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Reduced Susceptibilities of *Shigella sonnei* Strains Isolated from Patients with Dysentery to Fluoroquinolones

SANKICHI HORIUCHI,[1]* YOSHIO INAGAKI,[1] NAOKI YAMAMOTO,[1] NOBORU OKAMURA,[2] YATSUKA IMAGAWA,[3] AND RINTARO NAKAYA[4]

Department of Microbiology[1] and School of Allied Health Sciences,[2] Faculty of Medicine, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113, College of Environmental Health, Azabu University, Kanagawa 229,[3] and Department of Food and Nutrition, School of Home Economics, Japan Women's University, Bunkyo-ku, Tokyo 112,[4] Japan

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Seven clinical isolates of *Shigella sonnei* with reduced susceptibilities to fluoroquinolones (sparfloxacin, ciprofloxacin, and ofloxacin) were obtained. The MICs of fluoroquinolones against these *S. sonnei* strains were 16 to 32 times higher than those obtained against typical strains that are highly susceptible to these agents. The kinetics of [^{14}C]ofloxacin accumulation in these clinical strains were not different from those in the fully susceptible strains. However, DNA synthesis was much less inhibited by ofloxacin in the strains with reduced susceptibility. Analysis of the in vitro activity of the partially purified DNA gyrase from these isolates showed that the decreased quinolone susceptibility of the *S. sonnei* strains was likely due to mutation of the DNA gyrase subunit A gene.

The fluoroquinolones, such as sparfloxacin, ciprofloxacin, and ofloxacin, are newly introduced broad-spectrum antimicrobial agents (3, 7, 9, 12). They have greater activity against gram-negative and gram-positive bacteria than do the older DNA gyrase inhibitors, such as nalidixic acid (9, 27). The newer agents are highly active against many members of the family *Enterobacteriaceae* (14, 23, 24), achieve high levels in feces (21), and possess good oral bioavailability. Because of these advantages, fluoroquinolones have been increasingly used clinically. However, along with their frequent use, quinolone-resistant strains have emerged, especially among gram-negative bacteria (1, 6, 13). Two main mechanisms of quinolone resistance, which may occur singly or in combination, have been recently characterized (11, 18). One is a mutation of the target, DNA gyrase-DNA complex (1, 22, 25, 28). DNA gyrase is composed of two proteins, subunit A and subunit B. The subunit A protein mediates the ability of the enzyme to introduce and rejoin double-strand breaks in DNA, while the subunit B protein mediates energy transduction and ATP hydrolysis (17, 26). The other mechanism of quinolone resistance is associated with mutations affecting outer membrane permeability, which inhibit accumulation of not only fluoroquinolones but other antimicrobial agents (5, 10).

We isolated seven *Shigella sonnei* strains with reduced susceptibilities to quinolones from enteritis patients. Only one patient had received treatment with quinolones before the pathogens were isolated. The mechanisms of fluoroquinolone resistance in other gram-negative bacteria have been studied (1, 11, 16, 18, 22). However, there have been no reports of fluoroquinolone resistance in the genus *Shigella*. We describe here the biological characteristics and the mechanisms responsible for reduced susceptibility to fluoroquinolones in these *S. sonnei* isolates.

We carried out antibacterial susceptibility tests of 43 strains of *Shigella* isolated from January 1989 to March 1990 by the Research Group of Infectious Enteritis for Clinical Trial with Sparfloxacin (12). Ofloxacin, ciprofloxacin and nalidixic acid were gifts from Daiichi Pharmaceuticals, Tokyo, Japan, and sparfloxacin was a gift from Dainihon Pharmaceuticals, Tokyo, Japan. MICs of quinolones were determined by an agar dilution method on sensitivity-test agar (Eiken Chemicals, Tokyo, Japan) at 37°C with inocula of 1×10^4 to 5×10^4 CFU. We detected seven strains with reduced susceptibilities to fluoroquinolones (Table 1). The MICs of fluoroquinolones against these *S. sonnei* strains were 0.36 to 1.56 μg/ml, or 16 to 32 times higher than those against typical quinolone-susceptible strains. Five of the patients infected with such *S. sonnei* strains traveled to Taipei, Taiwan, at the end of March 1989, and the other two patients were infected by a family member who traveled to Taipei during the same period. These strains carried seven plasmids with sizes of 250, 230, 150, 100, 90, 40, and 7.5 kb (data not shown) and were also resistant to ampicillin, chloramphenicol, streptomycin, sulfonamide, tetracycline, and trimethoprim. Experiments to detect transfer of drug resistance were carried out by the methods described by Nakaya and Rownd (19). All drug resistances in the seven *S. sonnei* strains, except resistance to quinolones, were transferred to *Escherichia coli* K-12 CSH-2 Rif^r (rifampin resistant). Resistance to ampicillin, streptomycin, sulfonamide, and trimethoprim was encoded on the 100-kb plasmid, while resistance to chloramphenicol, sulfonamide, trimethoprim, and tetracycline was transferred by the 90-kb plasmid (data not shown). Although we could not ascertain in detail the sources and the route of transmission of dysentery in these patients, these findings strongly indicated that these seven isolates of *S. sonnei* may have been derived from the same origin or related origins.

