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Research article

Antimicrobial susceptibility and virulence gene analysis of *Shigella* species causing dysentery in Iranian children: Implications for fluroquinolone resistance

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ABSTRACT

Shigella species significantly impact global health due to their role in diarrheal diseases. A 2019–2022 cross-sectional study on 432 stool samples from pediatric patients in Mashhad, Iran, identified *Shigella* spp. and tested their susceptibility to 12 antimicrobials by the disk diffusion method. The presence of virulence factors, namely *ipaH*, *virA*, *stx1*, and *stx2*, as well as plasmid-mediated quinolone resistance (PMQR) genes, including *qnrA*, *qnrB*, *qnrC*, *qnrD*, and *qnrS*, were ascertained through the utilization of polymerase chain reaction techniques. Sequencing of 15 isolates detected mutations within quinolone resistance-determining regions (QRDRs) at the *gyrA* and *parC* genes, indicating fluoroquinolone (FQ) resistance. 19.2 % (83/432) of stool samples contained *Shigella*, primarily *S. sonnei* (77.1 %), followed by *S. flexneri* (21.6 %) and *S. boydii* (1.2 %). Most isolates were from children under five (55.4 %). All strains had the *ipaH* gene, lacked *stx1* and *stx2*, and 86.7 % had *virA*. High resistance was noted for ampicillin and tetracycline (84.3 % each), trimethoprim-sulfamethoxazole (81.9 %), and azithromycin (60.2 %). 87.1 % of isolates were multidrug-resistant (MDR). The most common PMQR genes were *qnrA* and *qnrS* (41 % each). The *qnrD* gene, prevalent in 36.1 % of cases, is reported in Iran for the first time. The most common PMQR profile was *qnrADS* (15.7 %). Resistance to nalidixic acid and ciprofloxacin was 45.8 % and 12 %, respectively. The *Shigella* isolates exhibited mutations in the *gyrA* (at codons 83, 87, and 211) and *parC* (at codons 80, 84, 93, 126, 128, 129, and 132) genes. The D87Y mutation in the *gyrA* gene was the most common in *Shigella* isolates, occurring in 73 % of cases. The F93S and L132T mutations in the *parC* gene were unique to this study. Empirical FQ therapy in patients infected with MDR *Shigella*, possessing PMQR determinants and/or mutations in the QRDRs of *gyrA* and *parC*, may escalate the risks of secondary diseases, extended treatment duration, therapeutic failure, and resistance spread. Consequently, the necessity for continuous surveillance and genetic testing to detect FQ-resistant *Shigella* strains is of paramount importance.

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1. Introduction

Shigellosis significantly contributes to morbidity and mortality related to diarrheal diseases, particularly in low-income countries [1]. It is characterized by symptoms such as recurrent episodes of diarrhea and/or dysentery. The subject is manifesting symptoms such as the presence of mucus and blood in the stools, tenesmus, and abdominal discomfort [1,2]. The rapid spread of infection, combined with the low infectious dosage of less than 200 cells, contributes to the disease's high communicability [3]. Among the four species of *Shigella*, namely *Shigella dysenteriae* (*S. dysenteriae*), *Shigella boydii* (*S. boydii*), *Shigella flexneri* (*S. flexneri*), and *Shigella sonnei* (*S. sonnei*), it is *S. flexneri* and *S. sonnei* that account for over 90 % of global shigellosis incidences [4]. While *S. sonnei* is more prevalent in industrialized countries, *S. flexneri* is the most common kind of Shigellosis in developing nations, especially in Asia [5]. Globally, *Shigella* is believed to be responsible for around 80 to 165 million infections and 600,000 deaths per year, with the majority of cases and deaths occurring in children [6]. Some sources indicate a low shigellosis mortality rate in Iran across various settings and populations [7]. However, the incidence of shigellosis in Iran is still high, especially in children under 5 years of age, and varies by region, season, and serotype of *Shigella* [8–10].

The treatment modalities for shigellosis encompass rehydration and the administration of antibiotics [11]. Appropriate administration of the correct antibiotic medication can diminish the duration of symptoms and avert possibly lethal consequences [8]. The selection of appropriate antimicrobial therapy has become increasingly challenging due to the emergence of *Shigella* clinical isolates resistant to multiple medications. This resistance has been created by the overuse and misuse of antibiotics in the treatment of shigellosis over several decades [3,8–10]. During the periods of 2000–2020, researchers in Iran observed the high prevalence of antibiotic-resistant *Shigella* spp [12]. Empirical treatment no longer recommends the use of antibiotics such as ampicillin, tetracyclines, sulfonamides, and trimethoprim/sulfamethoxazole due to the increasing global resistance among *Shigella* isolates [13,14]. Currently, oral quinolones are the preferred medication for nearly all instances of confirmed or assumed shigellosis [15]. However, there have been documented cases of *Shigella* that are resistant to fluoroquinolones (FQs) in various regions globally. This issue is more prevalent, particularly in Asia [12,14,16]. Also, there has been a worldwide increase in the prevalence of FQ-resistant *Shigella* isolates, primarily caused by the excessive utilization of ciprofloxacin for the treatment of shigellosis. These strains are believed to have emerged from a shared ancestor in South Asia around 2007 and have since become more prevalent [17,18].

Research indicates that quinolone resistance is caused by changes in the activity of efflux pumps and specific genetic mutations in the quinolone resistance-determining region (QRDR) of DNA gyrase (*gyrA* and *gyrB*) and DNA topoisomerase (*parC* and *parE*) [19,20]. DNA gyrase is a specific form of DNA topoisomerase known as type II, which carries out the processes of DNA supercoiling, relaxation, decatenation, and unknotting by inducing a double-stranded break in the DNA molecule [21]. Quinolones stabilize a fragmented DNA complex by binding to these enzymes. Point mutations, such as amino acid alterations in the QRDR of target enzymes, can lead to a decrease in quinolone binding [19]. The plasmid-mediated quinolone resistance (PMQR) is conferred by the *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *aac(60)-Ib-cr*, and *qepA* genes, as reported [22,23]. Research has demonstrated that the qnr gene's 218-residue shields DNA gyrase from quinolone drug inhibition [24]. PMQR genes facilitate the selection of chromosomal changes that confer high-level resistance and reduce the susceptibility of bacteria to quinolones and FQs. Moreover, PMQR genes have the capacity to horizontally transfer to other bacteria, posing a significant risk to human health [25–27].

