Al-Khafaji *et al. European Journal of Medical Research (2024) 29:419*
https://doi.org/10.1186/s40001-024-02007-y

European Journal of Medical Research

RESEARCH | Open Access

Prevalence of plasmid-mediated quinolone resistance genes and biofilm formation in different species of quinolone-resistant clinical *Shigella* isolates: a cross-sectional study

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Abstract

Background The purpose of this study was to look into the presence of plasmid-mediated quinolone resistance (PMQR) genes and biofilm formation in several species of clinical *Shigella* isolates that were resistant to quinolones.

Methods The stool samples of 150 patients (younger than 10 years) with diarrhea were collected in this cross-sectional study (November 2020 to December 2021). After cultivation of samples on Hektoen Enteric agar and xylose lysine deoxycholate agar, standard microbiology tests, VITEK 2 system, and polymerase chain reaction (PCR) were utilized to identify *Shigella* isolates. The broth microdilution method was used to determine antibiotic susceptibility. PMQR genes including *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrE*, *qnrS*, *qnrVC*, *qepA*, *oqxAB*, *aac(6′)-Ib-cr*, and *crpP* and biofilm formation were investigated in quinolone-resistant isolates by PCR and microtiter plate method, respectively. An enterobacterial repetitive intergenic consensus polymerase chain reaction (ERIC-PCR) technique was used to determine the clonal relatedness of quinolone-resistant isolates.

Results A total of 95 *Shigella* isolates including *S. sonnei* (53, 55.8%), *S. flexneri* (39, 41.1%), and *S. boydii* (3, 3.2%) were identified. The highest resistance rates of the isolates were against ampicillin (92.6%, n = 88/95). Overall, 42 of 95 (44.2%) isolates were simultaneously resistant against two or more quinolones including 26 (61.9%) *S. sonnei* and 16 (38.1%) *S. flexneri*. All isolates were multidrug-resistant (resistance to more than 3 antibiotics). The occurrence of PMQR genes was as follows: *qnrS* (52.4%), *qnrA* and *aac(6′)-Ib-cr* (33.3%), and *qnrB* (19.0%). The prevalence in species was as follows: 61.5% and 37.5% (*qnrS*), 19.2% and 56.3% (*qnrA*), 38.5% and 25.0 (*aac(6′)-Ib-cr*), and 19.2% and 18.8% (*qnrB*) for *S. sonnei* and *S. flexneri*, respectively. The other PMQR genes were not detected. In total, 52.8% (28/53) of quinolone-susceptible and 64.3% (27/42) of quinolone-resistant isolates were biofilm producers. Biofilm formation was not significantly different between quinolone-resistant and quinolone-susceptible isolates (*P*-value = 0.299). Quinolone-resistant isolates showed a high genetic diversity according to the ERIC-PCR.

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Conclusion It seems that *qnrS*, *qnrA*, and *aac(6')-Ib-cr* play a significant role in the quinolone resistance among *Shigella* isolates in our region. Also the quinolone-resistant *S. flexneri* and *S. sonnei* isolates had a high genetic diversity. Hence, antibiotic therapy needs to be routinely revised based on the surveillance findings.

Keywords ERIC-PCR, Iraq, PMQR, Quinolone resistance, *Shigella*

Introduction

Apart from shigellosis, *Shigella* species are the pioneer cause of the bacillary dysentery in human. *Shigella*-associated diarrhea in children remains an important global human health problem, especially in developing countries [1]. It is estimated that 13.2% of all diarrhea-related mortality in the world are caused by *Shigella* species, making them the second most common cause of these deaths [1, 2]. *Shigella* is a member of the *Enterobacterales* family, and generally, four *Shigella* species, including *Shigella sonnei*, *Shigella boydii*, *Shigella dysenteriae*, and *Shigella flexneri* are responsible for shigellosis or milder forms of diarrheal disease [3]. Among them, *S. sonnei* is the most reported and sometimes dominant species in developed countries that causes mild, watery, or bloody diarrhea. While in low- and middle-income nations, *S. flexneri* is the major species that causes epidemic and endemic shigellosis. Likewise, the other two species (*S. boydii* and *S. dysenteriae*) are relatively rare [2, 4].

Fluoroquinolones belong to a family of synthetic broad-spectrum antibiotics. In addition to being effective antibiotics against multidrug-resistant bacteria, they are also among the most prescribed antibiotics for treating patients with shigellosis [4, 5]. Thus, quinolone-resistant *Shigella* species received a high rank among the global priority list of antibiotic-resistant bacteria published by the World Health Organization (WHO) [2, 3]. In recent years, fluoroquinolone-resistant *Shigella* species have emerged in many parts of the globe, particularly in Asia due to excessive use of these antibiotics [6].

Based on the available evidence, the resistance to fluoroquinolones in bacteria, including *Shigella* species, occurs mainly by point mutations in chromosomal genes encoding DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*) located in quinolone resistance-determining regions (QRDRs) [3, 5–7]. However, the plasmid-mediated quinolone resistance (PMQR) genes are also involved in this process through four main mechanisms, including the aminoglycoside acetyltransferase-encoding enzyme variant *aac(6')-lb-cr*, *qnr* genes, efflux-pump-encoding *qepA* and *oqxAB* genes, and a recently detected phosphorylase gene (*crpP*) [3, 5, 8]. The *aac(6')-lb-cr* gene codes a variant of the aminoglycoside acetyltransferase that acetylates aminoglycosides such as amikacin and kanamycin and fluoroquinolones such as ciprofloxacin and norfloxacin [9]. Currently, seven types of *qnr* genes have been demonstrated to be transferrable by plasmids, namely *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrE*, *qnrS*, and *qnrVC* [10]. A different qnr protein belonging to the pentapeptide repeat protein (PRP) family is encoded by each gene that alters the target of quinolones [11]. A 56-kb conjugative plasmid, pOLA52, encodes oqxAB efflux pump via *oqxA* and *oqxB* genes, which confers resistance to several compounds, including fluoroquinolone antibiotics [12]. The quinolone efflux pump A (*qepA*) gene was first described in an *Escherichia coli* isolate from a urine specimen in 2007 [13]. This gene encodes a protein of 511 amino acids that significantly reduces the susceptibility to quinolones [12]. ATP-dependent phosphorylation of ciprofloxacin by CrpP enzyme that encoded by *crpP* gene, leads to a reduction in ciprofloxacin susceptibility. The *crpP* gene have mostly been identified in *Pseudomonas aeruginosa*, with lesser frequency reported in *E. coli*, *Acinetobacter baumannii*, and *Klebsiella pneumoniae* [8].

Also, a substantial amount of research has focused on the association between antibiotic resistance and biofilm production in bacteria. However, researchers are still attempting to define the association among creation of biofilms and resistance to antibiotics, as there are inconsistent results among different bacteria species [14]. Moreover, few studies have investigated the association between the formation of biofilms in *Shigella* isolates and quinolone resistance.