We compared the accumulations of ofloxacin between quinolone-"resistant" and quinolone-susceptible strains of *S. sonnei* by the method described by Cohen et al. (5). Bacteria were grown at 37°C in L broth (15) supplemented with 0.2% glucose to an optical density at 530 nm of 0.5, washed with fresh medium, and suspended in L broth to an optical density at 530 nm of 10 (10^{10} cells per ml). Ofloxacin

* Corresponding author.

TABLE 1. MICs of quinolones for clinical isolates of quinolone-resistant and quinolone-susceptible *S. sonnei* strains

Strain	Date isolated (mo/day/yr)	Place of isolation (hospital)	MIC (μg/ml) of[a]:			
			SPFX	CPFX	OFLX	NA
Resistant strains						
PO18[b]	03/28/89	Hiroshima City	0.39	0.39	1.56	>100
PO20[b]	03/31/89	Hiroshima City	0.39	0.39	0.78	>100
PO27[b]	04/04/89	Hiroshima City	0.39	0.39	1.56	>100
PO26[c]	03/31/89	Hiroshima City	0.19	0.39	1.56	>100
PO28[d]	04/04/89	Hiroshima City	0.39	0.19	1.56	>100
PO29[d]	04/05/89	Hiroshima City	0.39	0.39	1.56	>100
PO30[c]	03/24/89	Kyoto City	0.19	0.19	0.78	>100
Susceptible strains						
PO01	01/12/89	Tokyo Metropolitan Ebara General	0.012	0.012	0.10	1.56
PO10	03/09/89	Kyoto City	0.024	0.024	0.10	1.56

[a] Abbreviations: SPFX, sparfloxacin; CPFX, ciprofloxacin; OFLX, ofloxacin; NA, nalidixic acid.
[b] The patients from whom these strains were isolated were in the same tour group during a visit to Taipei, Taiwan, in March 1989.
[c] The patients from whom these strains were isolated were in the same tour group during a visit to Taipei, Taiwan, in March 1989.
[d] Isolated from family members of the patient from whom PO26 was isolated.

accumulation was assayed by adding [^{14}C]ofloxacin (81.9 μCi/mg; Daiichi Pharmaceuticals) to a final concentration of 6.25 μg/ml, adjusting the mixture to a specific activity of 20 μCi/mg by the addition of unlabeled ofloxacin (stock solution; 1.0 mg/ml in 0.02 N NaOH), and incubating the mixture for 30 min at 30°C. The samples (0.1 ml) were taken at specified times and collected on glass fiber GF/C filters (Whatman Inc., Clifton, N.J.). Filters were washed immediately three times with 2-ml portions of medium and dried, and then the radioactivity was assayed with a liquid scintillation system (Beckman LS-6800). The results were expressed as counts of [^{14}C]ofloxacin accumulated per minute per 10^9 cells. Ofloxacin accumulation reached a steady-state level in less than 3 min in susceptible strains PO01 and PO10 and "resistant" strains PO18 and PO26. Although the MIC of ofloxacin against strain PO26 was 16 times higher than that against the susceptible strain PO01, no differences in accumulation were seen (Fig. 1). Similar results were seen at ofloxacin concentrations of 3.12 and 12.5 μg/ml. Our results suggest that the mechanism of reduced quinolone susceptibility in *S. sonnei* is not an alteration in the membrane permeability (1, 5, 10).

The inhibition of DNA synthesis by ofloxacin with the seven clinical and susceptible strains was measured by the method of Chow et al. (4), and the kinetics of DNA synthesis were examined. Bacteria were grown at 37°C in M9 medium, washed with M9 medium, and suspended in fresh medium to an optical density at 530 nm of 2.0. Samples (0.2 ml) were incubated with ofloxacin for 2 min at 37°C with shaking prior to the addition of 1 μCi of [6-^{3}H]thymidine (20.1 Ci/mmol; New England Nuclear Corp., Boston, Mass.) for 2 min at 37°C. The reaction was terminated by adding 4 ml of cold 10% trichloroacetic acid. The precipitates were collected on Whatman GF/C filters and washed three times with 4 ml of 10% trichloroacetic acid and once each with 4 ml of cold 0.01 N HCl and 4 ml of cold 95% ethanol, and then the filters were dried. The radioactivity was measured by using a Beckman LS-6800 liquid scintillation system. A linear relationship existed between the drug concentration and the extent of inhibition of DNA synthesis (Fig. 2). The 50% inhibitory concentrations of the clinical strains PO18 and PO26 were 1.2 to 1.6 μg/ml, or about 10 times higher than that of the fully susceptible strain PO01; a reasonable correlation between MIC and 50% inhibitory concentration existed for PO01 and PO18 or PO26.