Shigella possesses a wide range of virulence factors that enable it to enter colonic epithelial cells, avoid phagocytic vesicles, and ultimately destroy the mucosal cells that line the intestinal tract. Notable virulence factors comprise Shiga toxins (Stx1 and Stx2), invasion plasmid antigen H (IpaH), and virulence gene A (VirA) [28]. Shiga toxins play a crucial role in the pathogenesis of hemolytic uremic syndrome, a dangerous condition that individuals infected with *Shigella* spp. can manifest [29]. *Shigella* species release IpaH, an enzyme that attaches ubiquitin molecules to proteins and modulates the host's immune response. VirA, an auxiliary virulence determinant, plays a crucial role in the intracellular proliferation and survival of *Shigella* [30]. Together, these virulence characteristics enhance *Shigella*'s ability to cause disease, making it a significant threat to human health.

The primary objective of this study is to ascertain the magnitude of resistance to FQs, a category of antibiotics frequently employed in the treatment of shigellosis, and to pinpoint the genetic determinants responsible for this resistance. The study aims to determine the pathogenicity of the circulating strains by examining the frequency of virulence genes. The findings of this study have significant significance for public health, as they will provide valuable insights for treatment approaches and contribute to the control of drug-resistant *Shigella*, ultimately leading to a decrease in the prevalence of dysentery in children. The study's results will additionally enhance the worldwide comprehension of patterns in antimicrobial resistance and the development of harmful qualities among *Shigella* species. This knowledge is crucial for formulating efficient interventions and strategies to counteract the dissemination of resistant infections.

2. Material and methods

2.1. Study design

This study utilized a descriptive-cross design to investigate *Shigella* in Mashhad, Iran, from 2019 to 2022.

2.2. Sample processing

The fecal specimens of the patients were inoculated into the Cary-Blair transport medium after 2 h of collection. Subsequently, the

specimens were sent to our laboratory within 24 h. Prior to incubation at a temperature of 37 °C for a duration of 24 h, all feces were introduced into appropriate screening media such as MacConkey (Ibresco Life Science, Iran) and selective media like xylose lysine deoxycholate (QUELAB, Canada). The colonies that showed the features of *Shigella* spp. were identified and tested for species level using standard microbiological and biochemical procedures. The *Shigella* polyvalent antisera from the Statens Serum Institut (Baharafshan, Iran) was utilized according to the manufacturer's instructions for conducting latex agglutination serotyping. *Shigella* spp. isolates were preserved at a temperature of −70 °C in order to conduct antibiotic susceptibility testing and molecular analysis.

2.3. Antimicrobial susceptibility testing

Following the guidelines laid out by the Clinical and Laboratory Standards Institute in 2022 [31] and the orders of Iranian doctors, the Kirby-Bauer disk diffusion method was used to ascertain the antimicrobial susceptibility pattern of every single *Shigella* isolate. The antimicrobial drugs used in the study were ampicillin (AMP), ciprofloxacin (CIP), cefotaxime (CTX), azithromycin (AZT), trimethoprim/sulfamethoxazole (SXT), levofloxacin (LEV), ceftriaxone (CRO), meropenem (MEM), nalidixic acid (NA), norfloxacin (NOR), ofloxacin (OFL), and tetracycline (TET). The control strain utilized in the experiment was *Escherichia coli* ATCC 25922. In this study, the term multidrug-resistant (MDR) isolate refers to the *Shigella* species that is resistant to at least one antibiotic from three different classes, while extensively drug-resistant (XDR) isolates are those that are resistant to all but two or fewer categories of antibiotics, meaning they remain susceptible to only one or two categories.

2.4. Polymerase chain reaction (PCR) amplification and sequencing

PCR technique was employed to identify the presence of specific virulence genes, including *ipaH*, *virA*, *stx1*, and *stx2*, as well as PMQR genes *qnrA*, *qnrB*, *qnrC*, *qnrD*, and *qnrS*. The genes *gyrA* and *parC* were amplified and sequenced to discover mutations. The amplification process was carried out using a thermocycler (Eppendorf, Germany). A total of 15 samples were dispatched to Pishgam Biotech Company, located in Tehran, Iran, for the purpose of *gyrA* and *parC* sequencing. Sequence alignments and analyses were conducted utilizing Vector NTI Suite 9 (InforMax Inc., Bethesda, MD, USA), with the derived nucleotide sequences being cross-referenced against publicly accessible databases. Table 1 displays primer sequences, the PCR assay's thermal cycling parameters, and amplified fragment lengths.

2.5. Statistical analysis

The data were analyzed using the SPSS software (Version 26; Chicago, IL). In addition, GraphPad Prism 8 was used to generate a bar chart. In order to determine the statistical significance of the relationship, we conducted chi-square tests or Fisher's exact tests, depending on the anticipated frequencies in the cells. Statistical significance was established by employing two-sided p-values below 0.05 for all comparisons. However, if the p values were between 0.05 and 0.1, the term 'marginally significant' was used. A marginally significant result refers to a situation where the p-value is slightly higher than the traditional threshold of 0.05. This suggests that there is some evidence supporting the alternative hypothesis, but it is not strong enough to reject the null hypothesis. The strength of the link

Table 1
Primer oligonucleotides used in this study.

