ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Jan. 2000, p. 192–195 Vol. 44, No. 1
0066-4804/00/$04.00+0

Susceptibilities of *Neisseria gonorrhoeae* Isolates Containing Amino Acid Substitutions in GyrA, with or without Substitutions in ParC, to Newer Fluoroquinolones and Other Antibiotics

MASATOSHI TANAKA,[1]* HIROSHI NAKAYAMA,[2] MASASHI HARAOKA,[1] TAKESHI SAIKA,[3] INTETSU KOBAYASHI,[3] AND SEIJI NAITO[1]

Department of Urology, Faculty of Medicine, Kyushu University,[1] and Nakayama Urologic Clinic,[2] Fukuoka, and Chemotherapy Division, Mitsubishi-kagaku BCL, Tokyo,[3] Japan

Received 18 March 1999/Returned for modification 10 September 1999/Accepted 25 October 1999

We examined the antimicrobial susceptibilities of 85 *Neisseria gonorrhoeae* isolates, classified according to the presence of amino acid substitutions in the GyrA and ParC proteins, to 12 fluoroquinolones and 7 other antibiotics. Sitafloxacin and HSR-903 showed excellent activity against *N. gonorrhoeae*, including strains with both GyrA and ParC substitutions. Among the strains with various GyrA substitutions, strains with a serine-91-to-phenylalanine mutation required the highest MICs of all of the fluoroquinolones tested and were cross-resistant to structurally unrelated β-lactams.

To date, fluoroquinolones have shown excellent clinical efficacy in the treatment of gonorrhea, but the recent emergence of clinical isolates resistant to fluoroquinolones has become a major problem in the treatment of gonococcal infections in several countries, including Japan (6, 9). Clinical failure of gonorrhea treatment has been encountered not only with ciprofloxacin (11) but also with sparfloxacin and pazufloxacin, which have been developed recently (9, 10). We therefore were concerned as to whether the newly constructed fluoroquinolones would have excellent clinical efficacy against gonorrhea caused by fluoroquinolone-resistant isolates in Japan. We also wondered whether the fluoroquinolone-resistant strains were cross-resistant to antimicrobial agents other than fluoroquinolones. In the present investigation, we tested the antimicrobial susceptibilities of *Neisseria gonorrhoeae* isolates, including wild-type strains and strains containing *gyrA* mutations with or without *parC* mutations, to various fluoroquinolones and other antibiotics.

A total of 85 *N. gonorrhoeae* isolates obtained between February 1993 and February 1997 were evaluated. Of the 85 strains, 43 had amino acid substitutions in the quinolone resistance-determining region (QRDR) within the GyrA protein alone, 22 had substitutions in the QRDRs within both the GyrA and ParC proteins, and the remaining 20 had no substitutions within the QRDR in either the GyrA or the ParC protein (wild type). None were posttreatment isolates or repeat isolates from the same patient. The isolates tested were epidemiologically unrelated. *N. gonorrhoeae* strains were identified on the basis of being gram-negative diplococci and by their oxidase reaction and sugar utilization patterns. The PCR and direct DNA sequencing were performed, as described previously (9), to identify mutations in the *gyrA* and *parC* genes of the gonococcal strains. The oligonucleotide primers for the PCR amplification were designed to amplify the genes corresponding to the QRDR within the GyrA and ParC proteins (1, 9).

MICs for all isolates were determined by an agar dilution technique with a GC agar base (Becton Dickinson, Paramus, N.J.) containing 1% IsoVitaleX (Becton Dickinson) (8). Plates were inoculated with 5 μl of 10^6 CFU of each isolate per mL with a multipoint inoculator. World Health Organization reference *N. gonorrhoeae* strains A, B, C, D, and E and *N. gonorrhoeae* ATCC 49226 (8) were included as quality controls. The plates were incubated for 24 h at 35°C in a 5% CO_2 atmosphere. β-Lactamase production was assayed by using the chromogenic cephalosporin test (nitrocefin; Oxoid, Basingstoke, United Kingdom). The preexisting fluoroquinolones tested were norfloxacin, ciprofloxacin, levofloxacin, and sparfloxacin, and the newly constructed fluoroquinolones tested were pazufloxacin, prulifloxacin, grepafloxacin, trovafloxacin, gatifloxacin, sitafloxacin, moxifloxacin, and HSR-903. The nonfluoroquinolone antimicrobial agents were penicillin G, imipenem, ceftriaxone, cefixime, tetracycline, azithromycin, and spectinomycin. Antimicrobial susceptibilities of isolates to ciprofloxacin, penicillin G, tetracycline, ceftriaxone, and spectinomycin were judged by the breakpoint criteria as defined by the National Committee for Clinical Laboratory Standards (NCCLS) (8).

