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Molecular characterisation of quinolone-resistant *Shigella* strains isolated in Tehran, Iran

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ABSTRACT

Over the past few years, the number of *Shigella* strains resistant to nalidixic acid has increased and has made the selection of effective antimicrobial therapy more difficult. The purpose of this study was to investigate the molecular mechanism of quinolone resistance in *Shigella* strains. *Shigella* strains isolated from 1100 diarrhoeal patients in Tehran, Iran, were assessed for their susceptibility to nalidixic acid prior to PCR-RFLP and sequence analysis of their quinolone resistance genes. Among 73 *Shigella* strains isolated, 23 (31.5%) were resistant to nalidixic acid. The most common *Shigella* spp. was *Shigella sonnei* (54; 74.0%). Of the 23 quinolone-resistant isolates, 4 (17.4%) (including 2 *Shigella flexneri*, 1 *S. sonnei* and 1 *Shigella boydii*) contained the *qnrS* gene. However, none of the isolates harboured *qnrA* or *qnrB* genes. PCR-RFLP analysis of *gyrA* showed a mutation profile in two nalidixic acid-resistant strains, including one *S. sonnei* and one *S. flexneri*. Sequencing of mutant *gyrA* genes revealed a point mutation at position 83, resulting in the replacement of serine by leucine. In conclusion, molecular mechanisms of resistance to quinolones were identified in 6 of 23 *Shigella* isolates. Other possible mechanisms of resistance should also be investigated for better characterisation of quinolone-resistant *Shigella* isolates.

1. Introduction

Shigellosis is a major cause of diarrhoea-related morbidity and mortality, particularly in developing countries, and is characterised by symptoms including diarrhoea and/or dysentery with frequent mucous bloody stools, abdominal cramps and tenesmus [1,2]. The disease is highly communicable, in part because of the rapid spread of the pathogen and the low infectious dose of 10–200 cells [3]. Of the four *Shigella* spp., shigellosis is predominantly caused by *Shigella flexneri* in developing countries, especially in Asia, whereas *Shigella sonnei* is predominant in industrialised countries [4]. A recent (2014) World Health Organization (WHO) report indicated that, worldwide, an estimated 165 million cases of shigellosis occur annually with ca. 1 million deaths, the majority (69%) occurring in children under the age of 5 years [5]. In Iran, shigellosis is one of the major causes of morbidity in children with diarrhoea [6,7].

Treatment of shigellosis includes both rehydration and antimicrobial therapy [1,8]. Appropriate antimicrobial therapy reduces the duration of symptoms and prevents life-threatening complications [9]. Unfortunately, over the past few decades, overuse and misuse of antibiotics in the treatment of shigellosis has led to emergence of multidrug-resistant clinical isolates of *Shigella* and has made the selection of effective antimicrobial therapy more difficult [9–12]. For instance, a comparative study between 2001–2003 and 2004–2006 conducted in Iran showed that the antibiotic resistance rate of *Shigella* spp. increased from 13.8% to 15.5% [12].

Increased resistance to commonly used antibiotics including sulfonamides, tetracyclines, ampicillin and trimethoprim/sulfamethoxazole among *Shigella* isolates exists worldwide and these agents are no longer recommended for empirical treatment [12,13]. Most authorities now recommend an oral quinolone for proven or suspected shigellosis [13,14]. However, cases of fluoroquinolone-resistant *Shigella* have been reported in many areas of the world and are increasingly prevalent, particularly in Asia [15,16].

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Resistance to quinolones appears to be due to chromosomal point mutations in the quinolone resistance-determining region (QRDR) of DNA gyrase (*gyrA* and *gyrB*) and DNA topoisomerase (*parC* and *parE*) as well as changes in the expression of efflux pumps [11,16,17]. DNA gyrase, a type II DNA topoisomerase, creates a double-stranded break in DNA to catalyse DNA supercoiling, relaxation, decatenation and unknotting [18]. Quinolones bind to these enzymes and stabilise a drug–enzyme–cleaved DNA complex. Point mutations such as amino acid substitutions in the QRDR of target enzymes can diminish quinolone binding [19]. Generally, multiple mutations are required for clinically important resistance in Enterobacteriaceae, including *Shigella* spp. [20].

Plasmid-mediated quinolone resistance (PMQR) conferred by *qnrA*, *qnrB*, *qnrS*, *aac(6′)-Ib*-cr and *qepA* genes has also been described [15,21–23]. In 1998, the first PMQR gene (*qnr*) was discovered in a clinical strain of *Klebsiella pneumoniae* from the southern USA [24]. Since then, other PMQR genes have been detected in *Shigella* [15]. The *qnr* gene encodes a 218-residue protein called Qnr. Purified Qnr protein has been shown to protect DNA gyrase against the inhibitory effects of quinolone drugs [25].

Given the relative frequency of drug-resistant *Shigella* strains in clinical infections worldwide, it is important to understand the molecular mechanisms of antimicrobial resistance in this important pathogen for the implementation of intervention strategies. The increase in drug-resistant *Shigella* strains and the lack of studies on the causative genetic mutations in Iran prompted us to investigate the molecular mechanism of quinolone resistance in *Shigella* strains isolated from patients in Tehran, Iran.