Purification of DNA gyrase was carried out as described by Aoyama et al. (1). Bacterial cells grown in 20 liters of L broth were collected, and the cells (ca. 20 g) were lysed by freezing and then subjected to centrifugation. The supernatant was precipitated with ammonium sulfate and fractionated by novobiocin-Sepharose and heparin-Sepharose CL-6B column chromatography (Pharmacia Fine Chemicals). The assay of DNA gyrase activity was carried out as described previously (17) with plasmid pBR322 DNA as the substrate. One unit of supercoiling activity was defined as the amount that brought 50% of relaxed pBR322 to the

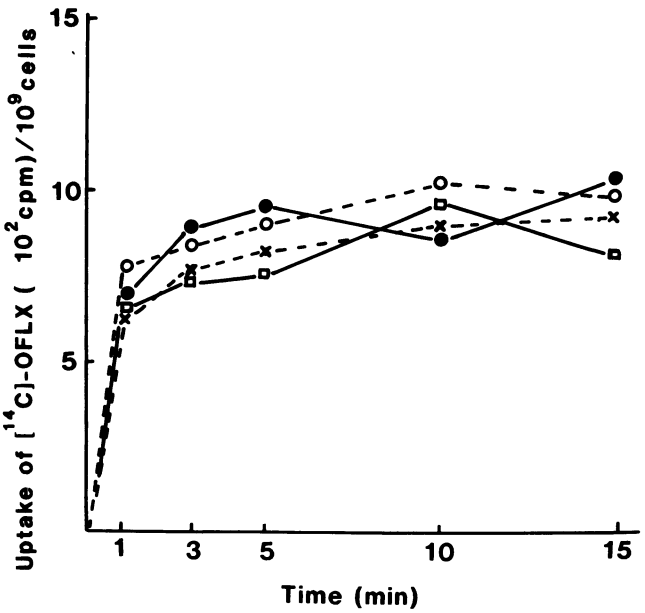

FIG. 1. Accumulation of [^{14}C]ofloxacin (OFLX) by quinolone-susceptible strains PO01 (●) and PO10 (□) and relatively resistant strains PO18 (○) and PO26 (×) of *S. sonnei*. Details are given in the text.

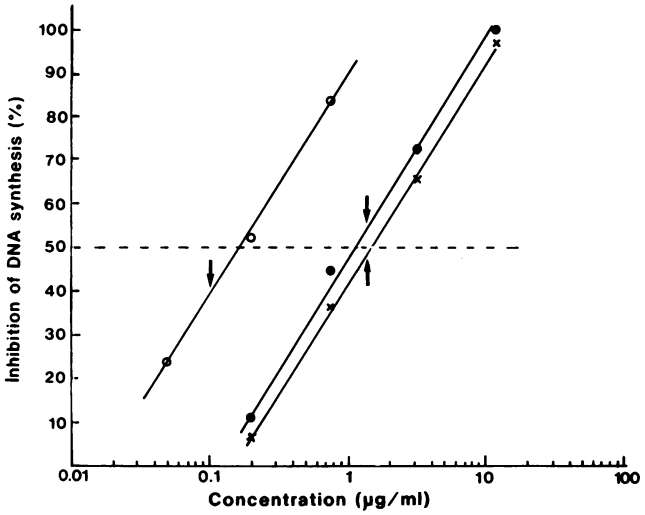

FIG. 2. Percent inhibition of DNA synthesis in *S. sonnei* PO01 (○), PO18 (●), and PO26 (×) by ofloxacin, measured by the method of Chow et al. (4). The broken line intersects the 50% inhibitory concentration, and the arrows show the MIC of ofloxacin against each strain.

