Profiles of gyrA Mutations and Plasmid-Mediated Quinolone Resistance Genes in Shigella Isolates with Different Levels of Fluoroquinolone Susceptibility

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Purpose: Fluoroquinolone-resistant Shigella is considered a serious public health problem and has been put on the WHO global priority list of antibiotic-resistant bacteria. This study was aimed to investigate the fluoroquinolone resistance in Shigella and its relevant genetic mechanisms.

Materials and Methods: Shigella isolates that were isolated from diarrheal patient’s feces in Ningbo China from 2011 to 2018 were tested for susceptibility to ampicillin, gentamicin, tetracycline, nalidixic acid, ciprofloxacin, and cefotaxime. Genes related to quinolone resistance were amplified by PCR.

Results: A total of 118 Shigella isolates were collected, including 76 S. flexneri isolates, 40 S. sonnei isolates, and 2 S. boydii isolates. Ciprofloxacin susceptibility test identified 10 (9%) susceptible, 65 (55%) intermediate, and 43 (36%) resistant isolates. Of 76 S. flexneri isolates, 37 were ciprofloxacin resistant, a prevalence significantly higher than 6 of 40 S. sonnei isolates (P=0.01). The isolates collected during 2014–2018 displayed a significant increase in the prevalence of ciprofloxacin resistance (P=0.05) than those collected during 2011–2013. All the ciprofloxacin-intermediate and resistant isolates had mutations of gyrA(S83L) and parC (S80I), whereas only the ciprofloxacin-resistant isolates had gyrA (D87N) mutation and qnrB gene. Additionally, 30% of the ciprofloxacin-resistant isolates were positive for aac(6’)-Ib-cr gene.

Conclusion: This study shows the currently increasing prevalence of ciprofloxacin resistance. The reduced fluoroquinolone susceptibility is highly associated with gyrA (S83L) and parC (S80I) mutations, while the fluoroquinolone resistance is highly associated with gyrA (D87N) mutation, qnrB gene and perhaps aac(6’)-Ib-cr gene.

Keywords: Shigella, ciprofloxacin resistance, gyrA mutations, plasmid-mediated quinolone resistance, PMQR, quinolone resistance-determining region, QRDR

Background

Shigellosis is a diarrheal disease in humans caused by four Shigella species: Shigella sonnei (S. sonnei), Shigella flexneri (S. flexneri), Shigella boydii (S. boydii) and Shigella dysenteriae (S. dysenteriae). This disease has become a major public health problem in developing countries. Several antimicrobial agents, most often fluoroquinolones and β-lactams, are usually used to treat shigellosis. Fluoroquinolone is one of the quinolones, which are a family of synthetic broad-spectrum antibiotic drugs. The first quinolone was nalidixic acid that was introduced in 1962 and is considered to be the
predecessor of all members of the quinolone family. Among a number of fluoroquinolones, ciprofloxacin is preferred in treating many bacterial infections, including shigellosis, because of its safety and low cost.

Fluoroquinolone resistance in the Enterobacteriaceae family, of which Shigella is a member, has been proved to be mainly caused by point mutations in genes encoding DNA gyrase and topoisomerase IV, such as gyrA, gyrB, and parC genes in quinolone resistance-determining regions (QRDRs). Accordingly, Shigella often acquires fluoroquinolone resistance through point mutations in gyr genes and transferable plasmid-mediated quinolone resistance (PMQR) genes that consist of the pentapeptide repeat protein-encoding qnr, the efflux-pump-encoding qepA and the aminoglycoside acetyltransferase-encoding enzyme variant aac(6’)-Ib-cr. These complicated mechanisms of quinolone resistance, plus extensive use of quinolones, lead to the increased prevalence of fluoroquinolone resistance in Shigella.