Target gene	Primer sequences (5′–3′)	Thermal parameters		Amplicon size (bp)	Ref.
gyrA	F:TACACCGGTCAACATTGAGG R:TTAATGATTGCCGCCGTCGG	30 cycles	Denaturation 94 °C/45s Annealing 64 °C/30s Extension 72 °C/1 m	648	[32]
parC	F:GTCTGAACTGGGCCTGAATGC R:AGCAGCTCGGAATATTTC	30 cycles	Denaturation 94 °C/1 m Annealing 68 °C/30s Extension 72 °C/1 m	249	[32]
qnrA	F:CAGCAAGAGGATTTCTCACG R:AATCCGGCAGCACTATTACTC	30 cycles	Denaturation 94 °C/1 m Annealing 63 °C/1 m Extension 72 °C/1.5 m	630	[33]
qnrB	F:GGCTGTCAGTTCTATGATCG R:GAGCAACGATGCCTGGTAG DR: SAKCAACGATGCCTGGTAG			488	[33]
qnrC	F:GCAGAATTCAGGGGTGTGAT R:AACTGCTCCAAAAGCTGCTC			118	[33]
qnrD	F:CGAGATCAATTTACGGGGAATA R:AACAAGCTGAAGCGCCTG			581	[33]
qnrS	F:GCAAGTTCATTGAACAGGGT R:GTCAGGAWAAACAACAATACC			518	[34]
virA	F:CTGCATTCTGGCAATCTCTTCACATC R: TGATGAGCTAACTTCGTAAGCCCTCC	30 cycles	Denaturation 94 °C/30s Annealing 58 °C/30s	215	[35]
ipaH	F: TGGAAAAACTCAGTGCCTCT R: CCAGTCCGTAAATTCATTCT		Extension 72 °C/1 m	423	[36]
Stx1	F:CATCGCGAGTTGCCAGAAT R:GCGTAATCCCACGGACTCTTC	35 cycles	Denaturation 94 °C/1 m Annealing 63 °C/1 m Extension 72 °C/1 m	78	[37]
Stx2	F:CCGGAATGCAAATCAGTC R:CAGTGACAAAACGCAGAACT	40 cycles	Denaturation 94 °C/45s Annealing 60 °C/45s Extension 72 °C/1 m	113	[37]

was evaluated using the Cramér's V coefficient and 95 % confidence intervals (CI). To assess the practical importance and precision of the results, it is necessary to interpret the data analysis using effect size and confidence interval (CI) in null hypothesis significance testing (NHST), instead of relying exclusively on statistical significance. Statistical significance quantifies the probability of observing the results due to random chance, whereas practical significance assesses the magnitude and meaningfulness of the influence in the actual world.

3. Result

3.1. Shigella isolation and serotyping in the study population

Out of 5312 stool samples, 432 (0.08 %) were selected for investigation due to >10 white blood cells per microscope area. Of these, 83 (19.2 %) tested positive for *Shigella* spp., with 77.1 % (n = 64) as *S. sonnei*, 21.6 % (n = 18) as *S. flexneri*, and 1.2 % (n = 1) as *S. boydii*. No *S. dysenteriae* strain was detected. Table 2 presents the *Shigella* isolate distribution by gender, admission type, and age groups.

The study revealed a gender-based distribution of *Shigella* species, with a higher prevalence in males. The overall prevalence was 60.2 % for males and 39.8 % for females. *S. sonnei* was the most prevalent, found in 72 % of males and 84.8 % of females. *S. flexneri* was present in 26 % of males and 15.1 % of females, while *S. boydii* was found in one male. Statistical analysis, based on a sample size of N = 83, showed a weak correlation between gender and *Shigella* prevalence, indicated by a Cramér's V value of 0.16, a 95 % CI of [0.02, 0.35], and a p-value of 0.33.

Our examination of *Shigella* hospital admissions revealed that inpatients were predominantly *S. sonnei* (71.5 %) and *S. flexneri* (28.5 %), with no *S. boydii* cases. Outpatient cases were led by *S. sonnei* (85.3 %). The distribution was 59 % inpatients and 41 % outpatients. A modest to moderate correlation with marginal significance (Cramér's V = 0.23, 95 % CI [0.04, 0.40], p = 0.05) was observed between *Shigella* species and admission type.

Our study, with an average mean age of 5.17 years (SD = ± 3.05), revealed that *Shigella* infection was most prevalent in preschoolers (39.8 %, n = 33) and least prevalent in toddlers (10.8 %, n = 9). Preschoolers had the highest incidence of *S. sonnei* 75.8 % and *S. flexneri* 21.2 %. S. boydii cases were found in one preschooler. A weak correlation (Cramér's V = 0.12, 95 % CI [0.08, 0.33], p = 0.87) was observed between *Shigella* species and age groups.

The patients had a range of symptoms, including dysentery (19.2 %, n = 16), watery diarrhea (90.3 %, n = 75), stomach discomfort (72.2 %, n = 60), vomiting (25.3 %, n = 21), and fever (60.2 %, n = 50).

3.2. Antimicrobial resistance profiles of Shigella spp.

Table 3 presents the antibiotic resistance characteristics of 83 *Shigella* isolates obtained from pediatric patients. With the exception of one sample, every isolate showed phenotypic resistance to at least one tested antibiotic. In non-FQ classes, AMP and TET had the highest resistance (84.3 %, 70/83), followed by SXT (81.9 %, 68/83) and AZT (60.2 %, 50/83). Among FQ, NA (45.8 %, 38/83), CIP (12 %, 10/83), and OFL (6 %, 5/83) exhibited the most resistance. *S. flexneri* (n = 18) had the highest rates of resistance, with 88.8 % (n = 16) being resistant to TET. In *S. sonnei* (n = 64), the highest resistance rates were observed in 87.5 % (n = 56) of cases for SXT. A single *S. boydii* isolate was resistant to both antibiotics.

