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Reduced Clinical Efficacy of Pazufloxacin against Gonorrhea Due to High Prevalence of Quinolone-Resistant Isolates with the GyrA Mutation

MASATOSHI TANAKA,[1]* TETSURO MATSUMOTO,[1] MISAO SAKUMOTO,[1] KOICHI TAKAHASHI,[1] TAKESHI SAIKA,[2] INTETSU KABAYASHI,[2] JOICHI KUMAZAWA,[1] AND THE PAZUFLOXACIN STD GROUP

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

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Forty-two men with gonococcal urethritis were treated with an oral dosage of 200 mg of pazufloxacin, a new fluoroquinolone, three times daily for 3 days. Only 28 of the 42 men (66.7%) had negative culture results for *Neisseria gonorrhoeae* during follow-up. Of the 42 isolates, 41 could be recultured for antibiotic susceptibility testing and DNA sequencing. In 26 of the 41 isolates (63.4%), GyrA mutations with or without ParC mutations were identified. Among the 26 isolates, 23 contained a single GyrA mutation, 1 contained two GyrA mutations, and 2 contained three mutations including double GyrA and single ParC mutations. A single Ser-91-to-Phe mutation, which was detected in 14 of the 26 isolates, was the most common GyrA mutation, followed by an Ala-75 to Ser mutation and an Asp-95 to Asn or Gly mutation in GyrA. All three isolates with two or three mutations contained the Ser-91-to-Phe GyrA mutation. Eleven of the 14 isolates with the single Ser-91-to-Phe mutation within GyrA and all 3 isolates with two or three mutations persisted after pazufloxacin treatment. On the other hand, all 15 wild-type and 9 mutant isolates with a substitution at codon Ala-75 or Asp-95 were eradicated. The mean MIC of pazufloxacin for mutants with the single Ser-91-to-Phe mutation in GyrA was 66-fold higher than that for the wild type. The results obtained in this study suggest that a high prevalence of fluoroquinolone-resistant gonococcal isolates with the Ser-91-to-Phe mutation in GyrA reduced the efficacy of pazufloxacin as treatment for gonococcal urethritis.

The increasing frequency of occurrence of *Neisseria gonorrhoeae* isolates with plasmid- and chromosome-mediated resistance to penicillin or tetracycline is a serious problem worldwide. Fluoroquinolones such as norfloxacin, ofloxacin, and ciprofloxacin demonstrate excellent in vitro activities against *N. gonorrhoeae*, including penicillin- and tetracycline-resistant strains, and are highly effective for the oral treatment of gonococcal infections caused not only by strains sensitive to penicillin or tetracycline but also by strains resistant to these antibiotics (4, 6, 20). Therefore, over the past decade, fluoroquinolone regimens have increasingly been used in various countries for the treatment of gonococcal infections. Although the emergence of isolates of *N. gonorrhoeae* showing reduced susceptibility to fluoroquinolones has recently been reported in several countries including Japan (3, 5, 7, 9–12, 22, 23), high failure rates for fluoroquinolone treatment of gonococcal urethritis have not yet been reported.

Pazufloxacin (T-3761) is a new orally administered fluoroquinolone which has excellent in vitro activity against β-lactamase-producing and -nonproducing clinical isolates of *N. gonorrhoeae*, with an MIC at which 50% of isolates are inhibited (MIC_{50}) of <0.006 μg/ml and an MIC_{90} of 0.1 μg/ml (16). A single oral dose of 200 mg of pazufloxacin results in a mean peak level in plasma of 2.0 μg/ml in 1.2 h, with the half-life averaging 1.9 h (17). These data suggest that pazufloxacin might be effective for the treatment of gonococcal urethritis in men. An open clinical trial was undertaken to assess the efficacy and safety of pazufloxacin in men with acute urethritis caused by *N. gonorrhoeae*. However, a high failure rate was obtained for the treatment of gonococcal urethritis with pazufloxacin. Thus, we examined the prevalence of clinically fluoroquinolone-resistant *N. gonorrhoeae* isolates with mutations in the GyrA protein with or without mutations in the ParC protein, both of which confer quinolone resistance (2, 24), and investigated the relationship between the clinical efficacy of pazufloxacin and mutations within the GyrA and ParC proteins of these bacteria.