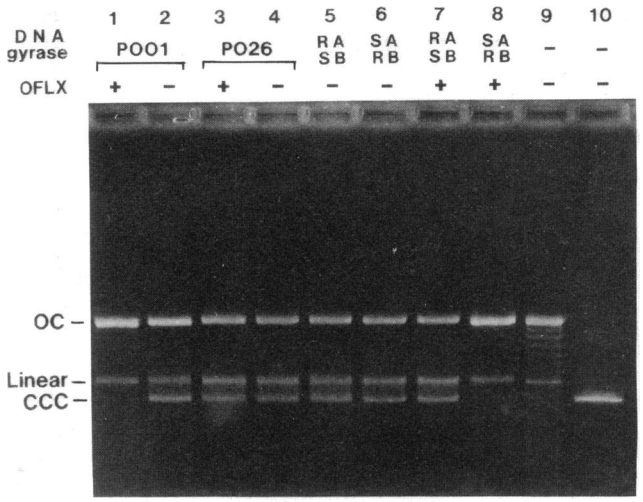

FIG. 3. Comparative inhibitory effects of ofloxacin on supercoiling activities of reconstituted subunit A and B proteins from *S. sonnei* PO01 and PO26. After relaxed pBR322 DNA was incubated with reconstituted DNA gyrases in the presence of ofloxacin (OFLX), the relaxed (OC) and supercoiled (CCC) DNAs were separated on an agarose gel and gyrase activities were determined following staining with ethidium bromide. Lanes 1 and 2, subunits A and B from PO01; lanes 3 and 4, subunits A and B from PO26; lanes 5 and 7, subunit A (RA) from PO26 and subunit B (SB) from PO01; lanes 6 and 8, subunit A (SA) from PO01 and subunit B (RB) from PO26; lane 9, reaction mixture without DNA gyrase (relaxed pBR322 DNA); lane 10, supercoiled pBR322 DNA. Lanes 1, 3, 7, and 8 contain ofloxacin at the concentration of 1.56 μg/ml; lanes 2, 4, 5, 6, 9, and 10 contain no ofloxacin.

supercoiled form as detected by agarose gel electrophoresis as described by Gellert et al. (8).

Inhibition of DNA gyrase supercoiling activity was assayed in a manner similar to that described by Sato et al. (22). The reaction mixture (20 μl) contained 40 mM Tris hydrochloride (pH 7.5), 20 mM KCl, 4 mM $MgCl_2$, 2 mM spermidine hydrochloride, 20 μg of *E. coli* tRNA per ml, 2 mM ATP, 2 mM dithiothreitol, 0.3 μg of relaxed pBR322 DNA (treated with topoisomerase I [Bethesda Research Laboratories]), drug solution, and DNA gyrase subunits A and B (1 U each). After incubation for 1 h at 37°C, the reaction was stopped by the addition of 5 μl of proteinase K (1 mg/ml). The reaction mixture was subjected to 0.8% agarose gel electrophoresis in TBE buffer (0.1 M Tris borate and 1 mM EDTA [pH 8.0]). The inhibitory effects of ofloxacin on the activities of the reconstituted DNA gyrases from strains PO01 and PO26 were determined (Fig. 3). Ofloxacin at the concentration of 1.56 μg/ml completely inhibited pBR322 DNA supercoiling by DNA gyrase isolated from susceptible strain PO01 (Fig. 3, lane 1) but did not inhibit the DNA supercoiling by DNA gyrase isolated from the clinical strain PO26 (Fig. 3, lane 3).

The reconstituted DNA gyrase of subunit A (SA) from PO01 and subunit B (RB) from PO26 was inhibited by 1.56 μg of ofloxacin per ml (Fig. 3, lane 8), whereas the reconstituted DNA gyrase of subunit A (RA) from PO26 and subunit B (SB) from PO01 was not inhibited at this concentration (Fig. 3, lane 7). These results suggest that alterations in DNA gyrase subunit A contributed to the reduced susceptibilities of these clinical strains but do not exclude the possibility that other mutations contributed as well.

The MICs of fluoroquinolones against the seven isolates tested in the present study did not exceed the breakpoint for susceptibility as determined by the National Committee for Clinical Laboratory Standards (for example, the breakpoint for susceptibility to ofloxacin is a MIC of ≤2 μg/ml) (20). Therefore, these isolates would not have been classified as quinolone resistant by standard clinical laboratory criteria. The seven patients from whom these isolates had been obtained before antibiotic therapy were then treated with sparfloxacin for a clinical trial (12). However, treatment with sparfloxacin for three of these seven patients was not effective in terms of a bacteriological cure (negative culture). In contrast, the clinical efficacy of sparfloxacin by the same criteria for 36 patients from whom typical fluoroquinolone-susceptible isolates were obtained was 100%. We also tested the susceptibilities of 43 isolates of *Shigella* spp. to fluoroquinolones in this clinical trial (12). The MICs of sparfloxacin and ciprofloxacin against 36 susceptible isolates were in the range of 0.006 to 0.024 μg/ml, whereas the MICs against the remaining seven isolates we used in the present study were 0.19 to 0.39 μg/ml, or 16 to 32 times higher than those against the susceptible strains. It is also interesting that an increasing occurrence of resistance to nalidixic acid has been reported for *Shigella* isolates (especially *Shigella dysenteriae* type 1) in Bangladesh, where fluoroquinolones are being considered as an alternative antimicrobial therapy (2).

Our present results may suggest that such nalidixic acid-resistant *Shigella* strains comprise mutants for which fluoroquinolone MICs are higher than for typical susceptible strains but remain below the breakpoint for susceptibility.

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