Consequently, the purpose of this study was to examine the frequency of PMQR genes and biofilm development in various species of clinical quinolone-resistant *Shigella* isolates. Also, the enterobacterial repetitive intergenic consensus polymerase chain reaction (ERIC-PCR) technique was used to examine the clonal relatedness of quinolone-resistant isolates.

Materials and methods

Ethics statement

Ethics approval was obtained from the University of Babylon, Hilla, Iraq (Project No: M220601) compliant with the Declaration of Helsinki. All methods were performed in accordance with the relevant guidelines and regulations of the University of Babylon, Hilla, Iraq. Written consent was obtained from parents or guardians of all participants.

Study design and sample collection

In this cross-sectional study, stool samples were collected from 150 pediatric patients (younger than 10 years) with diarrhea referred to the private hospitals and treatment centers from November 2020 to December 2021. Exclusionary criteria were antibiotic use within 72 h of sample collection and being over 10 years in age, while inclusion criteria included any manifestation of fever, abdominal pain, cramps, vomiting or diarrhea. Sterile plastic containers were used to collect stool samples, which were immediately transferred to the microbiology laboratory for analysis.

Bacterial isolation and identification

A portion of all samples were inoculated on Hektoen Enteric (HE) Agar and Xylose Lysine Desoxycholate (XLD) Agar (Condalab, Madrid, Spain) and incubated at 37 ºC for 24 h. Also, a small percentage of specimens were enriched in 5 ml of Gram-negative (GN) broth (Condalab, Madrid, Spain) for six hours at 37 °C. The enriched specimens were sub-cultured on HE and XLD plates and placed at 37 ºC for 24 h [4]. The isolates that appeared green and red on HE and XLD, respectively, were considered presumptive *Shigella* isolates and identified using standard biochemical tests, including sulfide indole motility (SIM), citrate and urea hydrolysis, methyl red (MR) and Voges–Proskauer (VP), Kligler Iron Agar (KIA), and Lysine Iron Agar (LIA) [15]. *Shigella* isolates were confirmed by the VITEK 2 Compact system (bioMérieux, Marcy l'Étoile, France) according to the manufacturer protocol [16]. All isolates were further confirmed by polymerase chain reaction (PCR) of putative integrase as a specific marker for *Shigella* genus using previously described primers (Bioneer, South Korea) (Table 1) [17]. The QIAamp DNA Minikit (Qiagen GmbH, Hilden, Germany) was used for DNA extraction from *Shigella* isolates following the manufacturer instructions. PCR reaction (25 µL) was performed with the following program: 95 °C for 5 min, 35 cycles of 95 °C for 40 s, 60 °C for 30 s, 72°C for 30 s, and final extension at 72 °C for 5 min. The PCR products (5 µL) were loaded into 1% agarose gel and placed in 1X tris–acetate-EDTA (TAE) buffer contained ethidium bromide (0.5 µg/mL). The electrophoresis was performed for 45 min and amplicons were analyzed in a Bio-Rad gel documentation system (USA). In all experiments, *S. sonnei* ATCC® 25,931™ and *S. flexneri* ATCC® 29,903™ were used as quality control.

Table 1 Primer sequences that were used in this study

Genes	Sequences (5'–3')	Annealing (°C)	Product size (bp)	References
Putative integrase	F- TCGCATTTCTCTCCCCACCACG R- CCGGATGTGTCTCGGGCAATC	60	159	[17]
qnrA	F- CAGCAAGAGGATTTCTCACG R- AATCCGGCAGCACTATTACTC	53	630	[19]
qnrB	F- GGCTGTCAGTTCTATGATCG R- GAGCAACGATGCCTGGTAG	55	488	[19]
qnrC	F- GCAGAATTCAGGGGTGTGAT R- AACTGCTCCAAAAGCTGCTC	55	118	[19]
qnrD	F- CGAGATCAATTTACGGGGAATA R- AACAAGCTGAAGCGCCTG	54	581	[19]
qnrS	F- GCAAGTTCATTGAACAGGGT R- TCTAAACCGTCGAGTTCGGCG	57	428	[19]
oqxAB	F- CCGCACCGATAAATTAGTCC R- GGCGAGGTTTTGATAGTGGA	52	313	[19]
aac(6')-Ib-cr	F- TTGGAAGCGGGGACGGAM R- ACACGGCTGGACCATA	57	260	[19]
qepA	F- GCAGGTCCAGCAGCGGGTAG R- CTTCCTGCCCGAGTATCGTG	55	218	[19]
qnrVC	F- AATTTTAAGCGCTCAAACCTCCG R- TCCTGTTGCCACGAGCATATTTT	55	521	[20]
qnrE	F- GGCATTGATTTTTGAAGGCGA R- GTGGGTAAAATTGGCCGCTC	56	517	[21]
crpP	F- CGAGCTGCTGTTGCTGCTCCTGG R- CGACCGGTACCGACAAGCTGGAC	63	177	[22]
ERIC-PCR	F- AAGTAAGTGACTGGGGTGAGCG R- ATGTAAGCTCCTGGGGATTCAC	40	Variable	[23]

Antimicrobial susceptibility testing (AST)

The minimal inhibitory concentrations (MICs) of ciprofloxacin (32–0.03125 μg/mL), levofloxacin (32–0.03125 μg/mL), gatifloxacin (64–0.0625 μg/mL), ofloxacin (64–0.0625 μg/mL), imipenem (64–0.0625 μg/mL), meropenem (64–0.0625 μg/mL), ampicillin (512–0.05 μg/mL), amoxicillin/clavulanic acid (512–0.05 μg/mL), azithromycin (512–0.05 μg/mL), cefotaxime (64–0.0625 μg/mL), ceftriaxone (64–0.0625 μg/mL), ceftazidime (256–0.025 μg/mL), tetracycline (256–0.025 μg/mL), chloramphenicol (512–0.05 μg/mL), trimethoprim/sulfamethoxazole (64–0.0625 μg/mL), and cefepime (256–0.025 μg/mL) (Sigma-Aldrich, Darmstadt, Germany) were determined by broth microdilution method according to the Clinical Laboratory Standard Institute (CLSI) 2021 principles [18]. The Mueller–Hinton broth (Merck, Darmstadt, Germany) was used for AST. The breakpoints for resistance or susceptibility to each antibiotic were determined according to the CLSI 2021 criteria [18]. Multidrug-resistant (MDR) isolates were defined as those resistant to three or more antibiotics belong to different category [14]. *Escherichia coli* ATCC® 25,922™ was used as the control strain.