Table 1 shows the antimicrobial susceptibilities of 85 *N. gonorrhoeae* isolates, classified by the presence of amino acid substitutions in GyrA and ParC, to various fluoroquinolones and other antibiotics. The MICs at which 50% of the isolates tested are inhibited (MIC_{50}) and MIC_{90} of the fluoroquinolones for the strains with both GyrA and ParC substitutions were much higher than those for the wild type and strains with GyrA substitutions alone. However, the MIC_{50} and MIC_{90} of sitafloxacin and HSR-903 for the strains with both GyrA and ParC substitutions were substantially lower than those of the other fluoroquinolones. All of the strains with GyrA substitutions alone and the wild type were susceptible to ciprofloxacin (MIC $\leq$ 0.06 μg/ml), while 14 (63.6%) of the 22 isolates with both GyrA and ParC substitutions were resistant to ciprofloxacin (MIC $\geq$ 1 μg/ml). The MIC_{90} of the β-lactams for the strains with GyrA substitutions alone were four- or eightfold

* Corresponding author. Mailing address: Department of Urology, Faculty of Medicine, Kyushu University, 3-1-1, Maidashi, Higashi-ku, Fukuoka 812-8582, Japan. Phone: 81-92-642-5603. Fax: 81-92-642-5618. E-mail: masatosh@uro.med.kyushu-u.ac.jp.

TABLE 1. Antimicrobial susceptibilities of *N. gonorrhoeae* isolates, classified by the presence of amino acid substitutions in GyrA and ParC, to fluoroquinolones and other antibiotics