2. Materials and methods

2.1. Bacterial isolates

Over a period of 2 years between 2008 and 2010, a total of 73 *Shigella* clinical strains were isolated from 1100 diarrhoeal patients in Tehran, Iran. Individual isolates were analysed by standard biochemical and serological tests as described previously [26]. Serogrouping of the isolates was confirmed using the slide agglutination test (Mast Diagnostics, Bootle, UK). Isolates were stored in trypticase soy broth (Merck KGaA, Darmstadt, Germany) with 25% (v/v) glycerol until use.

All ethical issues were considered. Life, health, dignity, integrity, right to self-determination, privacy and confidentiality of personal information of research subjects were protected in this study.

2.2. Antibiotic susceptibility testing

Antimicrobial susceptibility to nalidixic acid (30 μg; Oxoid Ltd., Basingstoke, UK) was determined by the Kirby–Bauer disk diffusion method on Mueller–Hinton agar (Oxoid Ltd.) in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines [27]. To ensure the accuracy of the results obtained by susceptibility testing, *Escherichia coli* ATCC 25922 was used for quality control.

2.3. PCR-restriction fragment length polymorphism (RFLP) analysis

Shigella isolates were grown on Luria–Bertani agar (Merck KGaA) and were incubated overnight at 37 °C. A single colony was removed from the plate, was suspended in 200 μL of distilled water and was heated at 100 °C for 10 min [28]. Following centrifugation at 8000 × *g* for 8 min, the supernatant was transferred into a new tube and was used for subsequent PCR analysis.

Table 1
PCR primers used in this study.

Primer/ target amplicon	Primer sequence (5′→3′)	Annealing temperature (°C)	Reference
DgyrA	F: AAC CGT TGA TGA CTT CCG TC R: TAT GCG ATG TCG GTC ATT GT	55	This study
LgyrA	F: TAC ACC GGT CAA CAT TGA GG R: TTA ATG ATT GCC GCC GTC GG	55	[29]
qnrS	F: ACG ACA TTC GTC AAC TGC AA R: TAA ATT GGC ACC CTG TAG GC	53	[30]
qnrA	F: ATT TCT CAC GCC AGG ATT TG R: GAT CGG CAA AGG TTA GGT CA	53	[30]
qnrB	F: GAT CGT GAA AGC CAG AAA GG R: ACG ATG CCT GGT AGT TGT CC	53	[30]

The QRDR of the target DNA gyrase (*gyrA*) and the PMQR determinants (*qnrS*, *qnrA*, and *qnrB*) were amplified using the primer sets described in Table 1. All isolates presenting resistance to nalidixic acid were screened for these determinants. Amplification was performed using a thermal cycler (Eppendorf, Hamburg, Germany) for 30 cycles. Thermal cycling parameters for the PCR assays consisted of denaturation at 94 °C for 2 min, annealing of primers at 53–55 °C for 2 min, and primer extension at 72 °C for 1 min. To ascertain expected sizes of the amplicons, the PCR products were run on 1.5% agarose gels, were stained with ethidium bromide (Sigma–Aldrich, Steinheim, Germany) and were visualised using an ultraviolet transilluminator (Tanon, Shanghai, China).

To investigate the probable mutation in the *gyrA* gene of nalidixic acid-resistant strains, RFLP analysis was used. PCR-amplified *gyrA* fragments were digested with *Hinf*I restriction enzyme (MBI, Fermentas, Lithuania). Restriction analysis was performed overnight at 37 °C using the buffer and temperature recommended by the manufacturer. Restriction fragments were subjected to agarose gel electrophoresis, followed by ethidium bromide staining.

2.4. Sequencing

PCR products of mutant strains identified by enzymatic digestion were subjected to DNA sequencing. PCR products of non-mutant strains were used as controls.

3. Results

3.1. Antimicrobial susceptibility testing

In total, 73 *Shigella* spp. isolates were isolated during the study period, of which 23 (31.5%) were resistant to nalidixic acid. Serological analysis showed that *S. sonnei* (*n* = 54; 74.0%) was the most common, followed by *S. flexneri* (*n* = 14; 19.2%), *Shigella boydii* (*n* = 4; 5.5%) and *Shigella dysenteriae* (*n* = 1; 1.4%). The age distribution data revealed that *Shigella* was isolated from 40 cases (54.8%) in the 1–5-year-old age group, 32 (43.8%) in the 6–12-year-old age group and 1 (1.4%) in >12-year-old age group. The male/female ratio was 1:0.73.

3.2. PCR results

The results of DNA amplification by the PCR method based on the primers described in Table 1 showed the presence of 648-bp and 511-bp fragments for the *gyrA* gene amplified by LgyrA and DgyrA primers, respectively (Fig. 1). Of the 23 quinolone-resistant isolates, 4 (17.4%) (including 2 *S. flexneri*, 1 *S. sonnei* and 1 *S. boydii*) contained the *qnrS* gene. None of the isolates harboured the *qnrA* or *qnrB* genes.