PMQR genes detection by PCR method

Uniplex PCR method was used to detect 11 PMQR genes in quinolone-resistant isolates using previously described primers (Bioneer, South Korea) as follows: *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrE*, *qnrS*, *qnrVC*, *aac(6')-Ib-cr*, *qepA*, *oqxAB*, and *crpP* [19–22]. The PCR reaction (25 μL) was performed in a Bio-Rad thermocycler (USA) with the following temperatures: 95 °C for 5 min, 35 cycles of 95 °C for 30 s, annealing (Table 1) for 30 s, 72°C for 30 s, and final extension at 72 °C for 5 min. The electrophoresis and visualization of the PCR products were performed similar to the previous stage.

Biofilm formation

A 96-well microtiter plate was used to assess the biofilm formation of quinolone-resistant and quinolone-susceptible isolates based on the previously described method with minor modifications [14]. The isolates were cultivated overnight in tryptic soy broth (Condalab, Madrid, Spain) and then were adjusted to a 0.5 McFarland standard turbidity. A total of 150 μL of these suspensions was dispensed in microtiter plate wells and incubated overnight at 37 °C. An initial three washes with phosphate-buffered saline (PBS) were done, followed by 20 min of fixation with methanol and 15 min of staining with 0.1% (v/v) crystal violet solution. Biofilm formation was measured at 550 nm optical density (OD_{550}) using a microtiter plate reader (Thermo Fisher Scientific, USA). The isolates were categorized into four groups: non-biofilm ($OD \leq ODc$), low-biofilm ($ODc < OD \leq 2$ ODc), medium-biofilm (2 $ODc < OD \leq 4$ ODc), and strong-biofilm ($OD > 4$ ODc) [14].

Clonal association by ERIC-PCR

The clonal association of quinolone-resistant isolates were investigated by ERIC-PCR. ERIC-PCR was performed in 25 μL volume using following program: 95 °C (15 min), 35 cycles [95 °C (30 s), 40 °C (45 s), and 72 °C (6 min)], and a final extension at 72 °C (10 min). The ERIC-PCR primers are shown in Table 1 [23]. Using a standard 1-kb DNA ladder (Fermentas, USA) as a reference, the ERIC-PCR patterns were evaluated by comparing positions of band lanes in each agarose gel (2%). To analyze the ERIC-PCR patterns, dendrograms were generated based on unweighted pair group method with arithmetic mean (UPGMA) and the Dice similarity coefficient using GelJ version 2.0 software [23, 24]. *Shigella* isolates with 90.0% or more similarity were considered to be clonally related.

Statistical analysis

Descriptive statistical analysis (frequency and prevalence of antibiotic resistance rates and PMQR genes) were performed using Statistical Package for the Social Sciences (SPSS) version 20.0 (SPSS Inc., Chicago, IL, USA). Fisher's exact test or Chi-square test were used to determine any significant correlations (P-value < 0.05) between variables.

Results

Shigella isolates

During the study period, a total of 95 presumptive *Shigella* isolates were collected and primarily identified using standard phenotypic and biochemical tests. Using VITEK 2 Compact system, the *Shigella* isolates were confirmed on species level in the following order: *S. sonnei* (53, 55.8%), *S. flexneri* (39, 41.1%), and *S. boydii* (3, 3.2%). No *S. dysenteriae* isolate was detected. All identified isolates showed the 159 bp specific band of putative integrase of *Shigella* genus in PCR assay. *Shigella* isolates were mostly retrieved from male patients (59, 62.1%) including 31 (52.5%) *S. sonnei*, 25 (42.4%) *S. flexneri*, and 3 (5.1%) *S. boydii*. The remaining were collected from females (36, 37.9%). There was no significant differences between the prevalence of *Shigella* species and gender of patients (P-value $= 0.334$). Also, most of the isolates were identified in pediatric patients aged from 4–6 years old (42, 44.2%), followed by 2–4 years old (28, 29.5%), 6–8 years old (13, 13.7%), < 2 years old (9, 9.5%), and 8–10 years old (3, 3.2%).

Antibiotic resistance rates

The AST revealed that 98.9% (n = 94/95) and 97.9% (n = 93/95) of *Shigella* isolates were susceptible to imipenem and meropenem, respectively (Table 2). *S. sonnei*, *S. boydii*, and *S. flexneri* showed the susceptibility rates of 100.0% (n = 53/53), 100.0% (n = 3/3), and 97.4% (n = 38/39) towards imipenem, respectively. The highest resistance rates of the *Shigella* isolates were against ampicillin (92.6%, n = 88/95), followed by amoxicillin/clavulanic acid (85.3%, n = 81/95), trimethoprim/sulfamethoxazole (76.8%, n = 73/95), ceftriaxone (56.8%, n = 54/95), and cefotaxime (50.5%, n = 48/95). All three species *S. boydii* (100.0%, n = 3/3), *S. sonnei* (90.6%, n = 48/53), and *S. flexneri* (94.9%, n = 37/39) were highly resistant to ampicillin, with no significant difference ($P > 0.05$). In total, 42 of 95 (44.2%) *Shigella* isolates were quinolone-resistant including 26 (61.9%) *S. sonnei* and 16 (38.1%) *S. flexneri*. No *S. boydii* isolate was quinolone-resistant. All 42 quinolone-resistant isolates were simultaneously resistant against two or more quinolones (Table 2). The highest co-resistance rate was observed against levofloxacin–ofloxacin (23.8%, n = 10/42), followed by ciprofloxacin–levofloxacin (21.4%, n = 9/42). The highest resistance rate was against ciprofloxacin (31.6%, n = 30/95) followed by ofloxacin (28.4%, n = 27/95), levofloxacin (26.3%, n = 25/95), and gatifloxacin (15.8%, n = 15/95), respectively. All isolates including 42 quinolone-resistant strains were MDR.

Prevalence of PMQR genes

In total, the PMQR genes were detected in 92.9% (39/42) of quinolone-resistant *Shigella* isolates including all quinolone-resistant *S. flexneri* (100.0%, n = 16/16) and 88.5% (n = 23/26) of quinolone-resistant *S. sonnei*. Four different PMQR genes were found to be associated with both quinolone-resistant *S. sonnei* and *S. flexneri* isolates in this study (Table 3, Fig. 1, Fig. 2, and Fig. 3). The occurrence of these genes in their descending order of positivity was as follows: *qnrS* (52.4%) > *qnrA* and *aac(6′)-Ib-cr* (33.3%) > *qnrB* (19.0%). The prevalence of different genes in *Shigella* species was as follows: 61.5% and 37.5% (*qnrS*), 19.2% and 56.3% (*qnrA*), 38.5% and 25.0 (*aac(6′)-Ib-cr*), and 19.2% and 18.8% (*qnrB*) for *S. sonnei* and *S. flexneri*, respectively. The co-incidence of PMQR determinants was detected in 40.5% (17/42) of quinolone-resistant isolates (Table 3). Statistically, there was no significant difference ($P > 0.05$) in the prevalence of the four PMQR genes in both *Shigella* species. The *qnrC*, *qnrD*, *qnrE*, *qnrVC*, *qepA*, *oqxAB*, and *crpP* genes were not detected in any isolate.

