Case Report
Clinical Microbiology

Ann Lab Med 2012;32:366-369
http://dx.doi.org/10.3343/alm.2012.32.5.366
ISSN 2234-3806 • eISSN 2234-3814

ANNALS OF LABORATORY MEDICINE

Quinolone-resistant *Shigella flexneri* Isolated in a Patient Who Travelled to India

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We report a recent case in which ciprofloxacin-resistant *Shigella flexneri* was isolated from a 23-yr-old female patient with a history of travel to India. Prior to her admission to our internal medicine department, she experienced symptoms of high fever and generalized weakness from continuous watery diarrhea that developed midway during the trip. *S. flexneri* was isolated from the stool culture. Despite initial treatment with ciprofloxacin, the stool cultures continued to show *S. flexneri* growth. In the susceptibility test for antibiotics of the quinolone family, the isolate showed resistance to ciprofloxacin (minimum inhibitory concentration [MIC], 8 μg/mL), norfloxacin (MIC, 32 μg/mL), ofloxacin (MIC, 8 μg/mL), nalidixic acid (MIC, 256 μg/mL), and intermediate resistance to levofloxacin (MIC, 4 μg/mL). In molecular studies for quinolone resistance related genes, plasmid borne-quinolone resistance genes such as *qnrA, qnrB, qnrS, aac(6')-Ib-cr, qepA,* and *oqxAB* were not detected. Two mutations were observed in gyrA (248C→T, 259G→A) and 1 mutation in *parC* (239G→T). The molecular characteristics of the isolated *S. flexneri* showed that the isolate was more similar to the strains isolated from the dysentery outbreak in India than those isolated from Korea.

Key Words: Quinolone resistance, *Shigella flexneri*, Ciprofloxacin resistance

Received: January 9, 2012
Revision received: March 10, 2012
Accepted: May 25, 2012

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INTRODUCTION

Shigella flexneri is an endemic organism in most developing countries and causes severe illnesses; the mortality rate of *S. flexneri* infections is higher than those of infections with other *Shigella* species [1]. Patients are often treated with antimicrobial agents to reduce the duration of the illness, and the treatment is known to reduce the period of *Shigella* excretion [2]. Quinolones, especially ciprofloxacin, are commonly used for the treatment of shigellosis. However, in recent years, some *Shigella* strains have rapidly developed resistance to antimicrobial agents [3]. In India, one of the endemic areas for *S. flexneri*, this organism is one of the predominant species isolated from cultures and shows 45.6% resistance to ciprofloxacin [4]. In 8 Asian countries, 12% of the *S. flexneri* isolates obtained from 2001 to 2004 were documented to show resistance to ciprofloxacin [5]. In Korea, the National Institute of Health reported 2 quinolone-resistant strains of *S. flexneri* among 5,938 *Shigella* spp. in 2008 [6]. Here, we present a recent case in which quinolone-resistant *S. flexneri* was isolated from a patient with a history of travel to India.

CASE REPORT

A 23-yr-old woman had a history of high fever with a chief complaint of general weakness induced by repeated watery diarrhea. She had been in India for 10 days prior to her admission to our hospital. She experienced these symptoms midway

through her trip and subsequently received fluid treatment. When she visited our hospital, diarrhea and other symptoms were absent; however, she was examined for further evaluation. Her physical examination showed that the heart rate was 64 beats/min, respiratory rate was 20 breaths/min, and the blood pressure was 110/60 mmHg. Results of laboratory investigations were as follows: hemoglobin, 11.9 g/dL; white blood cell count, 5.72×10^9 cells/L (37% segmented neutrophils, 48% lymphocytes, 5% monocytes, 8% eosinophils, and 2% atypical lymphocytes); and serum sodium concentration, 141 mEq/L. Her stool occult blood cultures yielded no growth.