In recent years, multidrug-resistant (MDR) and even extensively drug-resistant (XDR) Shigella strains that are resistant to most often administered antibiotics, such as fluoroquinolones and β-lactams, have been reported in many parts of the world. Therefore, fluoroquinolone-resistant Shigella has been put on the World Health Organization (WHO) global priority list of antibiotic-resistant bacteria. Previous literature usually classified Shigella into two groups of fluoroquinolone-resistant and -susceptible isolates and studied molecular mechanisms in each group. However, our surveillance on the quinolone resistance in Shigella found a lot of Shigella isolates exhibiting intermediate fluoroquinolone resistance. These isolates were typically nalidixic acid-resistant and fluoroquinolone-intermediate, which is distinct from resistance and susceptibility of both nalidixic acid and fluoroquinolone. This level of intermediate resistance represents reduced fluoroquinolone susceptibility and reflects a step towards fluoroquinolone resistance and lacks a systematic analysis.

Several previous studies in China were conducted to investigate the mechanisms of fluoroquinolone resistance in Shigella through testing norfloxacin susceptibility. However, seeing the clinical preference of ciprofloxacin, more research is required to analyze mechanisms of fluoroquinolones resistance in terms of ciprofloxacin. Hence, this study was aimed to investigate the fluoroquinolone (ciprofloxacin) resistance and relevant molecular mechanisms in 118 Shigella isolates that were classified based on three levels of fluoroquinolone resistance. We compared the molecular mechanisms between the isolates with reduced fluoroquinolone susceptibility and the fluoroquinolone-resistant isolates, intending to clarify what further molecular mechanisms lead to the fluoroquinolone resistance. This comparison may shed light on the dynamic step from intermediate fluoroquinolone resistance to high resistance.

**Methods**

**Bacterial Isolates**

A total of 118 Shigella isolates were isolated from diarrheal patient’s feces in Ningbo China from 2011 to 2018. The isolates consisted of *S. flexneri* (76 isolates), *S. sonnei* (40 isolates), and *S. boydii* (2 isolates). All isolates were identified by API20E biochemical identification system (bioMérieux, Paris, France) and were serotyped by slide agglutination using Shigella-specific antisera (Denka Seiken, Japan). The isolates were kept in brain heart infusion broth containing 40% glycerol at −80 °C.

**Antimicrobial Susceptibility Testing (AST)**

All isolates were tested for minimal inhibitory concentrations (MICs) of ampicillin, gentamicin, tetracycline, nalidixic acid (NAL), ciprofloxacin (CIP), and ceftoxime by Etest (Oxoid, ThermoFisher, US). The MIC of trimethoprim-sulfamethoxazole (SXT) (1:19) was determined by the broth microdilution method. AST results were interpreted according to the guidelines in the Clinical and Laboratory Standards Institute (CLSI) document M02-A13. Confirmation of extended-spectrum β-lactamase (ESBL) on ceftoxime-resistant isolates was performed using the disc diffusion method specified in the CLSI document M02-A13. Isolates with ≥5 mm increase in the zone diameter for ceftoxime-clavulante versus the zone diameter for ceftoxime were defined as ESBL-producing.

**Genetic Determinants Related to Quinolone Resistance**

All isolates were tested for the quinolone resistance genes in QRDRs, including gyrA, gyrB, and parC, using primers listed in the previous literature. The amplicons ofthese genes were sequenced, and their mutations were determined by aligning the amplified sequences against the homologous sequence of *E. coli* ATCC25922. The genes in PMQR, including four
and ciprofloxacin susceptibility, the *Shigella* isolates were classified into susceptible (susceptible to both nalidixic acid and ciprofloxacin), intermediate (resistant to nalidixic acid and while intermediate-susceptible to ciprofloxacin) and resistant (resistant to both nalidixic acid and ciprofloxacin) isolates. The association between the levels of quinolone resistance and the genetic determinants was investigated. We also compared the prevalence of ciprofloxacin resistance and the genetic determinants between the two groups stratified by isolation time.

