. 135:365.

27. Ruiz, J., D. Castro, P. Goni, J. Santamaria, J. Borrego, and J. Vila. 1997. Analysis of the mechanism of quinolone resistance in nalidixic acid-resistant clinical isolates of *Salmonella* serotype Typhimurium. J. Med. Microbiol. 46:623–628.

28. Pu, X.-Y., J.-C. Pan, H.-Q. Wang, W. Zhang, Z.-C. Huang, and Y.-M. Gu. 2009. Characterization of fluoroquinolone-resistant *Shigella flexneri* in Hangzhou area of China. J. Antimicrob. Chemother. 63:917–920.

29. Das, A., M. Natarajan, and J. Mandal. 2016. The emergence of quinolone resistant *Shigella sonnei*, Pondicherry, India. PLoS One 11:e0160290.

30. Cui, X., J. Wang, C. Yang, B. Liang, Q. Ma, S. Yi, H. Li, H. Lui, P. Li, Z. Wu, J. Xie, L. Jia, R. Hao, L. Wang, Y. Hua, S. Qiu, and H. Song. 2015. Prevalence and antimicrobial resistance of *Shigella flexneri* serotype 2 variant in China. Front. Microbiol. 6:435.

31. Liu, Y., L. Hu, Y. Pan, J. Cheng, Y. Zhu, Y. Ye, and J. Li. 2012. Prevalence of plasmid-mediated quinolone resistance determinants in association with β-lactamases, 16S rRNA methylase genes and integrons amongst clinical isolates of *Shigella flexneri*. J. Med. Microbiol. 61:1174–1176.

32. Sethuvel, D.P.M., S. Anandan, N.K.D. Ragupathi, B. Veeraraghavan, O. Vinod, and K. Walia. 2015. Association of blaCTX-M-15 and qnr genes in multidrug-resistant *Salmonella* Typhimurium and *Shigella* spp. from India. J. Infect. Dev. Count. 9:1294–1297.

33. Schmiedel, J., L. Falgenhauer, E. Domann, R. Bauerfeind, E. Prenger-Berninghoff, C. Imirzalioglu, and T. Chakraborty. 2014. Multiresistant extended-spectrum β-lactamase-producing Enterobacteriaceae from humans, companion animals and horses in central Hesse, Germany. BMC Microbiol. 14:187.

34. González-López, J.J., N. Piedra-Carrasco, F. Salvador, V. Rodríguez, A. Sánchez-Montalvá, A.M. Planes, I. Molina, and M.N. Larrosa. 2014. ESBL-producing *Salmonella enterica* serovar typhi in traveler returning from Guatemala to Spain. Emerg. Infect. Dis. 20:1918.

35. Veldman, K., C. Dierikx, A. van Essen-Zandbergen, W. van Pelt, and D. Mevius. 2010. Characterization of multidrug-resistant, qnrB2-positive and extended-spectrum-β-lactamase-producing *Salmonella* Concord and *Salmonella* Senftenberg isolates. J. Antimicrob. Chemother. 65:872–875.

36. Dolejska, M., L. Villa, H. Hasman, L. Hansen, and A. Carattoli. 2013. Characterization of IncN plasmids carrying blaCTX-M-1 and qnr genes in *Escherichia coli* and *Salmonella* from animals, the environment and humans. J. Antimicrob. Chemother. 68:333–339.

37. Xiong, Z., J. Li, T. Li, J. Shen, F. Hu, and M. Wang. 2010. Prevalence of plasmid-mediated quinolone-resistance determinants in *Shigella flexneri* isolates from Anhui Province, China. J. Antibiot. (Tokyo) 63:187–189.

38. Park, C.H., A. Robicsek, G.A. Jacoby, D. Sahm, and D.C. Hooper. 2006. Prevalence in the United States of aac (6′)-Ib-cr encoding a ciprofloxacin-modifying enzyme. Antimicrob. Agents Chemother. 50:3953–3955.

39. Izumiya, H., Y. Tada, K. Ito, T. Morita-Ishihara, M. Ohnishi, J. Terajima, and H. Watanabe. Characterization of *Shigella sonnei* isolates from travel-associated cases in Japan. J. Med. Microbiol. 58:1486–1491.

40. Cattoir, V., L. Poirel, and P. Nordmann. 2008. Plasmid-mediated quinolone resistance pump QepA2 in an *Escherichia coli* isolate from France. Antimicrob. Agents Chemother. 52:3801–3804.

Address correspondence to:
Mohammad Mehdi Soltan Dallal, PhD
Food Microbiology Research Center
Tehran University of Medical Sciences
Tehran 1588774185
Iran

E-mail: msoltandallal@gmail.com