The study reveals that 87.5 % of *S. sonnei* isolates are SXT-resistant, compared to 61.1 % of *S. flexneri*, indicating species variation in resistance with a modest degree of association (Cramér's V = 0.28, 95 % CI [0.06, 0.51], p = 0.01). AZT data analysis shows higher resistance in *S. sonnei* (68.7 %) than *S. flexneri* (27.7 %), with a significant moderate-to-strong connection (Cramér's V = 0.34, 95 % CI [0.13, 0.52], p = 0.02). CTX resistance is 23.4 % in *S. sonnei* and 44.4 % in *S. flexneri*, with a marginally significant difference and weak association (Cramér's V = 0.19, 95 % CI [0.01, 0.42], p = 0.08). CRO resistance is low in *S. sonnei* (3.1 %) but high in *S. flexneri* (27.7 %), with a significant disparity and moderate-to-strong connection (Cramér's V = 0.36, 95 % CI [0.07, 0.61], p = 0.001).

The study demonstrates that 89.1 % of isolates show MDR, emphasizing the challenge of treating related illnesses. *S. sonnei* has a higher MDR prevalence (93.8 %) than *S. flexneri* (72.2 %), with a significant difference (p = 0.02). However, the Cramér's V value of

Table 2
Distribution of *Shigella* isolates based on gender, hospital admission, and age categories during 2019–2022 in Mashhad. Iran.

Shigella species	Gender		Hospital admission		Age groups*			
	Male No. (%)	Female No. (%)	Inpatient No. (%)	Outpatient No. (%)	Infant No. (%)	Toddler No. (%)	Preschool No. (%)	School age child No. (%)
S. sonnei	36 (72.0)	28 (84.8)	35 (71.5)	29 (85.3)	12 (85.7)	6 (66.7)	25 (75.8)	21 (77.8)
S. flexneri	13 (26.0)	5 (15.1)	14 (28.5)	4 (11.7)	2 (14.3)	3 (33.3)	7 (21.2)	6 (22.2)
S. boydii	1 (2.0)	0 (00.0)	0 (00.0)	1 (3.0)	0 (00.0)	0 (00.0)	1 (3)	0 (00.0)
Total	50 (60.2)	33 (39.8)	49 (59)	34 (41)	14 (16.9)	9 (10.8)	33 (39.8)	27 (32.5)
Data analysis	(V = 0.16, 95 % CI [0.02, 0.35], p = 0.33, N = 83)		(V = 0.23, 95 % CI [0.04, 0.40], p = 0.05, N = 83)		(V = 0.12, 95 % CI [0.08, 0.33], p = 0.87, N = 83)			

Abbreviations: V, Cramer's V effect size; CI, confidence interval. p, p-value; * Infant, age ≤1 year; Toddler, 1 < age ≥3. Preschool, 3 < age ≥6; School age child, 6 < age ≥12.

Table 3
Antimicrobial resistance distribution of *Shigella* isolates recovered from pediatric patients in Mashhad, Iran, 2019–2022.

Antibiotics	*S. sonnei* No. (%), *n* = 64	*S. flexneri* No. (%), *n* = 18	*p*-value	Cramér's V (95 % CI)	*S. boydii* No. (%), n = 1	Total of *Shigella* Strains No. (%), *n* = 83
Non-fluroquinolones						
AMP	55 (85.9)	14 (77.7)	0.4	–	1 (100.0)	70 (84.3)
TET	53 (82.8)	16 (88.8)	0.53	–	1 (100.0)	70 (84.3)
SXT	56 (87.5)	11 (61.1)	0.01	0.28 (0.06, 0.51)	1 (100.0)	68 (81.9)
AZT	44 (68.7)	5 (27.7)	0.02	0.34 (0.13, 0.52)	1 (100.0)	50 (60.2)
CTX	15 (23.4)	8 (44.4)	0.08	0.19 (0.01, 0.42)	0 (00.0)	23 (27.7)
CRO	2 (3.1)	5 (27.7)	0.001	0.36 (0.07, 0.61)	0 (00.0)	7 (8.4)
MEM	0 (00.0)	0 (00.0)	–	–	0 (00.0)	0 (00.0)
Fluroquinolones						
NA	32 (50.0)	6 (33.3)	0.21	–	0 (00.0)	38 (45.8)
CIP	8 (14.0)	2 (11.1)	0.33	–	0 (00.0)	10 (12.0)
OFL	4 (6.2)	1 (2.2)	0.91		0 (00.0)	5 (6.0)
NOR	2 (3.1)	1 (5.5)	0.62		0 (00.0)	3 (3.6)
LEV	1 (1.5)	1 (5.5)	–		0 (00.0)	1 (2.4)
FQ resistance	32 (50.0)	6 (33.3)	0.21	0.12 (0.006, 0.34)	0 (00.0)	38 (45.8)
MDR	60 (93.8)	13 (72.2)	0.02	0.28 (0.03, 0.54)	1 (100.0)	74 (89.1)

Abbreviations: AMP, ampicillin; TET, tetracycline; SXT, trimethoprim-sulfamethoxazole; AZT, azithromycin; CTX, cefotaxime; CRO, ceftriaxone MEM, Meropenem; NA, nalidixic acid; CIP, ciprofloxacin; OFL, ofloxacin; NOR, norfloxacin; LEV, levofloxacin; FQ, Fluroquinolones; MDR, Multidrug resistance.

0.28 with a 95 % CI [0.006, 0.34] suggests a moderate association between species type and MDR, despite the statistical significance.

Resistance to FQ is observed in 45.8 % of the cases, a significant statistic given the importance of these antibiotics in treatment protocols. Resistance is found in 50 % of *S. sonnei* and 33.3 % of *S. flexneri*. A p-value of 0.21 means that the difference in resistance between the two types of *Shigella* might not be a consistent difference, but rather something random. This is supported by a Cramér's V value of 0.12 and a 95 % confidence interval of [0.006, 0.34].