Biofilm formation

In total, 52.8% (28/53) of quinolone-susceptible isolates including 55.6% (15/27) of *S. sonnei*, 43.5% (10/23) of *S. flexneri*, and 100.0% (3/3) of *S. boydii* were low-biofilm producers. None of the quinolone-susceptible isolates were strong-biofilm producers or medium-biofilm producers. However, 64.3% (27/42) of quinolone-resistant isolates showed the biofilm formation phenomenon including 28.6% (12/42) strong-biofilm producers, 23.8% (10/42) medium-biofilm producers, and 11.9% (5/42) low-biofilm producers. Also, 15 isolates (35.7%, 15/42) were non-biofilm producers. In terms of *Shigella* species, 30.8% (8/26), 23.1% (6/26), 11.5% (3/26), and 34.6% (9/26) of quinolone-resistant *S. sonnei* were strong-biofilm producers, medium-biofilm producers, low-biofilm producers, and non-biofilm producers, respectively. Meanwhile, 25.0% (4/16), 25.0% (4/16), 12.5% (2/16), and 37.5% (6/16) of quinolone-resistant *S. flexneri* were strong-biofilm producers, medium-biofilm producers, low-biofilm producers, and non-biofilm producers, respectively. Biofilm formation was not significantly different between quinolone-resistant *S. sonnei* and quinolone-resistant *S. flexneri* isolates (P-value > 0.999). Moreover, the biofilm formation was not significantly different between quinolone-resistant and quinolone-susceptible *Shigella* isolates (P-value = 0.299).

Clonal association

Based on the ERIC-PCR, 16 quinolone-resistant *S. flexneri* isolates were distributed to 4 clusters (A to D) including 2 to 3 isolates and 6 singletons with 12 various ERIC-types (E1 to E12), indicating a high genetic diversity among the isolates (Fig. 4). The ERIC-PCR patterns included 3 to 8 bands with various sizes ranging from 100 to 1000 bp for each isolate. Also, the 26 quinolone-resistant *S. sonnei* isolates were distributed to 3 clusters (A to C) including 2 to 3 isolates and 19 singletons with 24 various ERIC-types (E1 to E24), showing a high genetic diversity among the isolates (Fig. 5). The ERIC-PCR patterns included 3 to 9 bands with various sizes that ranged from 100 to 1300 bp for each isolate.

Discussion

In this study, *S. sonnei* (58.0%) was the most common cause of the shigellosis followed by *S. flexneri* (41.1%) and *S. boydii* (3.2%). In contrast to this study, previous reports suggested *S. flexneri* as the leading species of shigellosis in developing countries [25, 26]. However, most developing countries, including those in Southeast Asia, are recording a shift in the major etiological agent of shigellosis to *S. sonnei* [27]. In line with the current study, Teimourpour et al. [28] from Iran, reported *S. sonnei* (69.9%) as the most predominant species followed by *S.*

Table 2 Antibiotic resistance rates and quinolone co-resistance of the different *Shigella* species

Antibiotic	***Shigella sonnei*** **n = 53 (55.8%)**			***Shigella flexneri*** **n = 39 (41.1%)**			***Shigella boydii*** **n = 3 (0.32%)**			**Total** **n = 95 (100.0%)**		
	S	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**
		***n* (%)**			***n* (%)**			***n* (%)**			***n* (%)**	
Ciprofloxacin	32 (60.4)	2 (3.8)	19 (35.8)	26 (66.7)	2(5.1)	11 (28.2)	3 (100.0)	0 (0.0)	0 (0.0)	61 (64.2)	4 (4.2)	30 (31.6)
Levofloxacin	38 (71.7)	3 (5.7)	12 (22.6)	26 (66.7)	0 (0.0)	13 (33.3)	3 (100.0)	0 (0.0)	0 (0.0)	67 (70.5)	3 (3.2)	25 (26.3)
Gatifloxacin	43 (81.1)	3 (5.7)	7 (13.2)	31 (79.5)	0 (0.0)	8 (20.5)	3 (100.0)	0 (0.0)	0 (0.0)	77 (81.1)	3 (3.2)	15 (15.8)
Ofloxacin	37 (69.8)	1 (1.9)	15 (28.3)	27 (69.2)	0 (0.0)	12 (30.8)	3 (100.0)	0 (0.0)	0 (0.0)	67 (70.5)	1 (1.1)	27 (28.4)
Imipenem	53 (100.0)	0 (0.0)	0 (0.0)	38 (97.4)	0 (0.0)	1 (2.6)	3 (100.0)	0 (0.0)	0 (0.0)	94 (98.9)	0 (0.0)	1 (1.1)
Meropenem	52 (98.1)	0 (0.0)	1 (1.9)	38 (97.4)	0 (0.0)	1 (2.6)	3 (100.0)	0 (0.0)	0 (0.0)	93 (97.9)	0 (0.0)	2 (2.1)
Ampicillin	4 (7.5)	1 (1.9)	48 (90.6)	1 (2.6)	1 (2.6)	37 (94.9)	0 (0.0)	0 (0.0)	3 (100.0)	5 (5.3)	2 (2.1)	88 (92.6)
Amoxicillin/clavulanic acid	6 (11.3)	0 (0.0)	47 (88.7)	8 (20.5)	0 (0.0)	31 (79.5)	0 (0.0)	0 (0.0)	3 (100.0)	14 (14.7)	0 (0.0)	81 (85.3)
Azithromycin	32 (60.4)	3 (5.7)	18 (34.0)	21 (53.8)	3 (7.7)	15 (38.5)	2 (66.7)	0 (0.0)	1 (33.3)	55 (57.9)	6 (6.3)	34 (35.8)
Cefotaxime	25 (47.2)	3 (5.7)	25 (47.2)	17 (43.6)	2 (5.1)	20 (51.3)	0 (0.0)	0 (0.0)	3 (100)	42 (44.2)	5 (5.3)	48 (50.5)
Ceftriaxone	22 (41.5)	4 (7.5)	27 (50.9)	13 (33.3)	1 (2.6)	25 (64.1)	1 (33.3)	0 (0.0)	2 (66.7)	36 (37.9)	5 (5.3)	54 (56.8)
Ceftazidime	26 (49.1)	1 (1.9)	26 (49.1)	17 (43.6)	3 (7.7)	19 (48.7)	1 (33.3)	0 (0.0)	2 (66.7)	44 (46.3)	4 (4.2)	47 (49.5)
Tetracycline	34 (64.2)	2 (3.8)	17 (32.1)	25 (64.1)	2 (5.1)	12 (30.8)	1 (33.3)	2 (66.7)	0 (0.0)	60 (63.2)	6 (6.3)	29 (30.5)
Chloramphenicol	39 (73.6)	0 (0)	14 (26.4)	27 (69.2)	0 (0.0)	12 (30.8)	3 (100)	0 (0.0)	0 (0.0)	69 (72.6)	0 (0.0)	26 (27.4)
Trimethoprim/sulfamethoxazole	13 (24.5)	2 (3.8)	38 (71.7)	7 (17.9)	0 (0.0)	32 (82.1)	0 (0.0)	0 (0.0)	3 (100.0)	20 (21.1)	2 (2.1)	73 (76.8)
Cefepime	24 (45.3)	7 (13.2)	22 (41.5)	14 (35.9)	1 (2.6)	24 (61.5)	3 (100.0)	0 (0.0)	0 (0.0)	41 (43.2)	8 (8.4)	45 (47.4)

