Mutation in DNA gyrase of norfloxacin-resistant clinical isolates of *Neisseria gonorrhoeae*

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Background and Objectives: Recently a rapid decrease in the susceptibility of *Neisseria gonorrhoeae* isolates to fluoroquinolones has occurred and gonococcal fluoroquinolone resistance is now a significant problem in the treatment of gonorrhoea in Japan. Thus, in order to investigate the quinolone resistance mechanisms in clinical isolates of *N gonorrhoeae* we studied an alteration in the DNA gyrase subunit A (GyrA) which is well-known as a common mechanism of bacterial quinolone resistance.
Materials and methods: Four clinical isolates of *N gonorrhoeae* resistant to norfloxacin and 5 strains susceptible to norfloxacin, including 2 clinical isolates and 3 WHO reference strains, were tested in this study. To identify mutations in the GyrA genes of gonococcal strains, polymerase chain reaction and direct DNA sequencing were performed.
Results: A single base change (serine codon TCC changed to phenylalanine codon TTC), which resulted in an amino acid change in GyrA at position 91, was identified in all 4 norfloxacin-resistant strains for which the MICs of norfloxacin ranged from 1·0 to 8·0 μg/ml, while no mutation within GyrA was detected in 5 norfloxacin-susceptible strains for which the MICs of norfloxacin ranged from 0·004 to 0·063 μg/ml.
Conclusions: The results from this study suggest that the serine-91 to phenylalanine substitution in GyrA is probably an essential mutation in fluoroquinolone resistance in clinical isolates of *N gonorrhoeae*.
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Keywords: *Neisseria gonorrhoeae*; quinolone resistance; mutation; DNA gyrase

Introduction

Fluoroquinolones such as norfloxacin, ofloxacin, and ciprofloxacin exert excellent in vitro antimicrobial activity against *Neisseria gonorrhoeae*, including penicillin- and tetracycline-resistant strains. Fluoroquinolones have proven to be highly effective as oral single-dose treatment for gonococcal infections. Thus, in the past decade, fluoroquinolone regimens have been used increasingly in developed countries for therapy of gonorrhoea, as well as genitourinary tract infections and other bacterial infections. However, the emergence of gonococcal isolates showing reduced susceptibility to fluoroquinolones has recently been reported in several countries.[1–7] In Japan, gonococcal fluoroquinolone resistance is now a significant problem in the treatment of gonorrhea.[8 9] It is therefore important to study quinolone resistance mechanisms in clinical isolates of *N gonorrhoeae*.

In several bacterial species such as *Escherichia coli* and *Staphylococcus aureus*, two main mechanisms by which the bacteria acquire resistance to fluoroquinolones have been proposed. First, mutations in DNA gyrase subunit A (GyrA) and subunit B (GyrB) genes of the target DNA gyrase proteins lead to lower inhibitory activities of fluoroquinolones against DNA gyrase. In *E coli*, mutation at serine-83 in the GyrA is most commonly found in quinolone-resistant strains.[10 11] Second, an alteration in drug permeation is also known to be a role of resistance to quinolones. In *E coli*, this phenomenon has been associated with mutations leading to decreasing amounts of the outer membrane protein OmpF.[12–14] Furthermore, an active efflux system has recently been reported as the quinolone resistance mechanism in gram-negative bacteria.[15]

However, less is known about quinolone resistance mechanisms in gonococci.[16 17] Our previous investigation[16] demonstrated that an alteration in drug permeability was associated with quinolone resistance in 2 of 4 norfloxacin-resistant isolates, while the remaining 2 isolates perhaps possess other quinolone mechanisms such as mutations in the target GyrA, because the 2 isolates accumulated norfloxacin as well as norfloxacin-susceptible strains. Thus, in this study, we analysed mutations within the GyrA gene in clinical isolates of *N gonorrhoeae* showing in vitro resistance to norfloxacin.

Materials and methods

Bacterial strains

Six clinical strains of *N gonorrhoeae* isolated from men with acute urethritis between February and July 1992 in Japan and three WHO *N gonorrhoeae* reference strains (kindly supplied by JW Tapsall, Department of Microbiology, The Prince of Wales Hospital, Randwick, Australia) were used in this study. None of the six clinical strains were posttreatment isolates or repeat isolates from the same patients. All the clinical isolates tested were epidemiologically unrelated. *N gonorrhoea* was identified as gram-negative diplococci and by oxidase reaction and sugar utilisation patterns.