Table 4 illustrates the MDR antibiotic resistance pattern in *Shigella* isolates between two age categories. The data reveals a high incidence of MDR, with the predominant patterns being AMP-TET-SXT-AZT, AMP-TET-SXT, AMP-TET-SXT-AZT-NA, and AMP-TET-SXT-AZT-NA-CTX, affecting 23 %, 10.8 %, 8.1 %, and 6.8 % of strains, respectively. One isolate exhibited XDR, indicating resistance to AMP-TET-SXT-AZT-NA-CIP-CTX-CRO. The most frequent core pattern in MDR isolates was AMP-TET-SXT, observed in 62.6 % of cases. The highest frequency of resistance to a specific number of antibiotics among the MDR strains was four antibiotics, accounting for 38 % (28/74) of cases. In the study of MDR *Shigella* isolates, an equal distribution of MDR strains was observed across two age groups: pre-school (under 5 years) and school age (over 5 years), with slight variations. The pre-school group exhibited a higher prevalence (23 %, 17/74) of bacteria resistant to 5 and more antibiotics compared to the school age group (18 %, 13/74).

Fig. 1 illustrates the antibiotic resistance profiles against non-FQ for both FQ-resistant and FQ-sensitive *Shigella* strains. There were slight differences in resistance to TET, AMP, and SXT among the groups. The FQ-resistant group exhibited enhanced resistance to AZT and CTX, while the FQ-sensitive group displayed increased resistance to CRO. A marginally significant variation in CTX resistance was observed ($p = 0.08$), indicating a weak association (Cramér's $V = 0.18$, 95 % CI [0.01, 0.39]).

3.3. Characterization of qnr and virulence genes

PCR identified the *ipaH* gene, the primary virulence factor, in all *Shigella* strains, while the *stx1* and *stx2* genes were absent. The *virA* gene was found in 86.7 % of the isolates, with no significant correlation to FQ resistance ($p > 0.05$).

Table 5 depicts the FQ resistance traits and amino acid changes in fifteen *Shigella* isolates. Nalidixic acid resistance was found in 45.8 % (n = 38) of isolates, while ciprofloxacin resistance was noted in 12 % (n = 10). Among the 83 *Shigella* isolates examined, 38 (45.8 %) possessed PMQR determinants, all of which exhibited resistance to nalidixic acid (NA), and 10 isolates (26.3 %) demonstrated resistance to ciprofloxacin (CIP). Specifically, 34 (41 %) carried *qnrA*, 34 (41 %) carried *qnrS*, 30 (36.1 %) carried *qnrD*, and 15 (18.1 %) contained *qnrB* determinants. The *qnrC* gene was not detected in any of the isolates. The *qnrABDS* profile was detected in 13.3 % (n = 11) of the isolates, while the *qnrADS* was found in 15.7 % (n = 13). From the 38 NA-resistant isolates, 15 were chosen for sequencing to detect *gyrA* and *parC* amino acid substitutions based on their antibiotic resistance profiles. Primarily, the *qnrABDS* profile and the *gyrA* gene displayed a mutation at position 87 (73 %, 11/15), substituting aspartic acid with tyrosine, which is chiefly linked to NA and CIP resistance. Resistance to NOR, LEV, and OFL appears to correlate with *gyrA* mutations, specifically the substitution of serine to leucine at position 83, aspartic acid to asparagine or glycine at position 87, and serine to isoleucine at position 80 in the *parC* gene. Uncommon mutations, excluding the frequently reported, Y128F, F93S, S126T, S129T, and L132T in the *parC* gene and H211Y in the *gyrA* gene, were identified.

4. Discussion

Shigella-induced diarrhea, particularly in children under 5, is a significant health issue in developing countries and was reported as

Table 4
Multi-drug resistance (MDR) patterns in *Shigella* isolates from pediatric patients in Mashhad, Iran, between 2019 and 2022, based on two age categories.