Antibiotic	Type of substitution (no. of isolates)	MIC (μg/ml)[a]		
		Range	MIC_{50}	MIC_{90}
Sitafloxacin	Wild type (20)	≤0.001–0.004	0.002	0.004
	GyrA alone (43)	≤0.001–0.063	0.008 (4×)	0.031 (8×)
	GyrA and ParC (22)	0.008–0.25	0.063 (32×)	0.25 (64×)
HSR-903	Wild type (20)	≤0.001–0.004	0.002	0.004
	GyrA alone (43)	≤0.001–0.063	0.008 (4×)	0.031 (8×)
	GyrA and ParC (22)	0.008–0.5	0.063 (32×)	0.5 (128×)
Gatifloxacin	Wild type (20)	0.002–0.008	0.008	0.008
	GyrA alone (43)	0.008–0.5	0.031 (4×)	0.063 (8×)
	GyrA and ParC (22)	0.031–2	0.5 (64×)	2 (256×)
Grepafloxacin	Wild type (20)	≤0.001–0.063	0.004	0.008
	GyrA alone (43)	0.008–0.5	0.031 (8×)	0.125 (16×)
	GyrA and ParC (22)	0.063–4	0.25 (64×)	4 (512×)
Sparfloxacin	Wild type (20)	≤0.001–0.008	0.004	0.008
	GyrA alone (43)	0.008–0.5	0.063 (16×)	0.125 (16×)
	GyrA and ParC (22)	0.125–8	0.25 (64×)	4 (512×)
Moxifloxacin	Wild type (20)	0.002–0.063	0.008	0.008
	GyrA alone (43)	0.016–1	0.063 (8×)	0.125 (16×)
	GyrA and ParC (22)	0.063–4	0.5 (64×)	4 (512×)
Trovafloxacin	Wild type (20)	≤0.001–0.031	0.008	0.008
	GyrA alone (43)	0.004–0.5	0.063 (8×)	0.25 (32×)
	GyrA and ParC (22)	0.063–8	0.5 (64×)	8 (1,024×)
Prulifloxacin	Wild type (20)	0.002–0.031	0.008	0.008
	GyrA alone (43)	0.008–0.5	0.063 (8×)	0.5 (64×)
	GyrA and ParC (22)	0.125–8	0.5 (64×)	8 (1,024×)
Ciprofloxacin (MIC ≥ 1 μg/ml)[b]	Wild type (20)	0.004–0.016	0.008	0.008
	GyrA alone (43)	0.008–0.5	0.063 (8×)	0.5 (64×)
	GyrA and ParC (22)	0.125–16	2 (256×)	8 (1,024×)
Levofloxacin	Wild type (20)	0.008–0.016	0.008	0.016
	GyrA alone (43)	0.016–0.5	0.125 (16×)	0.5 (32×)
	GyrA and ParC (22)	0.25–16	2 (256×)	8 (512×)
Pazufloxacin	Wild type (20)	0.008–0.063	0.016	0.016
	GyrA alone (43)	0.031–1	0.125 (8×)	0.5 (32×)
	GyrA and ParC (22)	0.5–16	4 (256×)	16 (1,024×)
Norfloxacin	Wild type (20)	0.008–0.063	0.031	0.063
	GyrA alone (43)	0.063–4	0.5 (16×)	4 (64×)
	GyrA and ParC (22)	0.5–16	8 (256×)	16 (256×)
Penicillin G[c] (MIC ≥ 2 μg/ml)[b]	Wild type (19)	0.031–1	0.063	0.5
	GyrA alone (38)	0.016–2	0.25 (4×)	2 (4×)
	GyrA and ParC (22)	0.063–1	0.5 (8×)	1 (2×)
Imipenem	Wild type (20)	0.031–0.125	0.125	0.125
	GyrA alone (43)	0.031–0.5	0.25 (2×)	0.5 (4×)
	GyrA and ParC (22)	0.063–0.5	0.25 (2×)	0.5 (4×)
Ceftriaxone (MIC ≤ 0.25 μg/ml)[d]	Wild type (20)	0.002–0.031	0.004	0.016
	GyrA alone (43)	0.002–0.25	0.016 (4×)	0.125 (8×)
	GyrA and ParC (22)	0.008–0.125	0.063 (16×)	0.125 (8×)
Cefixime	Wild type (20)	0.002–0.031	0.008	0.016
	GyrA alone (43)	0.004–0.25	0.016 (2×)	0.125 (8×)
	GyrA and ParC (22)	0.004–0.125	0.063 (8×)	0.063 (4×)
Tetracycline (MIC ≥ 2 μg/ml)[b]	Wild type (20)	0.063–8	0.25	0.5
	GyrA alone (43)	0.063–2	0.5 (2×)	1 (2×)
	GyrA and ParC (22)	0.125–1	0.5 (2×)	1 (2×)
Azithromycin	Wild type (20)	0.016–0.5	0.125	0.5
	GyrA alone (43)	0.031–0.5	0.125 (1×)	0.5 (1×)
	GyrA and ParC (22)	0.016–0.25	0.063 (0.5×)	0.25 (0.5×)
Spectinomycin (MIC ≥ 128 μg/ml)[b]	Wild type (20)	4–16	8	8
	GyrA alone (43)	4–16	8 (1×)	8 (1×)
	GyrA and ParC (22)	4–16	8 (1×)	8 (1×)

[a] Numbers in parentheses indicate fold change compared with wild type.
[b] NCCLS criteria for resistance (8).
[c] Only non-PPNG strains are shown.
[d] NCCLS criteria for sensitivity (8).

TABLE 2. Relationship between amino acid substitutions in GyrA and ParC and antimicrobial susceptibilities of *N. gonorrhoeae* to various fluoroquinolones and other antibiotics