Quinolone co-resistance in quinolone-resistant *Shigella* species	***Shigella sonnei*** **n = 26 (61.9%)** ***n* (%)**	***Shigella flexneri*** **n = 16 (38.1%)** ***n* (%)**	**Total** ***n* = 42 (100.0%)** ***n* (%)**
Ciprofloxacin–levofloxacin	5 (19.2)	4 (25.0)	9 (21.4)
Ciprofloxacin–ofloxacin	2 (7.7)	1 (6.3)	3 (7.1)
Ciprofloxacin–gatifloxacin	1 (3.8)	0 (0.0)	1 (2.4)
Levofloxacin–ofloxacin	7 (26.9)	3 (18.8)	10 (23.8)
Gatifloxacin–ofloxacin	6 (23.1)	2 (12.5)	8 (19.0)
Ciprofloxacin–levofloxacin–ofloxacin	3 (11.5)	1 (6.3)	4 (9.5)
Ciprofloxacin–gatifloxacin–ofloxacin	3 (11.5)	0 (0.0)	3 (7.1)
Levofloxacin–gatifloxacin–ofloxacin	0 (0.0)	1 (6.3)	1 (2.4)
Ciprofloxacin–levofloxacin–gatifloxacin–ofloxacin	0 (0.0)	5 (31.3)	5 (11.9)

flexneri (19.4%) and *S. boydii* (9.9%). On the other hand, the absence of *S. dysenteriae* in this study was not surprising, as the isolation of these species is relatively rare when compared to the other three species [4, 6]. Yu et al. [3] from China, also corroborated this result by recording a complete absence of *S. dysenteriae* in a study involving 118 *Shigella* isolates. These discrepancies may be due to the differences in the sample size, the race of studies population, and the hygiene situation of studied region.

Shigella infections are distributed differently based on age and gender worldwide. This study revealed the most incidence rate of *Shigella* isolates in males and in pediatric patients aged from 4–6 years old. In line with the current observations, Abbasi et al. [4] from Iran, Liu et al. [15] from China, and Jain et al. [25] from India reported a higher frequency rate of *Shigella* isolates in males than that of females. The descriptive epidemiology identified varying rates of shigellosis cases among males and females. Nevertheless, there is a lack of comprehensive explanations on the factors contributing to this disparity and the methods of transmission [29]. One of the reasons for this difference may be women's better personal hygiene habits [26, 29]. Moreover, males are more likely to be exposed to the environments contaminated with *Shigella* species [15]. Previous studies reported a more prevalence rate of *Shigella* infections in pediatric patients under 5 years which was consistent with our findings [15, 30]. Due to their low immunity and lack of prior exposure, children under five are more susceptible to shigellosis than older children [15].

Shigella species isolated in this study displayed a wide spectrum of antibiotic resistance. The highest resistance rates were found to ampicillin (92.6%), amoxicillin/clavulanic acid (85.3%), and trimethoprim/sulfamethoxazole (76.8%). *S. boydii* completely resisted ampicillin, amoxicillin/clavulanic acid, cefotaxime, and trimethoprim/sulfamethoxazole with a rate of 100.0%. Despite having a low prevalence of isolation, *S. boydii* could pose a serious health concern. Similar results were previously reported by Abbasi et al. [4], Zamanlo et al. [31] and Farahani et al. [32] from neighboring geographical area, Liu et al. [16] from China, and Phiri et al. [26] from Malawi. In another study carried out in Kenya by Leting et al. [33], the *Shigella* isolates displayed almost similar resistance rate against ampicillin (100.0%) and amoxicillin/clavulanic acid (84.2%). This implied that these group of antibiotics are no more suitable for the treatment of shigellosis. This could have resulted due to the extensive usage of these antibiotics, especially in the treatment of diarrhea leading to high degrees of resistance. According to Behruznia et al. [34], *Shigella* species have become resistant to first-line drugs (trimethoprim–sulfamethoxazole and ampicillin), so that they are no longer recommended for the treatment of shigellosis. The increase in resistance by *Shigella* species calls for concerns as the United Kingdom, Northern Ireland, and numerous other nations have all reported cases of extensively drug-resistant (XDR) *Shigella sonnei* [35]. Besides, our findings revealed that almost 50.0% of *Shigella* isolates were resistant to third generation cephalosporins that was in line with the previous reports from Iran [4, 31]. However, in contrast to our findings, Madhavan et al. [30] from India and Liu et al. [36] from China reported lower resistance rates for these antibiotics. The high resistance rate to third generation cephalosporins in this study may be due to the emergence of extended-spectrum beta-lactamases (ESBLs)-producing *Shigella* isolates [37–39]. In recent years, there have been reports of the emergence of *Shigella* strains carrying ESBL genes from some parts of the world including China, Iran, and England [37–39].

In this study, the focus was on quinolone-resistant strains. In total, 44.2% of *Shigella* isolates were simultaneously resistant against two or more quinolones including 61.9% *S. sonnei* and 38.1% *S. flexneri*. This resistance rate was much higher than a previous report from neighboring country Iran in which only 4.4% of isolates were quinolone-resistant [28]. Also, in this study, the most resistance rate among quinolones was related to ciprofloxacin (31.6%) that was in line with a previous report from China [6]. Due to the widespread use of fluoroquinolones as first-line antibiotics to treat diarrhea in recent years, quinolone resistance has increased considerably. A lack of local antimicrobial resistance surveillance, easy access to antibiotics, and inappropriate prescription of antibiotics in our region may all be contributing factors to the situation's deterioration. However, the high quinolone-resistant rate for *S. sonnei* in this study was in good parallel with a previous report that showed 76% (313/411) of *S. sonnei* isolates collected from several countries were quinolone-resistant [40]. A previous study from India has also reported quinolone-resistant *S. sonnei* brought on by a combination of *qnrB* and *gyrA* mutations [41]. Based on the available evidence, men who have sex with men (MSM) in Taiwan are frequently infected with quinolone-resistant *S. sonnei* that has the same QRDR mutations [42].