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

MATERIALS AND METHODS

Study population and design. Forty-seven men who presented with urethral discharge and whose urethral discharge showed gram-negative intracellular diplococci upon Gram staining and five or more polymorphonuclear leukocytes per high-power field were enrolled in this study. The men ranged in age from 19 to 47 years (mean age, 30.3 years). The diagnosis of gonococcal urethritis was confirmed by recovery of *N. gonorrhoeae* by culture. All participants provided oral or written informed consent before they were enrolled in the study.

Patients were treated with an oral dosage of 200 mg pazufloxacin three times daily for 3 days and were asked to return for reexamination on days 4 and 7 after the initiation of treatment. At each visit, patients were examined, urethral smears were collected for microscopy and the detection of *N. gonorrhoeae*, and first-void urine specimens were also obtained for the detection of the *Chlamydia trachomatis* antigen.

Microbiological examination. Two urethral specimens for the detection of *N. gonorrhoeae* and a first-void urine sample for the detection of the *C. trachomatis* antigen were collected from each patient. The detection of *N. gonorrhoeae* was performed by recovery by culture and a DNA probe test (Gen Probe). A commercial kit (IDEIA; Dako, Cambrigeshire, United Kingdom) was used for the *C. trachomatis* antigen assay. Specimens for culture of *N. gonorrhoeae* were immediately inoculated onto Thayer-Martin agar (Beckton Dickinson). The culture plates were incubated at 35°C for 24 to 48 h in a 5% CO_2 atmosphere. *N. gonorrhoeae* was identified by colony morphology, oxidase reaction, and appearance upon Gram staining and was confirmed by sugar utilization reactions. β-Lactamase production was assayed by the chromogenic cephalosporin technique (15, 18).

TABLE 1. Relationship between MIC and bacteriological response to pazufloxacin treatment

Pazufloxacin MIC (μg/ml)	No. of strains eradicated/no. of strains isolated (%)
0.006	2/2 (100)
0.013	12/12 (100)
0.025	1/1 (100)
0.05	3/3 (100)
0.1	5/5 (100)
0.2	2/3 (66.7)
0.39	1/5 (20)
0.78	1/3 (33.3)
1.56	0/4 (0)
3.13	0/2 (0)
6.25	0/0
12.5	0/1 (0)
ND[a]	1/1 (100)
Total	28/42 (66.7)

[a] ND, not determined.

N. gonorrhoeae isolates were stored at −70°C until antibiotic susceptibility testing. The MICs for all isolates were determined by an agar dilution technique with a GC agar base containing 1% IsoVitaleX (Beckton Dickinson) and twofold dilutions of the antibiotic (8). The plates were inoculated with 5 μl of 10^6 CFU of each isolate per ml with a multipoint inoculator. The plates were incubated at 35°C for 24 h in a 5% CO_2 atmosphere. The MICs were defined as the lowest antibiotic concentration that inhibited bacterial growth. Three fluoroquinolones were tested: pazufloxacin (Toyama Chemical Company, Tokyo, Japan), norfloxacin (Kyorin, Tokyo, Japan), and ciprofloxacin (Bayer, Osaka, Japan).

Molecular biology-based study. PCR and direct DNA sequencing were performed to identify mutations in the *gyrA* and *parC* genes of the gonococcal strains isolated before and after treatment. Chromosomal DNA was extracted by standard methods (1) and was subjected to PCR. The oligonucleotide primers for the PCR amplification were as follows: for the *gyrA* gene the forward primer was 5′-CGGCGCGTACTGTACGCGTTGAC-3′ and the reverse primer was 5′-AA TGTCTGCCAGCATTTCATGTGAGA-3′, and for the *parC* gene the forward primer was 5′-ATGCGCGATATGGGTTTGAC and the reverse primer was 5′-GGACAACAGCAATTCCGCAA. These primers were produced with a DNA synthesizer and were based on the sequences previously reported by Belland et al. (2). The *gyrA* gene sequence was determined from nucleotides 160 to 439, which correspond to amino acids 54 to 147 of the GyrA protein. This includes the quinolone resistance-determining region (amino acids 55 to 110 of the gonococcal GyrA protein) (2). The *parC* gene sequence was also determined from nucleotides 166 to 420, which correspond to amino acids 56 to 140 of the gonococcal ParC protein. This includes the quinolone resistance-determining region of the ParC protein (amino acids 66 to 119 of the gonococcal ParC protein) (2).