Antibiotic resistance profile	Age						No. of strains (%) N = 74
	Year ≤5 (55.4 %, N = 41)			Year >5 (44.6 %, N = 33)			
	S. sonnei No. (%)	*S. flexneri* No. (%)	*S. boydii* No. (%)	*S. sonnei* No. (%)	*S. flexneri* No. (%)	*S. boydii* No. (%)	
AMP-TET-SXT-AZT-NA-CIP-OFL-NOR	2 (4.8)	–	–	–	–	–	2 (2.7)
AMP-TET-SXT-AZT-NA-CIP-CTX-CRO	–	–	–	–	1 (3.1)	–	1 (1.3)
AMP-TET-SXT-AZT-NA-CIP-CTX	2 (4.8)	–	–	–	–	–	2 (2.7)
AMP-TET-SXT-AZT-NA-CTX	4 (9.5)	1 (2.3)	–	–	–	–	5 (6.8)
AMP-TET-SXT-AZT-NA-CIP	–	–	–	2 (6.3)	–	–	2 (2.7)
AMP-TET-SXT-AZT-CTX-CRO	–	–	–	–	1 (3.1)	–	1 (1.3)
AMP-TET-SXT-NA-CTX-CRO	–	–	–	1 (3.1)	–	–	1 (1.3)
AMP-TET-SXT-AZT-NA	2 (4.8)	–	–	4 (12.5)	–	–	6 (8.1)
AMP-TET-SXT-CTX-CRO	1 (2.3)	3 (7.1)	–	–	–	–	4 (5.4)
AMP-TET-SXT-AZT-CTX	–	–	–	1 (3.1)	–	–	1 (1.3)
AMP-TET-SXT-NA-CTX	–	–	–	1 (3.1)	–	–	1 (1.3)
AMP-TET-NA-CTX-OFL	1 (2.3)	–	–	–	–	–	1 (1.3)
AMP-TET-NA-CIP-OFL	–	–	–	–	1 (3.1)	–	1 (1.3)
AMP-SXT-AZT-NA-CTX	–	–	–	1 (3.1)	–	–	1 (1.3)
CTX-TET-NA-NOR-LEV	–	1 (2.3)	–	–	–	–	1 (1.3)
AMP-TET-SXT-AZT	7 (16.6)	–	1 (2.3)	8 (25)	1 (3.1)	–	17 (23.0)
AMP-TET-AZT-NA	1 (2.3)	1 (2.3)	–	–	–	–	2 (2.7)
AMP-SXT-AZT-NA	2 (4.7)	–	–	–	–	–	2 (2.7)
AMP-TET-SXT-NA	1 (2.3)	–	–	–	–	–	1 (1.3)
AMP-TET-SXT-CTX	1 (2.3)	–	–	–	–	–	1 (1.3)
AMP-TET-NA-OFL	–	–	–	1 (3.1)	–	–	1 (1.3)
AMP-SXT-AZT-CTX	1 (2.3)	–	–	–	–	–	1 (1.3)
SXT-AZT-NA-CTX	–	–	–	1 (3.1)	–	–	1 (1.3)
TET-SXT-AZT-CIP	1 (2.3)	–	–	–	–	–	1 (1.3)
TET-SXT-NA-CIP	–	–	–	1 (3.1)	–	–	1 (1.3)
AMP-TET-SXT	1 (2.3)	1 (2.3)	–	4 (12.5)	2 (6.2)	–	8 (10.8)
TET-SXT-NA	3 (7.1)	–	–	–	–	–	3 (4.0)
AMP-SXT-AZT	1 (2.3)	–	–	1 (3.1)	–	–	2 (2.7)
AMP-TET-AZT	1 (2.3)	–	–	–	–	–	1 (1.3)
AMP-AZT-NA	1 (2.3)	–	–	1 (3.1)	–	–	2 (2.7)
Total	33 (79.0)	7 (16.6)	1 (2.3)	27 (84.3)	6 (18.7)	0 (00.0)	74 (100.0)

Abbreviations: AMP, ampicillin; TET, tetracycline; SXT, trimethoprim-sulfamethoxazole; AZT, azithromycin; CTX, cefotaxime; CRO, ceftriaxone MEM, Meropenem; NA, nalidixic acid; CIP, ciprofloxacin; OFL, ofloxacin; NOR, norfloxacin; LEV, levofloxacin.

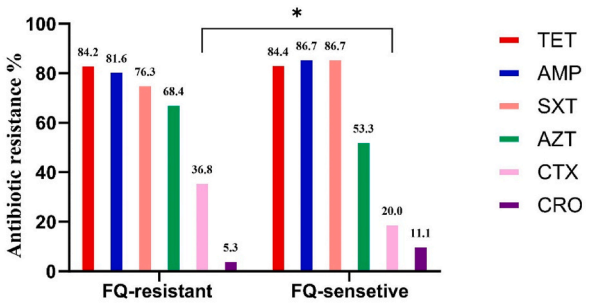

Fig. 1. Comparison between fluroquinolone-resistance and -sensitive *Shigella* strains isolated from pediatric patients in Mashhad, Iran, 2019–2022. The percentages on the bars represent the level of resistance. Abbreviations: TET, tetracycline; AMP, ampicillin; SXT, trimethoprim-sulfamethoxazole; AZT, azithromycin; CTX, cefotaxime; CRO, ceftriaxone. *, $p \leq 0.05$.

the second leading cause of diarrhea-related deaths in 2016 [38]. FQs are the primary treatment for multi-drug resistant *Shigella* infections. Prior studies largely attribute high FQ resistance to QRDR changes and PMQR determinant mechanisms [39–42]. Given the scarce data on the antibiotic-resistant and virulence profiles of *Shigella* in regions like Mashhad, Iran, this study aimed to investigate the

Table 5
FQ resistance characteristics and amino acid substitutions in fifteen *Shigella* isolates recovered from pediatrics patients in Mashhad, Iran, 2019–2022.

Isolate	Species	PMQR	Amino acid substitutions in QRDRs						Resistance profile
			GyrA			ParC			
Wild-type	*E. coli* K12	–	S83	D87	EM	S80	E84	UM	–
SHI-18	*S. sonnei*	A, B, S	–	Y		–	–		AMP-SXT-AZT-**NA**
SHI-5	*S. sonnei*	A, B, D, S	–	Y		–	–	Y128F	AMP-SXT-AZT-**NA**
SHI-3	*S. sonnei*	A, B, D, S	L	–		–	–	Y128F	SXT-AZT-CTX-**NA**
SHI-20	*S. sonnei*	A, B, D, S	–	Y		–	–		AMP-AZT-**CIP-NA**
SHI-13	*S. sonnei*	A, B, D, S	–	Y		–	–		AMP-AZT-**CIP-NA**
SHI-6	*S. sonnei*	A, B, D, S	–	Y		–	–		AMP-TET-SXT-AZT-**NA**
SHI-4	*S. sonnei*	A, B, D, S	–	Y		–	–		AMP-SXT-AZT-CTX-**NA**
SHI-14	*S. sonnei*	B, D, S	–	Y		I	–	F93S	AMP-TET-SXT-AZT-**NA**
SHI-21	*S. sonnei*	A, D, S	–	Y		–	–		AMP-TET-SXT-AZT-**NA**
SHI-16	*S. flexneri*	A, B, D, S	L	N		I	–		CTX-TET-**NOR-LEV-NA**
SHI-29	*S. flexneri*	D, S	L	N	H211Y	I	–		AMP-TET-**CIP-OFL-NA**
SHI-35	*S. sonnei*	A, D, S	–	Y		–	–		AMP-TET-SXT-AZT-CTX-**NA**
SHI-10	*S. flexneri*	A, B, D, S	–	Y		–	–	S126T, S129T	AMP-TET-SXT-AZT-CTX-**NA**
SHI-57	*S. sonnei*	A, D, S	L	G		–	–		AMP-TET-SXT-AZT-CTX-CRO-**CIP-NA**
SHI-49	*S. sonnei*	A, B, D	–	Y		–	–	S126T, S129T, L132T	AMP-TET-SXT-AZT-**CIP-OFL-NOR-NA**