The PMQR determinants have escalated into a serious global issue over the past few years [6]. To the best of our knowledge, this study was the first to investigate 11 different PMQR genes in quinolone-resistant *Shigella* isolates. The *qnrS* (52.4%) was the most frequent PMQR gene followed by *qnrA* and *aac(6')-Ib-cr* (33.3%), and *qnrB* (19.0%). The *qnrC*, *qnrD*, *qnrE*, *qnrVC*, *qepA*, *oqxAB*, and *crpP* genes were not detected in any isolate. In a previous study from India [41], 18.7% of *S. sonnei* isolates harbored *qnrC* gene and no isolate was positive for

Table 3 Prevalence of plasmid-mediated quinolone resistance (PMQR) genes in different species of quinolone-resistant clinical *Shigella* isolates

PMQR	*Shigella sonnei* (*n* = 26)		*Shigella flexneri* (*n* = 16)		Total (*n* = 42)	
	Positive	Negative	Positive	Negative	Positive	Negative
	n (%)		*n* (%)		*n* (%)	
qnrA	5 (19.2)	21 (80.8)	9 (56.3)	7 (43.8)	14 (33.3)	28 (66.7)
qnrB	5 (19.2)	21 (80.8)	3 (18.8)	36 (81.3)	8 (19.0)	34 (81.0)
qnrC	0 (0.0)	26 (100.0)	0 (0.0)	16 (100.0)	0 (0.0)	42 (100.0)
qnrD	0 (0.0)	26 (100.0)	0 (0.0)	16 (100.0)	0 (0.0)	42 (100.0)
qnrE	0 (0.0)	26 (100.0)	0 (0.0)	16 (100.0)	0 (0.0)	42 (100.0)
qnrS	16 (61.5)	37 (69.8)	6 (37.5)	33 (84.6)	22 (52.4)	20 (47.6)
qnrVC	0 (0.0)	26 (100.0)	0 (0.0)	16 (100.0)	0 (0.0)	42 (100.0)
qepA	0 (0.0)	26 (100.0)	0 (0.0)	16 (100.0)	0 (0.0)	42 (100.0)
aac(6')-Ib-cr	10 (38.5)	16 (61.5)	4 (25.0)	12 (75.0)	14 (33.3)	28 (85.3)
oqxAB	0 (0.0)	26 (100.0)	0 (0.0)	16 (100.0)	0 (0.0)	42 (100.0)
crpP	0 (0.0)	26 (100.0)	0 (0.0)	16 (100.0)	0 (0.0)	42 (100.0)

PMQR co-occurrence	*Shigella sonnei* (*n* = 26) *n* (%)	*Shigella flexneri* (*n* = 16) *n* (%)	Total (*n* = 42) *n* (%)
qnrA/aac(6')-Ib-cr	2 (7.7)	2 (12.5)	4 (9.5)
qnrS/aac(6')-Ib-cr	4 (15.4)	2 (7.7)	6 (14.3)
qnrA/qnrS	1 (3.8)	1 (6.3)	2 (4.8)
qnrB/qnrS	1 (3.8)	1 (6.3)	2 (4.8)
qnrB/aac(6')-Ib-cr	1 (3.8)	0 (0.0)	1 (2.4)
qnrA/qnrS/aac(6')-Ib-cr	1 (3.8)	0 (0.0)	1 (2.4)
qnrB/qnrS/aac(6')-Ib-cr	1 (3.8)	0 (0.0)	1 (2.4)

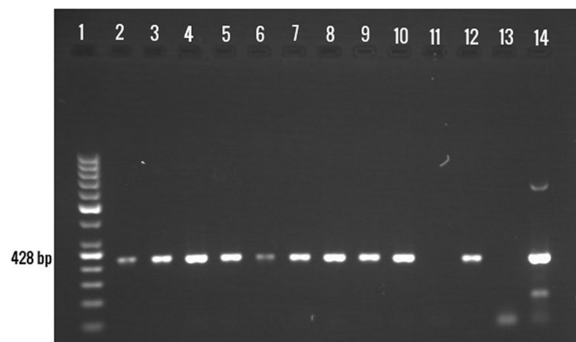


Fig. 1 Gel electrophoresis of *qnrS* gene (428 bp). Lane 1: DNA ladder (100 bp); Lane 2: Control positive; Lanes 3–8: *qnrS*-positive *Shigella sonnei* isolates; Lanes 9, 10, 12, and 14: *qnrS*-positive *Shigella flexneri* isolates; Lanes 11 and 13: *qnrS*-negative *Shigella sonnei*

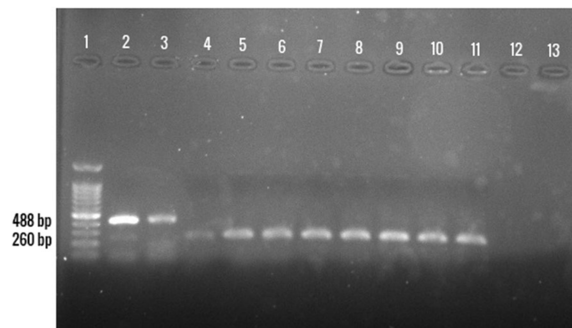


Fig. 2 Gel electrophoresis of *qnrB* (488 bp) and *aac(6')-Ib-cr* (260 bp). Lane 1: DNA ladder (100 bp); Lane 2: Positive control of *qnrB* gene; Lanes 3: *qnrS*-positive *Shigella sonnei* isolate; Lane 4: Negative control: DNA/RNA free water; Lane 5: Positive control of *aac(6')-Ib-cr* gene; Lanes 6–8: *aac(6')-Ib-cr*-positive *Shigella flexneri* isolates; Lanes 9–11: *aac(6')-Ib-cr*-positive *Shigella sonnei* isolates; Lanes 12 and 13: *aac(6')-Ib-cr*-negative *Shigella flexneri* isolates

qepA, *qnrS*, and *qnrA*. In another study from Iran, *qnrC* and *qnrD* genes were not detected in any *Shigella* isolates [43]. Also, in a previous research from China [44], *qnrA*, *qnrC*, *qnrD* and *qepA* genes were not found in *S. flexneri* isolates. However, Yang et al. [45] from China, reported the *qepA* gene in 2.05% of *Shigella* isolates. Moreover, based on the available evidence, there is no report of the presence of *qnrE*, *qnrVC*, and *crpP* genes in *Shigella* isolates from different countries.