**Results**

Overall, 118 *Shigella* isolates were collected, which consisted of 76 (64%) *S. flexneri* isolates, 40 (34%) *S. sonnei* isolates and two (2%) *S. boydii* isolates. Six *S. flexneri* serotypes were 2a (n=45, 38%), 4a (n=19, 16%), 2b (n=4, 4%), x (n=3, 3%), 1a (n=2, 2%), 6a (n=2, 2%) and untypeable (n=1, 1%).

Based on the different levels of resistance to nalidixic acid and ciprofloxacin, 118 *Shigella* isolates were classified into three groups, ie, 10 (9%) susceptible isolates, 65 (55%) intermediate-susceptible isolates and 43 (36%) resistant isolates (Table 1). The prevalence of resistance to ciprofloxacin (37/76) in the 76 *S. flexneri* isolates was significantly higher than that (6/40) in the 40 *S. sonnei* isolates (P=0.01) (Table 1). The resistance to ampicillin (100%) and nalidixic acid (92%) was most commonly observed (Table 1). Except for the moderate level of resistance to gentamicin (30%), the prevalence of resistance to cefotaxime, SXT, and tetracycline, as well as the prevalence of ESBL-production, was at high levels, varying from 64% to 76%.

We identified two types of *gyrA* mutations (S83L and D87N), one *parC* mutation (S80I), and two PMQR genes *qnrB* and *aac(6’)-Ib-cr* in the ciprofloxacin-intermediate and ciprofloxacin-resistant isolates (Table 2). No *gyrB* mutations were detected. Three groups of isolates with different levels of ciprofloxacin susceptibility each had distinct profiles of these resistance genes. The ciprofloxacin-resistant isolates all had *gyrA* (S83L), *parC* (S80I), and *gyrA* (D87N) mutations and *qnrB* genes; in addition, 30% of these isolates had *aac(6’)-Ib-cr*. The ciprofloxacin-resistant isolates all had *gyrA* (S83L) and *parC* (S80I) mutations. The ciprofloxacin-susceptible isolates had no relevant resistance genes. Altogether, *gyrA* (D87N) mutations, *qnrB* genes and *aac(6’)-Ib-cr* were only found in ciprofloxacin-resistant isolates, while *gyrA* (S83L) and *parC* (S80I) mutations existed in both the ciprofloxacin-intermediate and the ciprofloxacin-resistant isolates. Between two predominant *S. flexneri* serotypes 2a (n=45) and 4a (n=19), the prevalence of QRDR mutations (S83L, D87N, and S80I) was 91% and 84% with no statistical difference (P>0.05), and the prevalence of PMQR genes (*qnrB* and *aac(6’)-Ib-cr*) was 53% and 47% with no statistical difference (P>0.05).

When we compared the results between two periods of 2011–2013 and 2014–2018, two significant findings were the emergence of six ciprofloxacin-resistant *S. sonnei* isolates with *aac(6’)-Ib-cr* genes and the presence of *S. sonnei* isolates with predicted ESBL production (D87N and S80I) mutations.

**Table 1** Prevalence of Antimicrobial Resistance in Ciprofloxacin-Susceptible, Intermediate and Resistant *Shigella* Isolates