PCR amplification was performed with 25 μl of a reaction mixture which contained 2.5 μl of 10× *Taq* polymerase buffer (500 mM KCl, 100 mM Tris-HCl [pH 8.3], 15 mM $MgCl_2$, 0.1% gelatin), 0.25 μl of each of the two primers (25 pmol/μl), 0.5 μl of each of the four deoxynucleotide triphosphates (10 mM), 0.2 μl of *Taq* DNA polymerase (5 U/μl; Takara, Shiga, Japan), 2.5 μl of Triton X-100 (2 mg/ml), and 1.0 μl of template DNA (100 ng/μl). Thirty-five cycles were performed for each reaction. Each cycle consisted of 30 s at 93°C, 1 min at 52°C, and 1 min at 72°C.

The PCR amplification products were directly sequenced by the dideoxy-chain termination method (13) with the Taq DyeDeoxy Terminator Cycle Sequencing Kit and a model 373A autosequencer (ABI).

RESULTS

Clinical results. Of the 47 men enrolled in the study and treated with pazufloxacin, 5 could not be evaluated due to protocol violation. Among the 42 patients evaluated, only 1 had been treated with another fluoroquinolone before this study. The remaining 41 patients were not treated with any antimicrobial agent before pazufloxacin treatment. Seven were infected with penicillinase-producing *N. gonorrhoeae* strains. *N. gonorrhoeae* was not detected again in only 28 of the 42 patients (66.7%) during follow-up. Thus, the response rate to pazufloxacin treatment of gonococcal urethritis was very low. Table 1 presents the relationship between the MICs of pazufloxacin for *N. gonorrhoeae* isolates and the bacteriological responses. Forty-one isolates could be recultured for antibiotic susceptibility testing. All 23 isolates for which the MICs of pazufloxacin were 0.1 μg/ml or less were eradicated by treatment. Of the 11 isolates for which the MICs of pazufloxacin ranged from 0.2 to 0.78 μg/ml, only 4 were eradicated. All seven isolates for which the pazufloxacin MICs exceeded 1.56 μg/ml were persistent. The MIC of pazufloxacin for an isolate from the patient treated with another fluoroquinolone before this study was 1.56 μg/ml. These results indicate that the gonococcal isolates for which the MICs of pazufloxacin were higher than 0.2 μg/ml are possibly clinically resistant to pazufloxacin treatment. *C. trachomatis* was also present in 8 of the 42 patients (19.0%). The *C. trachomatis* antigen was not detected again in five of these eight patients during follow-up. One patient from whom *C. trachomatis* was eradicated had been given 200 mg of pazufloxacin three times daily for 7 days.

The treatment was well tolerated. No side effects related to the treatment were observed in these patients.

Prevalences of the *gyrA* and *parC* gene mutations. Table 2 lists the mutations within the *gyrA* and *parC* genes of 41 gonococcal strains isolated before treatment. In 26 of the 41 isolates (63.4%), GyrA mutations with or without ParC mutations were identified. Among these 26 isolates, 23 (56.1%) contained a single GyrA mutation, 1 (2.4%) contained two GyrA mutations, and 2 (4.9%) contained three mutations including double GyrA and single ParC mutations. A Ser-91-to-Phe mutation was the most common GyrA mutation, followed by an Ala-75-to-Ser mutation and an Asp-95-to-Asn or -Gly mutation in GyrA. The detected mutations within ParC were a Ser-88-to-Pro mutation and a Glu-91-to-Gly mutation. One type of silent mutation was frequently detected: the DNA sequence was substituted at codon 131, but the ParC protein sequence was unchanged.

Of the 41 strains, 18 were isolated in Tokyo and the remaining 23 were isolated in Fukuoka city, located in the northern part of Kyushu Island, which is far from Tokyo. There was no significant difference in the incidence of isolates with GyrA

TABLE 2. Mutations in the *gyrA* and *parC* genes in *N. gonorrhoeae* strains isolated before treatment

gyrA			*parC*			No. (%) of strains
Codon(s)	Nucleotide mutation	Amino acid mutation	Codon	Nucleotide mutation	Amino acid mutation	
75	GCG→TCG	Ala→Ser	131	CTC→CTG	Leu (silent)	5 (12.2)
91	TCC→TTC	Ser→Phe	131	CTC→CTG	Leu (silent)	14 (34.1)
95	GAC→AAC	Asp→Asn	131	None or CTC→CTG	None or Leu (silent)	2 (4.9)
95	GAC→GGC	Asp→Gly	131	None or CTC→CTG	None or Leu (silent)	2 (4.9)
91 and 95	TCC→TTC, GAC→GGC	Ser→Phe, Asp→Gly		None	None	1 (2.4)
91 and 95	TCC→TTC, GAC→GGC	Ser→Phe, Asp→Gly	91	GAG→GGG	Glu→Gly	1 (2.4)
91 and 95	TCC→TTC, GAC→AAC	Ser→Phe, Asp→Asn	88	TCC→CCC	Ser→Pro	1 (2.4)
	None	None	131	None or CTC→CTG	None or Leu (silent)	15 (36.6)