Abbreviations: PMQR, plasmid-mediated quinolone resistance; QRDRs, quinolone resistance-determining regions; S, serine; D, aspartic acid; E, glutamic acid; L, leucine; Y, tyrosine; N, asparagine; G, glycine; H, histidine; I, isoleucine; F, phenylalanine; T, threonine; AMP, ampicillin; TET, tetracycline; SXT, trimethoprim-sulfamethoxazole; AZT, azithromycin; CTX, cefotaxime; CRO, ceftriaxone; NA, nalidixic acid; CIP, ciprofloxacin; OFL, ofloxacin; NOR, norfloxacin; LEV, levofloxacin; EM, uncommon mutation.

antimicrobial susceptibility, FQ resistance mechanisms, and virulence genes of pediatric *Shigella* isolates.

While *S. flexneri* and *S. sonnei* are the predominant species in developing and industrialized countries, respectively [43]. A notable shift has been observed in Asian countries like China [42], Vietnam [44], Thailand [45], and Iran [39], where *S. sonnei* is now more prevalent than *S. flexneri*. Our findings on the prevalence of *Shigella* species (*S. sonnei* 77.1 % and *S. flexneri* 21.6 %) align with studies conducted in other regions of Iran [40,46,47], but diverge from the recent studies by Shoja [48] and Shiekh et al. [49]. These disparities may be attributed to factors such as geographical location, socio-economic conditions, and personal hygiene practices [49,50].

The existing WHO guidelines, which endorse the use of FQs as a first-line treatment and β-lactams and cephalosporins as second-line treatments, align with the current evidence and other international guidelines. There is no compelling evidence to suggest a need for altering these recommendations [51]. In Mashhad, Iran, the sole study was undertaken by Salimiyan-Rizi et al. [52]. When contrasted with this research, our study exhibited a higher resistance to all the examined antibiotics, with the exception of CRO. This comparison underscores the escalating antibiotic resistance observed in our study, highlighting the urgent need for continuous surveillance and effective antimicrobial stewardship.

In our study, concerning resistance to non-FQ antibiotics, both *S. sonnei* and *S. flexneri* exhibited comparable resistance rates against AMP, TET, and SXT, with over 60 % of all resistant isolates showing resistance (Fig. 1). Consequently, these antibiotics have lost their status as the primary choice for managing dysenteric syndrome and treating shigellosis, a significant shift from their recent past. This trend is consistent across most regions of Iran [39,48,49] and in other countries [42,53–55]. Our study observed high resistance to AZT in *S. sonnei* (68.7 %) and lower in *S. flexneri* (27.7 %), aligning with Karimi-Yazdi et al.'s findings in Iran [39] but differing from other reports [56,57]. Despite the WHO's recommendation of AZT as a secondary treatment for severe shigellosis [58], it remains the primary treatment for pediatric shigellosis in Iran [56]. However, this study found that approximately 60 % of *Shigella* species, notably *S. sonnei*, exhibited resistance to AZT, indicating caution in its usage.

Resistance to third-generation cephalosporins, such as cefotaxime and ceftriaxone, used as second-line treatments, was observed in 27.7 % and 8.4 % of cases, respectively. The comparatively lower resistance level compared to other studies may be attributed to the lesser usage of these antibiotics for treating diarrheal patients. In Mashhad, treatment predominantly involves the use of FQs and azithromycin [48,56].

The observed resistance to nalidixic acid in our study was medium-level resistance at 45.8 %, compared to recent studies [40,48]. This suggests a need for cautious administration of nalidixic acid. It's noteworthy that an increasing trend in resistance to nalidixic acid has been reported in Iran, a finding that aligns with our results [39,46]. Ciprofloxacin, a second-generation FQ currently recommended by the WHO as the primary treatment for dysentery patients resistant to third-generation cephalosporins and nalidixic acid, was found to be highly effective against 88 % of *Shigella* spp. in our study. Nevertheless, the resistance rate observed was relatively high compared to previous studies [39,40]. This could be due to the increased use of CIP in response to the high resistance of *Shigella* to third-generation cephalosporins [48]. Given that CIP is not advised for children under 18 due to its toxicity, its growing use in this demographic could pose future concerns.

Earlier research has shown that the elevated incidence of FQ resistance is predominantly attributed to changes in the QRDR and the mechanisms determined by PMQR [19,23]. Existing research concurs that the presence of two or more mutations in both the GyrA and ParC subunits, particularly at the highly conserved amino acids (Ser-83 and Asp-87) of *gyrA*, alters the structure of DNA gyrase and DNA topoisomerase IV. These changes are crucial for achieving moderate-to-high-level resistance to FQs [19,59]. The results of our study indicate that *Shigella* isolates harbored mutations in the *gyrA* (83, 87, 211 codon) and *parC* (80, 84, 93, 126, 128, 129, 132 codon)

genes. In alignment with the prevailing literature, our findings propose that all the observed modifications, excluding those at positions 93 and 132 in the *parC* gene, are linked to point mutations. These mutations result in the replacement of several amino acids in the GyrA and ParC subunits, thereby reducing the drug's affinity for its target and inducing high-level FQ resistance [59]. In the scope of our research, the mutation most commonly observed in *Shigella* isolates was D87Y in the *gyrA* gene (73 %). This mutation has been reported in some previous studies with varying prevalence rates [47,60,61]. It is plausible that this accelerated mutation could act as a catalyst for the development of resistance to various classes of FQs (Table 5). Historically, such singular mutations have been demonstrated to contribute incrementally to the emergence of completely resistant mutants, potentially signaling the risk of FQ resistance [62]. As per our understanding, the mutations F93S and L132T in the *parC* gene are distinctive features observed exclusively in this study.