| Ciprofloxacin MIC (μg/mL) | Serotype    | Number | Number of Isolates Resistant to Antimicrobial Agent (%) | Ampicillin | Gentamicin | Tetracycline | Nalidixic Acid | Cefotaxime | ESBL | SXT |
|---------------------------|-------------|--------|---------------------------------------------------------|------------|------------|--------------|---------------|------------|------|-----|
| Susceptible (<0.25)       | *S. flexneri* | 5      | 5 0 1 0 0 0 0 2 |            |            |              |               |            |      |     |
|                           | *S. sonnei*  | 5      | 5 0 0 0 0 0 0 1 |            |            |              |               |            |      |     |
| Intermediate (0.5)        | *S. flexneri* | 34     | 34 (100) 11 (32) 25 (74) 34 (100) 16 (47) 16 (47) 30 (88) |            |            |              |               |            |      |     |
|                           | *S. sonnei*  | 29     | 29 (100) 12 (41) 14 (48) 29 (100) 25 (86) 21 (72) 21 (72) |            |            |              |               |            |      |     |
|                           | *S. boydii*  | 2      | 2 0 0 2 0 0 0 0 |            |            |              |               |            |      |     |
| Resistant (≥1)            | *S. flexneri* | 37     | 37 (100) 10 (27) 30 (81) 37 (100) 30 (81) 28 (76) 30 (81) |            |            |              |               |            |      |     |
|                           | *S. sonnei*  | 6      | 6 (100) 2 (33) 4 (67) 6 (100) 6 (100) 6 (100) 6 (100) |            |            |              |               |            |      |     |
| Total                     |             | 118    | 118 (100) 35 (30) 76 (64) 108 (192) 77 (65) 71 (60) 90 (76) |            |            |              |               |            |      |     |

**Abbreviations:** SXT, trimethoprim-sulfamethoxazole; ESBL, extended-spectrum β-lactamase.
isolates during 2014–2018 and the significant increase in the prevalence of ciprofloxacin-resistant (MIC ≥1 μg/mL) isolates (P=0.01) (Table 3).

Discussion
In this study, 118 Shigella isolates were composed of 64% of S. flexneri and 34% of S. sonnei, a distribution of serotypes similar to a previous study in China that reported 78.7% of S. flexneri and 20.3% of S. sonnei in 301 isolates.18 No S. dysenteriae was found in this study. Of 118 isolates, 36% were ciprofloxacin-resistant, which was substantially higher than the prevalence of 2%–8% reported in the limited previous studies conducted in China, the United States and South Asia.19,20 In the study area, a previous study showed that the prevalence of ciprofloxacin resistance before 2013 remained at a high level of about 40.6–49.0%.21 We observed the significant increase (P=0.01) in the prevalence of ciprofloxacin resistance in S. flexneri and S. sonnei that were isolated during 2014–2018 than those isolated during 2011–2013 (Table 3). Of Shigella isolates that were collected during 2014–2018, 50% were ciprofloxacin-resistant and all were ciprofloxacin-non-susceptible. Regarding the major serotypes, besides the emerging ciprofloxacin-resistance in S. sonnei detected during 2014–2018, the proportion of ciprofloxacin-resistant S. flexneri isolates slightly increased from 36% (12/33) to 58% (25/43) between two periods (P=0.34) with the disappearance of ciprofloxacin-susceptible isolates (Table 3).

These isolates were also highly resistant to other drugs, especially β-lactams and SXT (Table 1). Between the ciprofloxacin-resistant isolates and the nonresistant isolates, there was no significant difference in the resistance rates of ampicillin, gentamicin, tetracycline, SXT and cefotaxime. This consistency indicates that the occurrence of ciprofloxacin resistance is independent of the resistance to other antimicrobials, such as β-lactams.

Table 2 Amino Acid Changes in gyrA and parC, and Quinolone Resistance-Related Genetic Determinants in S. flexneri and S. sonnei with Different Levels of Ciprofloxacin Susceptibility

| Ciprofloxacin MIC (μg/mL) | Serotype    | Number (n=118) | gyrA     | parC     | PMQR Genes  |
|--------------------------|-------------|----------------|----------|----------|-------------|
|                          |             |                |          |          | qnrA qnrB qnrS aac(6')-Ib-cr |
| Susceptible (≤0.25)      | S. flexneri | 5              | –        | –        | – – –      |
|                          | S. sonnei  | 5              | –        | –        | – – –      |
| Intermediate (0.5)       | S. flexneri | 34             | S83L     | S80I     | – – –      |
|                          | S. sonnei  | 29             | S83L     | S80I     | – – –      |
|                          | S. boydii  | 2              | S83L     | S80I     | – – –      |
| Resistant (≥1)           | S. flexneri | 37             | S83L, D87N | S80I     | – + –      + (27%) |
|                          | S. sonnei  | 6              | S83L, D87N | S80I     | – + –      + (33%) |