Amino acid substitution(s)		No. of isolates	Mean MIC (μg/ml) of antibiotics[a]:						
GyrA	ParC		SIFX	HSR-903	GFLX	GPFX	SPFX	MOFX	TVFX
Wild type	Wild type	20	0.002	0.002	0.006	0.007	0.005	0.01	0.009
A67S	Wild type	1	0.004	0.004	0.016	0.008	0.016	0.016	0.016
A75S	Wild type	5	0.009	0.009	0.019	0.016	0.016	0.028	0.044
A84P	Wild type	1	0.004	0.001	0.016	0.008	0.016	0.031	0.016
S91F	Wild type	21	0.025 (13×)	0.021 (11×)	0.052 (9×)	0.093 (13×)	0.09 (18×)	0.088 (9×)	0.14 (16×)
S91C	Wild type	1	0.001	0.002	0.008	0.008	0.008	0.016	0.016
D95G	Wild type	8	0.008	0.009	0.035	0.033	0.059	0.063	0.055
D95N	Wild type	5	0.009	0.007	0.031	0.025	0.047	0.057	0.041
S91F-D95G	Wild type	1	0.063	0.031	0.5	0.5	0.5	1	0.5
S91Y	R116H	1	0.016	0.016	0.063	0.063	0.125	0.125	0.125
S91F	A92G	1	0.031	0.031	0.063	0.25	0.125	0.125	0.5
S91F	A86N	4	0.02	0.014	0.071	0.19	0.19	0.125	0.75
S91F	S88P-E91G	1	0.008	0.016	0.063	0.25	0.25	0.063	0.5
S91F-D95N	E91G	1	0.13	0.063	0.5	2	1	1	0.5
S91F-D95N	S88P	9	0.089	0.072	0.6	0.72	0.69	1.2	0.99
S91F-D95N	S87I-S88P	1	0.25	0.5	1	8	4	4	8
S91F-D95N	S88P-E91K	2	0.25	0.5	2	6	6	4	6
S91F-D95N	S88P-E91G	1	0.125	0.25	1	2	4	2	1
S91F-D95N	S88P-E91Q	1	0.125	0.063	1	0.5	1	0.5	0.125

[a] SIFX, sitafloxacin; GFLX, gatifloxacin; GPFX, grepafloxacin; SPFX, sparfloxacin; MOFX, moxifloxacin; TVFX, trovafloxacin; PUFX, prulifloxacin; CPFX, ciprofloxacin; LVFX, levofloxacin; PZFX, pazufloxacin; NFLX, norfloxacin; PCG, penicillin G; IPM, imipenem; CTRX, ceftriaxone; CFIX, cefixime; TC, tetracycline; AZM, azithromycin; SPCM, spectinomycin. Numbers in parentheses indicate fold change compared with wild type, and for nonfluoroquinolones numbers are shown when threefold or more. NS, data not shown because all strains were PPNG.

higher than those for the wild type. The MIC_{90} of the β-lactams for strains with both GyrA and ParC substitutions were almost identical to those for the strains with GyrA substitutions alone. In general, however, the isolates with both GyrA and ParC substitutions were less susceptible to β-lactams at the MIC_{50} than the strains with GyrA mutations alone. Five (5.9%) isolates were penicillinase-producing *N. gonorrhoeae* (PPNG). Seven (8.2%) and three (3.5%) isolates had chromosomally mediated resistance to penicillin (MIC ≥ 2 μg/ml) and tetracycline (MIC ≥ 2 μg/ml), respectively. However, all of the isolates were susceptible to ceftriaxone (MIC ≤ 0.25 μg/ml) and spectinomycin (MIC ≤ 32 μg/ml).

We then investigated the relationship between antimicrobial susceptibility to fluoroquinolones and amino acid substitutions in GyrA and ParC in the gonococcal isolates. Among the strains with the various single GyrA substitutions, those containing a serine (Ser)-to-phenylalanine (Phe) mutation at position 91 (Ser-91 in *N. gonorrhoeae* GyrA corresponds to Ser-83 in *Escherichia coli* [1]) required the highest MICs of all of the fluoroquinolones (Table 2). The strains containing the Ser-91-to-Phe mutation in GyrA also exhibited resistance to structurally unrelated β-lactams (Table 2). All of the seven strains with chromosomally mediated resistance to penicillin contained the Ser-91-to-Phe mutation in GyrA. Five strains with the alanine-75-to-Ser mutation in GyrA required threefold-greater MICs of the cephems and azithromycin than those required by the wild type (Table 2). These five were all PPNG strains and may have originated in the same clone.

Among the various fluoroquinolones tested, the newly developed sitafloxacin and HSR-903 were more potent than other fluoroquinolones against not only isolates with GyrA alterations alone but also the strains with both GyrA and ParC alterations. The MIC_{50} and MIC_{90} of sitafloxacin against the strains containing both GyrA and ParC substitutions were 0.063 and 0.25 μg/ml, respectively, while those of HSR-903 were 0.063 and 0.5 μg/ml, respectively. Pharmacokinetic studies of these two fluoroquinolones have demonstrated that, after administration of a single 200-mg dose of sitafloxacin and HSR-903 to healthy volunteers, their serum drug concentrations peak at 1.86 and 0.86 μg/ml, respectively (reference 7 and K. Uemura, K. Mizuno, and M. Nakashima, Abstr. 36th Intersci. Conf. Antimicrob. Agents Chemother., abstr. F60, p. 100, 1996). These data corroborate that a single 200-mg-or-more dose of sitafloxacin or HSR-903 is likely to show excellent clinical efficacy against gonorrhea caused by fluoroquinolone-resistant *N. gonorrhoeae* carrying both GyrA and ParC substitutions. The increment in MIC of norfloxacin associated with the Ser-91-to-Phe mutation in GyrA is higher than is usually seen and suggests the possibility that other unidentified additional mutations such as efflux-type mutations (5) or porin gene mutations (4) contribute to resistance in these strains.