Statistically, there was no significant difference in the prevalence of the four PMQR genes between *S. sonnei* and *S. flexneri* species. In a previous study from China,

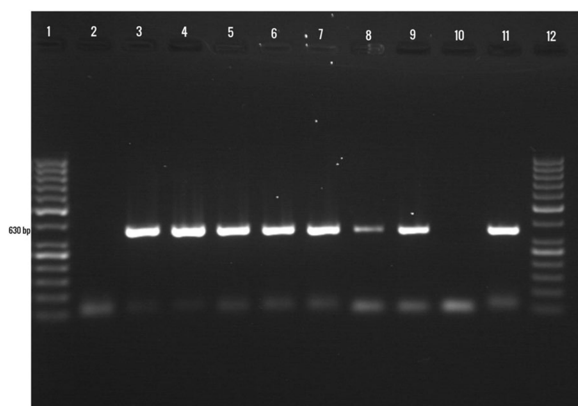
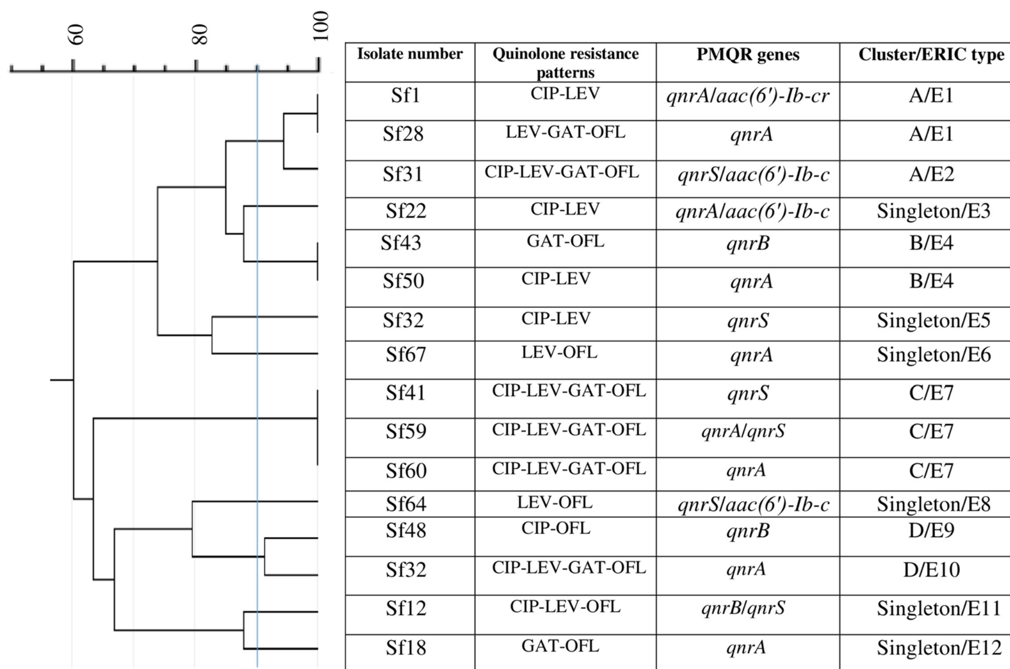


Fig. 3 Gel electrophoresis of *qnrA* gene (630 bp). Lane 1 and 12: DNA ladder (100 bp); Lane 2: Negative control: DNA/RNA free water; Lanes 3: Positive control of *qnrA* gene; Lanes 4–6: *qnrA*-positive *Shigella sonnei* isolates; Lanes 7, 8, 9, and 11: *qnrA*-positive *Shigella flexneri* isolates; Line 10: *qnrA*-negative *Shigella flexneri* isolates

aac (6′)-Ib-cr was the most frequent gene found in quinolone-resistant *Shigella* isolates [46]. The frequency of selecting chromosomal mutants to decrease ciprofloxacin activity by N-acetylation of its piperazinyl substituent has been shown to be significantly increased by *aac (6′)-Ib-cr* [46]. In this study, *qnrA* was detected in 19.2% and 56.3% of *S. sonnei* and *S. flexneri* isolates, respectively. However, in a previous studies from China [44, 46], the *Shigella* isolates were negative for this gene. Based on evidence, China had a majority prevalence of *qnrS* in *S. sonnei* and *S. flexneri*, while India had a majority prevalence of *qnrB* [47]. Also, similar to this study, *qepA* gene was not detected in *S. sonnei* isolates from China [48]. In a previous study from India, the *qnrS1* was detected in 47% of *S. boydii* isolate and no *S. sonnei* was positive for *qnr* genes that was in contrast to this study [49].

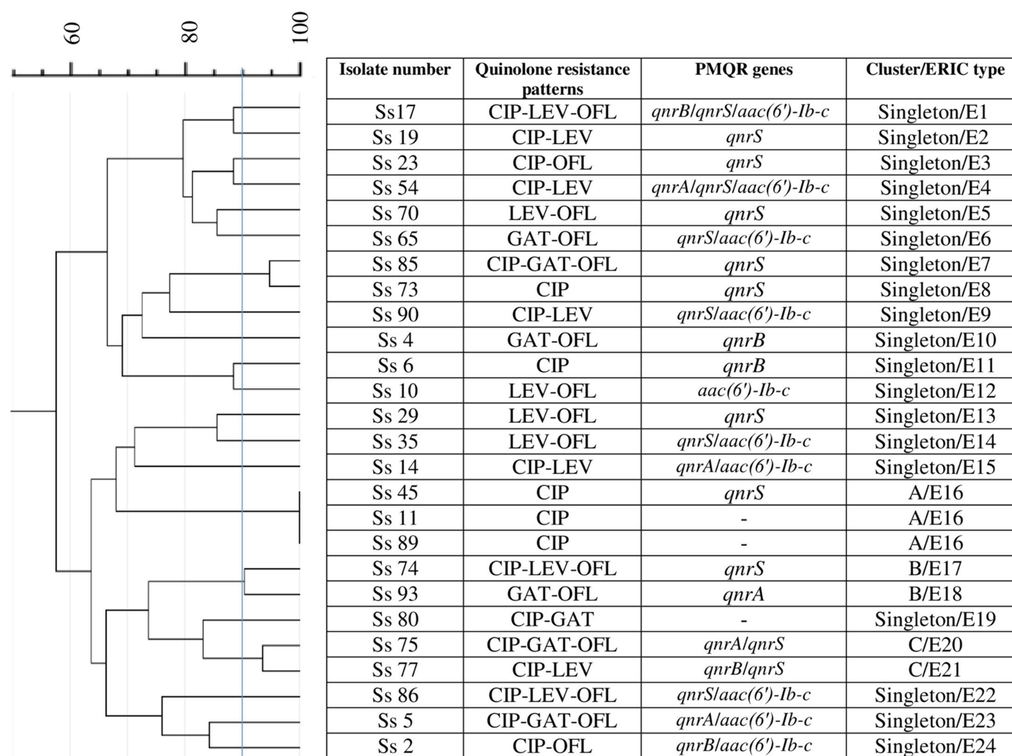
In this study, 52.8% of quinolone-susceptible and 64.3% of quinolone-resistant isolates were biofilm producers and the biofilm formation rates of resistant and susceptible isolates were not significantly different. In line with this study, no association was found between the biofilm formation and antibiotic resistance in *Pseudomonas aeruginosa* and *Acinetobacter baumannii* isolates in previous studies [50, 51]. However, several investigations have shown the positive correlation between biofilm production and antibiotic resistance [52, 53]. Despite extensive research, *Shigella* species have not been thoroughly characterized in terms of association of their biofilm formation with antimicrobial resistance. Hence, further studies are needed to reveal the molecular mechanisms involved in these processes.



