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ORIGINAL RESEARCH

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.[1,2] 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 is nalidixic acid that was introduced in 1962 and is considered to be the

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predecessor of all members of the quinolone family.[3] 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 *gyr*A, *gyr*B, and *par*C genes in quinolone resistance-determining regions (QRDRs).[4] Accordingly, *Shigella* often acquires fluoroquinolone resistance through point mutations in *gyr* genes[5] 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*.[6,7] 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 *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.[8,9] Therefore, fluoroquinolone-resistant *Shigella* has been put on the World Health Organization (WHO) global priority list of antibiotic-resistant bacteria.[10] Previous literature usually classified *Shigella* into two groups of fluoroquinolone-resistant and -susceptible isolates and studied molecular mechanisms in each group.[11] 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.[12,13] 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 (bioMerieux, 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 cefotaxime 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 M100-S30.[14] Confirmation of extended-spectrum β-lactamase (ESBL) on cefotaxime-resistant isolates was performed using the disc diffusion method specified in the CLSI document M02-A13.[15] Isolates with≥5 mm increase in the zone diameter for cefotaxime-clavulanate versus the zone diameter for cefotaxime were defined as ESBL-producing.

Genetic Determinants Related to Quinolone Resistance

All isolates were tested for the quinolone resistance genes in QRDRs, including *gyr*A, *gyr*B, and *par*C, using primers listed in the previous literature.[16] The amplicons of these 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

qnrA, qnrB, qnrS, and *aac(6′)-Ib-c*, were amplified using the primers previously described.[17]

Interpretation of the Results for Quinolone Susceptibility

Based on nalidixic acid 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-intermediate 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*

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	2
	S. sonnei	5	5	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	2	2	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.

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%)

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 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 [a]	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	10
	>32	28	14	2	43	21	<0.01	108

Note: [a]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.

References

1. Xia S, Xu B, Huang L, et al. Prevalence and characterization of human Shigella infections in Henan Province, China, in 2006. *J Clin Microbiol*. 2011;49(1):232–242. doi:10.1128/JCM.01508-10
2. von Seidlein L, Kim DR, Ali M, et al. A multicentre study of Shigella diarrhoea in six Asian countries: disease burden, clinical manifestations, and microbiology. *PLoS Med*. 2006;3(9):e353. doi:10.1371/journal.pmed.0030353
3. Andersson MI, MacGowan AP. Development of the quinolones. *J Antimicrob Chemother*. 2003;51(Suppl 1):1–11. doi:10.1093/jac/dkg212
4. Fabrega A, Madurga S, Giralt E, Vila J. Mechanism of action of and resistance to quinolones. *Microb Biotechnol*. 2009;2(1):40–61. doi:10.1111/j.1751-7915.2008.00063.x
5. Pu XY, Zhang Q, Pan JC, Shen Z, Zhang W. Spontaneous mutation frequency and molecular mechanisms of Shigella flexneri fluoroquinolone resistance under antibiotic selective stress. *World J Microbiol Biotechnol*. 2013;29(2):365–371. doi:10.1007/s11274-012-1190-3
6. Giraud E, Baucheron S, Cloeckaert A. Resistance to fluoroquinolones in Salmonella: emerging mechanisms and resistance prevention strategies. *Microbes Infect*. 2006;8(7):1937–1944. doi:10.1016/j.micinf.2005.12.025
7. Hopkins KL, Davies RH, Threlfall EJ. Mechanisms of quinolone resistance in Escherichia coli and Salmonella: recent developments. *Int J Antimicrob Agents*. 2005;25(5):358–373. doi:10.1016/j.ijantimicag.2005.02.006
8. Zhu J, Duan G, Yang H, Fan Q, Xi Y. Multi-drug resistance and characteristic of integrons in Shigella spp. isolated from China. *Biomed Environ Sci*. 2011;24(1):56–61. doi:10.3967/0895-3988.2011.01.007
9. Chang Z, Zhang J, Ran L, et al. The changing epidemiology of bacillary dysentery and characteristics of antimicrobial resistance of Shigella isolated in China from 2004–2014. *BMC Infect Dis*. 2016;16(1):685. doi:10.1186/s12879-016-1977-1
10. World Health Organization. Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics. Available from: http://www.who.int/mediacentre/news/releases/2017/bacteria-antibiotics-needed/en/. Accessed July 2, 2020.
11. Yang H, Duan G, Zhu J, Zhang W, Xi Y, Fan Q. Prevalence and characterisation of plasmid-mediated quinolone resistance and mutations in the gyrase and topoisomerase IV genes among Shigella isolates from Henan, China, between 2001 and 2008. *Int J Antimicrob Agents*. 2013;42(2):173–177. doi:10.1016/j.ijantimicag.2013.04.026
12. Gu B, Qin TT, Fan WT, et al. Novel mutations in gyrA and parC among Shigella sonnei strains from Jiangsu Province of China, 2002–2011. *Int J Infect Dis*. 2017;59:44–49. doi:10.1016/j.ijid.2017.03.023
13. Qin T, Qian H, Fan W, et al. Newest data on fluoroquinolone resistance mechanism of Shigella flexneri isolates in Jiangsu Province of China. *Antimicrob Resist Infect Control*. 2017;6:97. doi:10.1186/s13756-017-0249-1

14. CLSI. *Performance Standards for Antimicrobial Susceptibility Testing*. 30th ed. CLSI document M100. Wayne, PA: Clinical and Laboratory Standards Institute; 2020.
15. CLSI. *Performance Standards for Antimicrobial Disk Susceptibility Tests*. 13th ed. CLSI document M02. Wayne, PA: Clinical and Laboratory Standards Institute; 2018.
16. Eaves DJ, Randall L, Gray DT, et al. Prevalence of mutations within the quinolone resistance-determining region of gyrA, gyrB, parC, and parE and association with antibiotic resistance in quinolone-resistant Salmonella enterica. *Antimicrob Agents Chemother*. 2004;48(10):4012–4015. doi:10.1128/AAC.48.10.4012-4015.2004
17. Le TM, Baker S, Le TP, et al. High prevalence of plasmid-mediated quinolone resistance determinants in commensal members of the Enterobacteriaceae in Ho Chi Minh City, Vietnam. *J Med Microbiol*. 2009;58(Pt 12):1585–1592. doi:10.1099/jmm.0.010033-0
18. Zhang W, Luo Y, Li J, et al. Wide dissemination of multidrug-resistant Shigella isolates in China. *J Antimicrob Chemother*. 2011;66(11):2527–2535. doi:10.1093/jac/dkr341
19. Azmi IJ, Khajanchi BK, Akter F, et al. Fluoroquinolone resistance mechanisms of Shigella flexneri isolated in Bangladesh. *PLoS One*. 2014;9(7):e102533. doi:10.1371/journal.pone.0102533
20. Pu XY, Pan JC, Wang HQ, Zhang W, Huang ZC, Gu YM. Characterization of fluoroquinolone-resistant Shigella flexneri in Hangzhou area of China. *J Antimicrob Chemother*. 2009;63(5):917–920. doi:10.1093/jac/dkp087
21. Song Q, Lin W, Gao H, Yang Y, Xu J, Xu G. Changing antimicrobial resistance patterns and trends of Shigella isolates in Ningbo, Mid-East China, 2005–2013. *Int J Antimicrob Agents*. 2015;45(5):559–560. doi:10.1016/j.ijantimicag.2015.02.001