Interestingly, cross-resistance between the fluoroquinolones and the structurally unrelated β-lactams was observed in the gonococcal isolates. Other investigations have also reported such cross-resistance (2, 3). Among strains with the various substitutions, strains containing the Ser-91-to-Phe mutation showed significant resistance to penicillin G, imipenem, and cephems. The antimicrobial susceptibilities of isolates with other substitutions in GyrA alone to the antibiotics were comparable to those of the wild type. The susceptibilities of the strains with multiple substitutions to β-lactams were lower than those of the wild type but comparable to those of the isolates with the single Ser-91-to-Phe alteration. These results indicate that the Ser-91-to-Phe mutation may be important in cross-resistance to other structurally unrelated agents. We were unable to explain why strains containing the Ser-91-to-Phe mutation show cross-resistance to structurally unrelated β-lactams. Some of the strains with the Ser-91-to-Phe alteration in GyrA may have had efflux-type mutations (5) or porin gene mutations (4) that affect responses to quinolones and other drugs. Further study is necessary to investigate cross-resistance between fluoroquinolones and β-lactams.

TABLE 2—*Continued*

Mean MIC (μg/ml) of antibiotics[a]											
PUFX	CPFX	LVFX	PZFX	NFLX	PCG	IPM	CTRX	CFIX	TC	AZM	SPCM
0.008	0.007	0.012	0.016	0.028	0.23	0.11	0.008	0.01	0.69	0.17	7.6
0.008	0.016	0.031	0.063	0.125	0.016	0.063	0.002	0.008	0.063	0.063	8
0.025	0.031	0.069	0.069	0.25	NS	0.25	0.061 (8×)	0.031 (3×)	1.3	0.5 (3×)	7.2
0.016	0.016	0.031	0.063	0.125	0.063	0.063	0.004	0.004	0.063	0.125	8
0.22 (28×)	0.24 (34×)	0.34 (28×)	0.48 (30×)	1.6 (57×)	1.1 (5×)	0.41 (4×)	0.092 (12×)	0.074 (7×)	0.71	0.19	7.8
0.016	0.008	0.016	0.031	0.063	0.031	0.031	0.004	0.004	1	0.063	8
0.051	0.029	0.071	0.071	0.2	0.067	0.059	0.012	0.007	0.14	0.086	11
0.063	0.052	0.088	0.11	0.35	0.088	0.13	0.007	0.009	0.25	0.1	7.2
0.25	0.5	0.5	1	2	0.125	0.063	0.008	0.008	0.5	0.25	4
0.125	0.125	0.25	0.5	0.5	0.25	0.063	0.063 (8×)	0.008	0.25	0.063	8
0.25	0.25	0.5	1	2	1 (4×)	0.5 (5×)	0.063 (8×)	0.063 (6×)	1	0.25	8
0.5	0.22	0.38	1	1.8	0.75 (3×)	0.19	0.079 (10×)	0.055 (6×)	0.88	0.063	8
0.25	0.25	0.25	0.5	1	0.5	0.25	0.063 (8×)	0.063 (6×)	0.5	0.125	8
0.5	2	2	2	4	0.25	0.25	0.016	0.016	0.5	0.25	8
1.4	1.8	3.9	6	9.8	0.78 (3×)	0.28 (3×)	0.056 (7×)	0.063 (6×)	0.64	0.11	9.3
8	16	8	16	16	0.5	0.25	0.063 (8×)	0.063 (6×)	0.5	0.125	8
8	12	8	16	16	1 (4×)	0.19	0.094 (12×)	0.063 (6×)	0.31	0.063	8
4	4	16	16	16	0.5	0.25	0.031 (4×)	0.031 (3×)	0.5	0.063	8
2	2	4	8	8	0.063	0.125	0.008	0.031 (3×)	0.5	0.063	8

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