Isolate number	Quinolone resistance patterns	PMQR genes	Cluster/ERIC type
Sf1	CIP-LEV	*qnrA/aac(6′)-Ib-cr*	A/E1
Sf28	LEV-GAT-OFL	*qnrA*	A/E1
Sf31	CIP-LEV-GAT-OFL	*qnrS/aac(6′)-Ib-c*	A/E2
Sf22	CIP-LEV	*qnrA/aac(6′)-Ib-c*	Singleton/E3
Sf43	GAT-OFL	*qnrB*	B/E4
Sf50	CIP-LEV	*qnrA*	B/E4
Sf32	CIP-LEV	*qnrS*	Singleton/E5
Sf67	LEV-OFL	*qnrA*	Singleton/E6
Sf41	CIP-LEV-GAT-OFL	*qnrS*	C/E7
Sf59	CIP-LEV-GAT-OFL	*qnrA/qnrS*	C/E7
Sf60	CIP-LEV-GAT-OFL	*qnrA*	C/E7
Sf64	LEV-OFL	*qnrS/aac(6′)-Ib-c*	Singleton/E8
Sf48	CIP-OFL	*qnrB*	D/E9
Sf32	CIP-LEV-GAT-OFL	*qnrA*	D/E10
Sf12	CIP-LEV-OFL	*qnrB/qnrS*	Singleton/E11
Sf18	GAT-OFL	*qnrA*	Singleton/E12

CIP: ciprofloxacin, LEV: levofloxacin, GAT: gatifloxacin, OFL: ofloxacin

Fig. 4 The UPGMA dendrogram with the Dice coefficient of quinolone-resistant *Shigella flexneri* isolates in the basis of ERIC-PCR patterns



Isolate number	Quinolone resistance patterns	PMQR genes	Cluster/ERIC type
Ss17	CIP-LEV-OFL	*qnrB/qnrS/aac(6')-Ib-c*	Singleton/E1
Ss 19	CIP-LEV	*qnrS*	Singleton/E2
Ss 23	CIP-OFL	*qnrS*	Singleton/E3
Ss 54	CIP-LEV	*qnrA/qnrS/aac(6')-Ib-c*	Singleton/E4
Ss 70	LEV-OFL	*qnrS*	Singleton/E5
Ss 65	GAT-OFL	*qnrS/aac(6')-Ib-c*	Singleton/E6
Ss 85	CIP-GAT-OFL	*qnrS*	Singleton/E7
Ss 73	CIP	*qnrS*	Singleton/E8
Ss 90	CIP-LEV	*qnrS/aac(6')-Ib-c*	Singleton/E9
Ss 4	GAT-OFL	*qnrB*	Singleton/E10
Ss 6	CIP	*qnrB*	Singleton/E11
Ss 10	LEV-OFL	*aac(6')-Ib-c*	Singleton/E12
Ss 29	LEV-OFL	*qnrS*	Singleton/E13
Ss 35	LEV-OFL	*qnrS/aac(6')-Ib-c*	Singleton/E14
Ss 14	CIP-LEV	*qnrA/aac(6')-Ib-c*	Singleton/E15
Ss 45	CIP	*qnrS*	A/E16
Ss 11	CIP	-	A/E16
Ss 89	CIP	-	A/E16
Ss 74	CIP-LEV-OFL	*qnrS*	B/E17
Ss 93	GAT-OFL	*qnrA*	B/E18
Ss 80	CIP-GAT	-	Singleton/E19
Ss 75	CIP-GAT-OFL	*qnrA/qnrS*	C/E20
Ss 77	CIP-LEV	*qnrB/qnrS*	C/E21
Ss 86	CIP-LEV-OFL	*qnrS/aac(6')-Ib-c*	Singleton/E22
Ss 5	CIP-GAT-OFL	*qnrA/aac(6')-Ib-c*	Singleton/E23
Ss 2	CIP-OFL	*qnrB/aac(6')-Ib-c*	Singleton/E24

CIP: ciprofloxacin, LEV: levofloxacin, GAT: gatifloxacin, OFL: ofloxacin

Fig. 5 The UPGMA dendrogram with the Dice coefficient of quinolone-resistant *Shigella sonnei* isolates in the basis of ERIC-PCR patterns

Based on the ERIC-PCR, quinolone-resistant *S. flexneri* and *S. sonnei* isolates had a high genetic diversity. This incredibly complex pattern makes it possible that various strains of the same species circulate in our region. These results were in good parallel with the previous reports from Malawi [26], Peru [54], and Latin America [55]. To prove that the *Shigella* strains in this study were genetically unrelated, additional research utilizing more exact typing techniques, such as whole-genome sequencing (WGS) and multi-locus sequence typing (MLST), would be required [26]. This study had some limitations as the other quinolone resistance mechanisms (mutations in QRDRs) were not investigated.

Conclusion

This study demonstrated the predominance of *S. sonnei* and *S. flexneri* in our region. The *Shigella* isolates showed high resistance to ampicillin, amoxicillin/clavulanic acid, and trimethoprim/sulfamethoxazole and all isolates were MDR. It seems that *qnrS*, *qnrA*, and *aac(6')-Ib-cr* play significant role in the quinolone resistance among *Shigella* isolates in our region. There was no statistical association between biofilm formation and quinolone resistance. Also the quinolone-resistant *S. flexneri* and *S. sonnei* isolates had a high genetic diversity. Hence, antibiotic therapy needs to be routinely monitored based on the surveillance programs.

Abbreviations

ERIC-PCR	Enterobacterial repetitive intergenic consensus polymerase chain reaction
MIC	Minimal inhibitory concentration
MLST	Multi-locus sequence typing
PMQR	Plasmid-mediated quinolone resistance
QRDRs	Quinolone resistance-determining regions
WGS	Whole-genome sequencing

Acknowledgements

None.

Author contributions

NSKAK, BSAA, RMJE, and HOMAD: conceptualisation, data curation, formal analysis, investigation, methodology, project administration, writing—original draft preparation, writing— review and editing. SYA, AN, and DCN: data curation, formal analysis, writing-original draft preparation, writing—review and editing. MHGK, SSA, and MS: investigation, writing—review and editing. All authors reviewed the manuscript.

Funding

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Availability of data and materials

The datasets in the present study are accessible from the corresponding author, Saki M.

Declarations

Ethics approval and consent to participate
Ethics approval was obtained from the University of Babylon, Hilla, Iraq (Project No: M220601) compliant with the Declaration of Helsinki. All methods were performed in accordance with the relevant guidelines and regulations of the University of Babylon, Hilla, Iraq. Written consent was obtained from parents or guardians of all participants.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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Received: 11 September 2023 Accepted: 31 July 2024
Published online: 14 August 2024

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