S. flexneri were abundant in the first stool culture specimen. The micro-broth dilution assay with MicroScan WalkAway 96 SI (SIEMENS, Sacramento, CA, USA) showed that the isolate was resistant to ampicillin, ampicillin/sulbactam, ciprofloxacin, and trimethoprim-sulphamethoxazole, but was susceptible to amikacin, cefepime, cefotetan, cefoxitin, cephalothin, gentamicin, imipenem, and piperacillin/tazobactam. The minimum inhibitory concentration (MIC) of ciprofloxacin was 8 μg/mL. Initially, the patient received ciproctan (ciprofloxacin) 500 mg three times a day over 5 days. However, *S. flexneri* were still abundant in the follow-up stool culture, and the antibiotic-susceptibility test results also showed the same pattern from the previous results. After introduction of ceftriaxone in the treatment regimen for 5 days, *S. flexneri* was not isolated from the stool specimen.

Quinolone susceptibility of the isolates was tested in accordance with the CLSI guidelines (Table 1). The MICs of quinolones were tested on Mueller-Hinton broth (Becton Dickinson, Baltimore, MD, USA) using the micro-broth dilution method with a 96-well cell culture plate (SPL Lifescience, Pocheon, Korea) [7]. *E. coli* ATCC 25922 was used as the control strain. The quinolone-type antibiotics tested included ciprofloxacin (Sigma Chemical Co., St. Louis, MO, USA), norfloxacin (Sigma Chemical Co.), ofloxacin (Sigma Chemical Co.), sparfloxacin (Sigma Chemical Co.), nalidixic acid (Sigma Chemical Co.), and levofloxacin (Jeil Pharmaceutical Co., Seoul, Korea).

Table 1. Results of the quinolone susceptibility test by the micro-broth dilution method

Quinolone	MIC (μg/mL)	Interpretation
Ciprofloxacin	8	R
Norfloxacin	32	R
Ofloxacin	8	R
Sparfloxacin	8	-*
Levofloxacin	4	I
Nalidixic acid	256	R

*MIC level of sparfloxacin against *Shigella* is not mentioned in CLSI guideline. Abbreviations: MIC, minimum inhibitory concentration; R, resistance; I, Intermediate resistance.

Further molecular studies, which included inspection of mutations in the quinolone target genes and the genes responsible for the expression of the efflux pump (Table 2) were performed to determine the mechanism of resistance to quinolone. Each target gene was amplified using the PCR method, while the sequencing of *gyr*A [8], *gyr*B [9], *par*C [8], *parE* [9], *qnr*A [6], *qnr*B

Table 2. Primers used in this study

Target gene	Primer	Sequence (5′→3′)
gyrA [8]	Forward	AAATCTGCCCGTGTCGTTGGT
	Reverse	GCCATACCTACGGCGATA
gyrB [9]	Forward	ATGGATAAAGAAGGCTACAGCA
	Reverse	TCGACGTCCGCATCGGTCAT
parC [8]	Forward	CTGAATGCCAGCGCCAAATT
	Reverse	GCGAACGATTTCGGATCGTC
parE [9]	Forward	GACCGAAAGCTACGTCAACC
	Reverse	GTTCGGATCAAGCGTGGTTT
aac(6')-Ib-cr [6]	Forward	TTGCGATGCTCTATGAGTGGCTA
	Reverse	CTCGAATGCCTGGCGTGTTT
qnrA [6]	Forward	AGAGGATTTCTCACGCCAGGA
	Reverse	TTGAGATCGCACTCCCTGAA
qnrB [6]	Forward	GGCTGGCCGATTATGATTGGT
	Reverse	CGCGTGCGATGAGATAACC
qnrS [6]	Forward	TGCCACTTTGATGTCGCAGAT
	Reverse	CGCACGGAACTCTATACCGTAG
oqxA [10]	Forward	CTCGGCGCGATGATGCT
	Reverse	CCACTCTTCACGGGAGACGA
oqxB [10]	Forward	TTCTCCCCCGGCGGGAAGTAC
	Reverse	CTCGGCCATTTTGGCGCGTA
tolC [6]	Forward	CAGACCGATCAGCAAACCTTGA
	Reverse	GCCTGTGTATAGGAAAGAACGTCAA
	Probe	FAM CCGCGACCGCTTATT 5 NFQ
mdfA [6]	Forward	GGCTGGCCGATTATGATTGGT
	Reverse	CGCGTGCGATGAGATAACC
	Probe	FAM CAGCAGCGACCAATAG 5NFQ
ydhE [6]	Forward	CCGGCGATCCTCTTTGGT
	Reverse	GGAACCATTTAATTGCGCGATAACC
	Probe	FAM CAGCAGCGACCAATAG 5NFQ
16S rRNA	Forward	CCCCCTGGACGAAGACTGA
(control) [6]	Reverse	GTGGACTACCAGGGTATCTAATCCT
	Probe	FAM TCCCCACGCTTTCG 5 NFQ

Abbreviations: FAM, 6-carboxyfluorescein; NFQ, nonfluorescent quencher.