TABLE 3. Relationship between mutations in GyrA and ParC and bacteriological response

Mutation		Total no. of strains	Bacteriological response (no. [%] of isolates)	
GyrA	ParC		Eradicated	Persistent
Wild type	Wild type	15	15 (100)	0
Ala75Ser	Wild type	5	5 (100)	0
Ser91Phe	Wild type	14	3 (21.4)	11 (78.6)
Asp95Asn	Wild type	2	2 (100)	0
Asp95Gly	Wild type	2	2 (100)	0
Ser91Phe and Asp95Gly	Wild type	1	0	1 (100)
Ser91Phe and Asp95Gly	Glu91Gly	1	0	1 (100)
Ser91Phe and Asp95Asn	Ser88Pro	1	0	1 (100)

mutations between Fukuoka (69.6%) and Tokyo (61.1%). It seems that fluoroquinolone-resistant gonococci carrying GyrA mutations are widespread in Japan. We did not know whether the isolates carrying the Ser-91-to-Phe GyrA mutation or the double mutations were clonal.

We also analyzed the DNA sequences of isolates not eradicated by pazufloxacin treatment. All 14 strains which were isolated after treatment with pazufloxacin had GyrA mutations with or without ParC mutations. However, there were no differences in the GyrA and ParC mutation patterns or in susceptibility to pazufloxacin between pretherapy and posttherapy isolates from the same patient.

Bacteriological responses of mutants to pazufloxacin. The relationships between mutations in GyrA and ParC and the bacteriological responses to pazufloxacin are presented in Table 3. All 15 wild-type isolates, 5 containing the Ala-75-to-Ser mutation in GyrA and 4 containing the Asp-95-to-Asn or -Gly mutation in GyrA, were eradicated by pazufloxacin treatment. On the other hand, of the 14 isolates containing the single Ser-91-to-Phe mutation, only 3 (21.4%) were eradicated. Moreover, all three isolates with the double mutations in GyrA or the triple mutations involving both GyrA and ParC were persistent. These three mutants contained the Ser-91-to-Phe mutation in GyrA. These results indicate that although the mutants with the Ala-75-to-Ser, Asp-95-to-Asn, or Asp-95-to-Gly substitution in GyrA were still clinically susceptible to pazufloxacin treatment, the mutants containing the Ser-91-to-Phe substitution in GyrA with or without another substitution were clinically resistant to this treatment.

Susceptibilities of the mutants to fluoroquinolones. Table 4 describes relationships between mutations in GyrA and ParC and the pazufloxacin MICs for *N. gonorrhoeae*. The mean MICs of pazufloxacin for the mutants with the Ala-75-to-Ser, Ser-91-to-Phe, Asp-95-to-Asn, and Asp-95-to-Gly substitutions in GyrA were 0.08, 0.86, 0.2, and 0.075 μg/ml, respectively, while that for the wild-type isolate was 0.013 μg/ml. This result indicates that gonococcal isolates which acquired the single Ser-91-to-Phe mutation are more resistant to pazufloxacin than the other mutants with a single GyrA substitution. The mutants with double GyrA substitutions or triple substitutions involving GyrA and ParC were extremely resistant to pazufloxacin compared to the level of resistance of the wild type. These mutants were cross-resistant to other fluoroquinolones: norfloxacin and ciprofloxacin.

DISCUSSION

Until recently, fluoroquinolones have been shown to have excellent antimicrobial activities against *N. gonorrhoeae* isolates. Thus, fluoroquinolone regimens have increasingly been used in Japan. The emergence of gonococcal isolates showing reduced susceptibility to fluoroquinolones in vitro has recently been reported in Japan (22, 23). However, the widespread existence of gonococcal isolates clinically resistant to treatment with fluoroquinolones has not yet been recognized.