Studying PMQR in *Shigella* is essential for elucidating resistance mechanisms, guiding treatment protocols, and shaping public health strategies. It plays a role in moderating FQ resistance and affects the emergence of strains with enhanced resistance potential. In this investigation, 45.8 % of isolates harbored PMQR determinants, a prevalence higher than reported by Jomehzadeh et al. (35 %) [63] and Yaghoubi et al. (26 %) [47], yet lower than Abbasi et al.'s findings (60 %) [64] within Iran. The study identified all PMQR genes—*qnrA*, *qnrB*, *qnrD*, and *qnrS*—except for *qnrC*. Notably, *qnrA* and *qnrS* were the primary PMQR genes in *S. sonnei* (68.4 %) and *S. flexneri* (13.1 %), respectively, while *qnrB* was less prevalent in *S. sonnei* (31.5 %) and *S. flexneri* (7 %), contrasting with some research from Iran [63,64]. To our knowledge, this study marks the first reported discovery of the *qnrD* gene in Iranian *Shigella* isolates. The *qnrD* gene aids *Shigella*'s quinolone resistance, similar to other PMQR genes. Its unique role is less known, with *qnr* genes generally shielding key bacterial enzymes from quinolones. Varied effects across populations call for more focused research on *qnrD*, especially in Iranian isolates.

Regrettably, the current study revealed a high prevalence of multi-drug resistance (MDR) profiles, with 89.1 % (74 isolates) exhibiting such resistance. Regarding Iran, six separate studies have collectively identified 308 clinical isolates as MDR species [12]. This can be attributed to the misuse and irrational use of antibiotics, which can foster antibiotic resistance. To mitigate this, it is recommended to refrain from using antibiotics for *Shigella* infections, except in severe cases or those at risk of systemic infection. Our findings also indicated a higher incidence of MDR in *S. sonnei* compared to *S. flexneri*, corroborating the findings of Karimi-Yazdi et al. [39]. Alarmingly, high antibiotic resistance was also observed in isolates from water sources and food samples, with Shahin et al. reporting that all water-derived *Shigella* isolates and 89.5 % of food-derived isolates were MDR [65,66]. Given the varying levels of antimicrobial resistance among *Shigella* spp., it is advised that healthcare providers conduct susceptibility testing to identify the most effective antibiotics, rather than resorting to empirical treatment.

Exploring *Shigella* virulence factors along with antibiotic resistance profiles is crucial for enhancing treatment approaches and public health measures to manage Shigellosis. Species within the genus *Shigella* are capable of expressing a multitude of virulence factors, which play a significant role in the pathogenesis of Shigellosis. *Shigella* species possess a virulence plasmid and a chromosomal DNA, with the T3SS and *ipa/ipg* genes critical for epithelial invasion and Shigellosis pathogenesis [67]. These genes are located in the virulence plasmid's ipa-mxi-spa region. The *ipaH* gene, used for *Shigella* PCR identification, is present in multiple copies on the chromosome and plasmid. Our findings align with existing literature, confirming the presence of *ipaH* in all isolates [39,46,68]. In *Shigella* species, the *virA* gene is situated on the virulence plasmid, playing a pivotal role in bacterial intracellular dissemination and invasion, as well as the establishment of the entry region. Consistent with numerous prior studies [69–71] indicating the ubiquity of *virA* in *Shigella* isolates, our analysis also revealed a substantial prevalence of the *virA* gene (86.7 %).

5. Conclusion

This study on *Shigella* species causing dysentery in Iranian children has revealed a substantial level of antimicrobial resistance, particularly to non-FQ antibiotics and, to a lesser extent, to FQs, along with a notable presence of virulence genes. Empirical FQ therapy in MDR *Shigella*-infected patients with PMQR determinants and/or mutations in the QRDRs (*gyrA* and *parC*) may elevate the risks of secondary diseases, prolonged treatment, therapeutic failure, propagation of resistance, and increased fecal organism shedding. To mitigate this, there is a pressing need for ongoing monitoring, prudent use of antibiotics, and the formulation of potent treatment approaches to tackle *Shigella* infections resistant to drugs and reduce the incidence of dysentery among children. Hence, continuous surveillance and genetic testing are crucial for detecting FQ-resistant *Shigella* strains. The study offers critical insights beneficial for public health strategies and enhances our comprehension of antimicrobial resistance patterns and virulence evolution in *Shigella* species, which is vital for the worldwide management of infectious diseases.

Limitations

The research conducted provides a region-specific analysis of *Shigella* infections among pediatric patients in Mashhad, Iran. The dataset, comprising 432 stool samples collected from 2019 to 2022, offers valuable insights. However, it is important to note that this study's scope, while significant, may not encapsulate the global diversity of *Shigella* strains or the prevailing resistance patterns. Moreover, the investigation of 12 antimicrobials and selected virulence genes might not cover the comprehensive range of antibiotics and virulence factors pertinent to *Shigella* pathogenicity. Notably, the study did not determine the minimum inhibitory concentration (MIC) for ciprofloxacin for the isolated *Shigella* species. Therefore, these findings should be interpreted considering these limitations, providing a contextual understanding of the results. This approach ensures a balanced view of the study's contributions and its potential implications in the broader field of research.

Data availability statement

The data that support the findings of this study are available on request from the corresponding author.

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CRediT authorship contribution statement

Nafise Sadat Alavi Gonabadi: Writing – original draft, Software, Investigation. **Shaho Menbari:** Writing – review & editing, Software, Investigation, Formal analysis. **Hadi Farsiani:** Validation, Software, Methodology, Formal analysis, Data curation. **Hosein Sedaghat:** Methodology, Formal analysis, Data curation. **Mitra Motallebi:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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