[6], *qnr*S [6], *oqx*A [10], *oqx*B [10], and *aac(6')-Ib-cr* [6] genes was conducted by SCL (Seoul Clinical Laboratories), with a 3130XL DNA genetic analyzer (Applied Biosystems, Foster City, CA, USA). Plasmid-borne quinolone resistance genes such as *qnrA*, *qnrB*, *qnrS*, *aac(6')-Ib-cr*, *qepA*, and *oqxAB* were absent. Two mutations (248C→T, 259G→A) were found in *gyr*A and 1 mutation was found in *parC* (239G→T).

A laboratory test was conducted to study the expression of *mdfA, tolC,* and *ydhE*, which play a role in the encoding of the efflux pump genes, by the Korea Center for Disease Control and Prevention (KCDC) under the National Institute of Health (NIH) (Fig. 1). Isolates were cultured in 5 mL of Mueller-Hinton Broth (Becton Dickinson, Franklin Lakes, NJ, USA) with or without 5 μg/mL of ciprofloxacin at 37°C for 30 min [6]. Total RNA was isolated using the RNeasy Mini kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's protocol. Expression of each gene was determined by real-time PCR quantification, which was based on the relative expression levels of *mdfA*, *tolC*, and *ydhE* when compared with the expression levels of a reference gene for 16S rRNA. The sample was quantified by PCR in a final reaction volume of 20 μL, containing 2.5 μL of cDNA with TaqMan One-Step RT-PCR master mix (Applied Biosystems) [6]. Quantification was performed on the basis of the threshold cycle (CT) value, which was determined using the LC480 software system (Roche Diagnostics, Mannheim, Germany). Relative gene expression levels were calculated as $2\text{-}\Delta\Delta C_T$, where $\Delta\Delta C_T = \Delta C_T$ (sample) - ΔC_T (control), and ΔC_T is the C_T of the target gene subtracted from the C_T of the housekeeping gene [11]. When ciprofloxacin was included in the medium, the expression levels of *tolC* of the *S. flexneri* isolate increased to 3.7 times than the expression levels in the control isolate. *S. flexneri* isolated from NIH was used as the control isolate, which had only one mutation in *gyrA* (248C→T) with susceptibility to ciprofloxacin (MIC, 0.25 μg/mL).

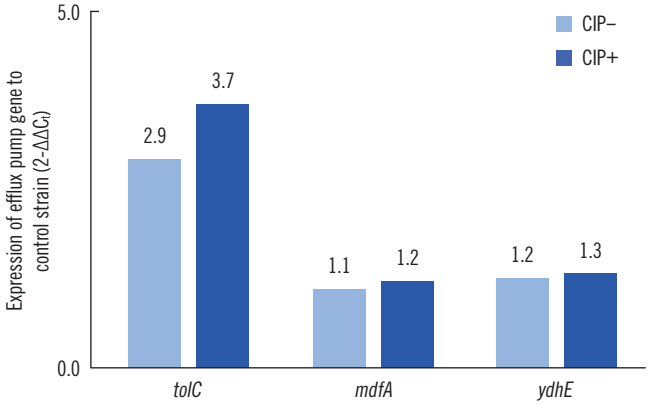

Fig. 1. Expression of the efflux pump genes in response to ciprofloxacin with reference to the expression levels in the control strain. Expression of *tolC*, *mdfA*, and *ydhE* was measured by real-time PCR in the absence and presence of ciprofloxacin. Each value represents the average of 5 culture replicates, each of which was evaluated twice. Relative gene expression levels were calculated as $2\text{-}\Delta\Delta C_T$, where $\Delta\Delta C_T = \Delta C_T$ (sample) - ΔC_T (control) [11].
CIP-: absence of ciprofloxacin; CIP+: presence of ciprofloxacin.