In this study, we evaluated the clinical efficacy of pazufloxacin, a new fluoroquinolone, for the treatment of gonococcal urethritis. This treatment eradicated *N. gonorrhoeae* isolates from only 66.7% of patients with gonococcal urethritis. This response rate would appear to be very low, because until recently the effectiveness rates of various fluoroquinolones for the treatment of uncomplicated gonorrhea were approximately 100% (4, 6, 14, 19–21). To determine the cause of this high rate of failure of pazufloxacin treatment, we investigated the frequency of occurrence of isolates with mutations in GyrA and ParC proteins which confer fluoroquinolone resistance on these bacteria. Surprisingly, in 26 (63.4%) of the 41 isolates tested, GyrA mutations with or without ParC mutations were identified. The identified mutations in GyrA were Ala-75-to-Ser, Ser-91-to-Phe, Asp-95-to-Asn, and Asp-95-to-Gly substitutions. Of these mutations, the Ser-91-to-Phe mutation was the most common GyrA mutation, and it was detected in 17 (65.4%) of the 26 isolates with GyrA substitutions. Among these 17 isolates, 14 contained the single Ser-91-to-Phe GyrA mutation, 1 contained two GyrA mutations, and 2 contained three mutations including double GyrA and single ParC mutations. The eradication rate for the isolates with the Ser-91-to-Phe mutation was only 17.6% (3 of 17 isolates), while that for wild-type isolates or isolates containing the other GyrA mutations was 100% (24 of 24 isolates). These results indicate that the Ser-91-to-Phe mutation in GyrA is a critical mutation which confers on *N. gonorrhoeae* clinical resistance to fluoroquinolones. Antibiotic susceptibility tests of these mutants corroborated the clinical significance of the Ser-91-to-Phe mutation in GyrA. The mean MICs of pazufloxacin for the mutants with the Ser-91-to-Phe substitution were increased 66-fold compared to that for the wild type. However, the level of reduction of the susceptibility of the mutants with substitutions at codon Ala-75 or Asp-95 was 6- or 15-fold less, respectively, than that of the wild type.

The ParC mutation was identified in only 2 of the 41 isolates. The double GyrA mutations coexisted in the two isolates with the ParC mutation. No isolates contained the ParC mutation without the simultaneous presence of at least one GyrA mutation. The mutants with both GyrA and ParC mutations were extremely resistant to fluoroquinolones. The ParC mutation

TABLE 4. Relationship between mutations in GyrA and ParC and the pazufloxacin MIC for *N. gonorrhoeae*

Mutation		No. of strains	Mean MIC (μg/ml)		
GyrA	ParC		Pazufloxacin	Norfloxacin	Ciprofloxacin
Wild type	Wild type	15	0.013	0.032	0.0069
Ala75Ser	Wild type	5	0.08 (6×)[a]	0.14 (4×)	0.019 (3×)
Ser91Phe	Wild type	14	0.86 (66×)	1.72 (54×)	0.28 (41×)
Asp95Asn	Wild type	2	0.2 (15×)	0.59 (18×)	0.075 (11×)
Asp95Gly	Wild type	2	0.075 (6×)	0.3 (9×)	0.038 (6×)
Ser91Phe and Asp95Gly	Wild type	1	3.13 (240×)	3.13 (98×)	0.39 (57×)
Ser91Phe and Asp95Gly	Glu91Gly	1	3.13 (240×)	12.5 (390×)	3.13 (454×)
Ser91Phe and Asp95Asn	Ser88Pro	1	12.5 (961×)	12.5 (390×)	3.13 (454×)

[a] The values in parentheses refer to the increase in the MIC compared with the MICs for the wild-type isolates.

may be essential for the bacteria to acquire high-grade fluoroquinolone resistance.

In this study we were not able to compare pazufloxacin therapy with ciprofloxacin therapy, because this study was undertaken as an open clinical trial. Treatment failure with ciprofloxacin has been reported for isolates for which MICs are $\geq$0.05 or 0.06 μg/ml (7, 9). Therefore, the isolates containing a single Ser-91-to-Phe GyrA mutation, double GyrA mutations, or triple mutations within GyrA and ParC may be refractory to ciprofloxacin treatment, because the ciprofloxacin MICs for these mutants ranged from 0.1 to 3.13 μg/ml.

To our knowledge, this is the first study to examine the relationship between the clinical efficacies of fluoroquinolones against gonorrhea and mutations involving the GyrA and ParC proteins of these bacteria. The high prevalence of fluoroquinolone-resistant *N. gonorrhoeae* isolates with the GyrA mutation limits the clinical efficacy of pazufloxacin against gonococcal infections in Japan.

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