Table 3 MICs of Quinolones in Shigella Isolates During 2011–2013 and 2014–2018

| MICs (μg/mL) | 2011–2013 (n=54) | 2014–2018 (n=64) | P * | Total |
|--------------|------------------|------------------|-----|-------|
|              | S. flexneri (n=33) | S. sonnei (n=19) | S. boydii (n=2) | S. flexneri (n=43) | S. sonnei (n=21) |       |       |
| Ciprofloxacin | ≤0.25 | 5      | 5      | –      | –      | –      | –      | 10    |
|              | 0.5   | 16     | 14     | 2      | 18     | 15     | 0.3    | 65    |
|              | ≥1    | 12     | –      | –      | 25     | 6      | 0.01   | 43    |
| Nalidixic acid| ≤32   | 5      | 5      | –      | –      | –      | –      | <0.01 |
|              | >32   | 28     | 14     | 2      | 43     | 21     | <0.01  | 108   |

Note: *P value between Shigella isolates collected during 2011–2013 and 2014–2018.
We identified five types of quinolone resistance-related genetic determinants, ie, two gyrA mutations S83L and D87N, a parC mutation S80I, as well as qnrB gene and aac(6’)-Ib-cr gene in PMQR (Table 2). Notably, the isolates with three different levels of ciprofloxacin susceptibility showed distinct profiles of antimicrobial resistance genes (Table 2). All five resistance genes were absent in ciprofloxacin-susceptible isolates, whereas ciprofloxacin-intermediate isolates only contained mutations of gyrA(S83L) and parC (S80I). By contrast, all ciprofloxacin-resistant isolates contained two mutations that existed in the ciprofloxacin-intermediate isolates and the gyrA (D87N) mutation, qnrB and aac(6’)-Ib-cr.

Such characteristic profiles were reported previously for gyrA and parC mutations between solely nalidixic acid-resistant isolates and both nalidixic acid- and norfloxacin-resistant strains.13 However, the different profiles regarding qnrB, and aac(6’)-Ib-cr between fluoroquinolone-intermediate and -resistant isolates were not reported in the preceding literature. The present results indicate that the gyrA (S83L) and parC (S80I) mutations in the ciprofloxacin-intermediate isolates may be the first step towards ciprofloxacin resistance. In contrast, gyrA (D87N), qnrB and aac(6’)-Ib-cr genes are the crucial factors of ciprofloxacin resistance. In previous literature, the prevalence of gyrA (S83L), gyrA (D87N) and parC (S80I) in ciprofloxacin-resistant Shigella isolates has been reported.18,19 Nevertheless, this study first demonstrated that gyrA (D87N), qnrB gene, and aac(6’)-Ib-cr gene were only present in ciprofloxacin-resistant S. flexneri and S. sonnei strains as in our study, suggesting that these genetic determinants were necessary causes of ciprofloxacin resistance in Shigella isolates.

Conclusion
This study shows the currently increasing prevalence of ciprofloxacin resistance. The characteristic profiles of gyrA mutations and PMQR genes are present among Shigella isolates with different levels of fluoroquinolone susceptibility. The reduced fluoroquinolone susceptibility is highly associated with gyrA (S83L) and parC (S80I) mutations. In contrast, the fluoroquinolone resistance is highly associated with gyrA (D87N) mutation, qnrB gene and perhaps aac(6’)-Ib-cr gene.

Ethics Approval and Consent to Participate
All procedures in the study involving patients were approved by the Institutional Ethical Committee of Ningbo City First Hospital, Ningbo, Zhejiang Province, China. Informed consent was waived.

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Disclosure
The authors declare no conflicts of interest.

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