DISCUSSION

Since 1998, cases of shigellosis caused by *Shigella sonnei* and *S. flexneri* have been steadily increasing in Korea [6], although cases of quinolone-resistant *S. flexneri* infections are still relatively rare. Several mechanisms are responsible for the development of quinolone resistance in gram-negative organisms. One of the prominent mechanisms is the mutation of the genes related to quinolone resistance. The target regions where mutations occur in the organism have been identified as topoisomerase IV (*parC* and *parE*) and DNA gyrase (*gyrA* and *gyrB*) [12]. Dynamic efflux of quinolone has also been observed, and efflux-modulated resistance has been reported in numerous pathogenic gram-negative bacteria [13]. Another mechanism depends on plasmid-mediated quinolone resistance (PMQR) determinants such as *qnr, aac(6')-Ib-cr*, and *qepA* [14].

In the 2 previously reported quinolone-resistant strains of *S. flexneri* in Korea [6], one strain was documented to contain 3 substitutions: 1 in *gyrA* (Ser83→Leu) and 2 in *parC* (Ser80→Ile and Arg91→Gln). The other strain had 4 substitutions: 2 in *gyrA* (Ser83→Leu and Asp87→Gly) and 2 in *parC* (Ser80→Ile and Arg91→Gln). Both the strains had the PMQR gene (*qnrS*) in common, and there was an increase in the expression levels of the efflux pump-encoding genes (*mdfA, tolC*, and *ydhE*), which were better expressed in the presence of ciprofloxacin [6] (Table 3). In the present case, *S. flexneri* showed the mutation of the genes related to quinolone resistance. Two mutations were documented in *gyrA*; 1 for nucleotide position 248C→T (Ser83→Leu) and the other for position 259G→A (Asp87→Asn). The change of amino acid codon 87 from aspartic acid to asparagine shows new amino acid substitution that has not been reported in the isolated strains in Korea. In *parC* gene, a mutation for nucleotide position 239G→T (Ser80→Ile) was also found. PMQR determinants were not found in the molecular analysis.

According to the reports on the dysentery outbreaks in India in 2008, all isolated *Shigella* spp. were susceptible to ceftriaxone, a few of the quinolone-resistant strains had the *aac(6')-Ib-cr* gene, and none of the documented strains had *qnrA, B*, and

http://dx.doi.org/10.3343/alm.2012.32.5.366

Table 3. Comparison between previously reported strains (strain number 1 and 2) and strain of present case (strain number 3)

Strain number	1*	2*	3
Mutation of *gyrA*	Ser83→Leu (248C→T)	Ser83→Leu (248C→T)	Ser83→Leu (248C→T)
		Asp87→Gly (260A→G)	Asp87→Asn (259G→A)
Mutation of *parC*	Ser80→Ile (239G→T)	Ser80→Ile (239G→T)	Ser80→Ile (239G→T)
	Arg91→Gln (272G→A, 273A→G)	Arg91→Gln (272G→A, 273A→G)	
Presence of PMQR gene	*qnrS*	*qnrS*	None

*Previously reported *S. flexneri* strains in Korea [6].

S genes [15]. In addition, quinolone-resistant *S. flexneri* strains had 2 documented mutations in *gyrA* and 1 mutation in *parC*, which is similar to the corresponding findings in our case [15]. When the strains were treated with CCCP (carbonyl cyamide-*m*-chlorophenyldrazone), norfloxacin or ciprofloxacin accumulation was noted in the cells. This suggests that efflux pumps are one of the factors responsible for the development of quinolone resistance. In our case, involvement of the efflux pump in quinolone resistance was evaluated by real-time PCR analysis. The expression levels of the efflux pump-encoding gene, *tolC*, were higher in the presence of ciprofloxacin than in its absence.

We report a case of shigellosis caused by quinolone-resistant *S. flexneri*, isolated from a patient who traveled to India. Although the mutation points in the *gyrA* and *parC* genes were identical to the mutation points in the strains isolated in Korea, the molecular characteristics of the isolate in our case were more similar to those of the quinolone-resistant strains isolated in India because amino acid substitutions in the strains are different and there were no PMQR genes.

Authors' Disclosures of Potential Conflicts of Interest

No potential conflicts of interest relevant to this article were reported.

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