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Antibiotic susceptibility testing

The minimum inhibitory concentration (MIC) for the 9 strains was determined using an agar dilution technique with a GC agar base (Becton Dickinson, USA) containing 1% Iso VitaleX (Becton Dickinson) and 2-fold dilutions of antibiotic. Plates were inoculated with 5 μl of 10^6 colony-forming units (cfu)/ml of each isolate by a multipoint inoculator. The plates were incubated for 24 hours at 35°C in 5% CO_2 atmosphere. MICs were read as the lowest concentration of antibiotic that inhibited bacterial growth. The antibiotics tested were norfloxacin (Kyorin Pharmaceutical Co Ltd, Japan), ofloxacin (Daiichi Pharmaceutical Co Ltd, Japan), ciprofloxacin (Bayer Yakuhin Ltd, Japan), penicillin G (Meiji Seika, Japan), ceftriaxone (Hoffman-LaRoche Inc, USA), tetracycline (Sigma, USA), and spectinomycin (Upjohn Co, Canada). All of the antibiotics were obtained as powders of stated potency from their manufacturers. β-lactamase production was tested by an acidometric assay (β-check; Pfizer Pharmaceuticals Inc, Japan).

Extraction of DNA and synthesis of oligonucleotides

Chromosomal DNA was extracted by the rapid boiling method described previously[18] and subjected to polymerase chain reaction (PCR). To amplify a DNA fragment of the *GyrA* gene corresponding to amino acids positions 53 to 179, which includes the region of *N gonorrhoeae* GyrA termed "the quinolone resistance-determining region" corresponding to amino acids positions 55 to 110 of gonococcal GyrA,[17] two oligonucleotides (NGGYRA F1, 5′-TGCACCGGCGCGTACTGTAC-3′; NGGYRA R1, 5′-ACGAGCCGTTGACGAGCAGT-3′) which were purchased from Takara Shuzo Co Ltd Biochemical Group (Shiga, Japan) were used. These primers were located within consensus amino acids of the bacterial GyrA proteins, and yielded a product of 384 bp DNA fragment after PCR amplification.

PCR

PCR amplification was performed in 20 μl of a reaction mixture containing 5 pmol each of the two primers, 20 nmol each of the four deoxynucleotide triphosphates, 10 mM Tris-HCl (pH 8·3), 50 mM KCl, 1·5 mM $MgCl_2$, 0·001% gelatin, and 0·5 U of Taq DNA polymerase (Takara, Japan). Thirty cycles were performed for each reaction, with one cycle consisting of 15 s at 94°C, 30 s at 60°C, and 30 s at 74°C.

Direct DNA sequencing

PCR-amplified DNA was sequenced with synthetic oligonucleotide primer (NGGYRA F1 or NGGYRA R1) by the dideoxy-chain termination method,[19] using the BcaBEST dideoxy sequencing kit dCTP version (Takara, Japan) and [a-^{35}S] dCTP (Amersham, UK). The samples were electrophoresed in 5% Long Ranger Gel (AT Biochem, USA) containing 7M urea at 1800 V. The gel was dried and exposed to *x* ray film (Fuji Photo Film, Japan).

Results

The antimicrobial susceptibilities of the nine gonococcal strains to norfloxacin, ofloxacin, ciprofloxacin, penicillinG, ceftriaxone, tetracycline, and spectinomycin are summarised in table 1. All of the nine strains were negative for β-lactamase production. The isolates A-10 (MIC; 8·0 μg/ml) and A-22 (MIC; 4·0 μg/ml) were highly resistant to norfloxacin and A-55 (MIC; 1·0 μg/ml) and A-161 (MIC; 2·0 μg/ml) were moderately resistant to norfloxacin. The strains A-37, A-69, WHO-A, WHO-B, and WHO-D were susceptible to norfloxacin, for which MICs of norfloxacin were 0·063, 0·004, 0·004, 0·031, and 0·063 μg/ml, respectively. The four norfloxacin-resistant isolates showed 16- to 2000-fold higher MIC values of the antibiotic, as compared with those of the 5 norfloxacin-susceptible strains, and were cross-resistant to ofloxacin and ciprofloxacin. Furthermore, the high-level norfloxacin-resistant isolates (A-10 and A-22) showed less susceptibilities to penicillin, ceftriaxone, and tetracycline than did the norfloxacin-susceptible isolates (A-37 and A-69).

In the previous study,[16] to research the other quinolone resistance mechanism of alteration in drug permeability in gonococci, we measured intracellular accumulation of norfloxacin in the same six clinical and three WHO strains tested in the present investigation. Among the four norfloxacin-resistant iso-

Table 1 Antimicrobial susceptibility of clinical isolates of N gonorrhoeae and norfloxacin accumulation by those gonococcal cells

Strain	MIC (μg/ml)*							Accumulation of NFLX (ng/mg of dry cells, after 20 min)†
	NFLX	OFLX	CPFX	PCG	CTRX	TC	SPCM	
A-10	8·0	1·0	0·5	0·5	0·031	2·0	16·0	0
A-22	4·0	1·0	0·5	0·5	0·016	2·0	16·0	48
A-55	1·0	0·5	0·125	0·25	0·016	2·0	16·0	54
A-161	2·0	0·25	0·125	0·063	0·016	0·5	16·0	0
A-37	0·063	0·063	0·016	0·063	0·008	1·0	32·0	20
A-69	0·004	0·002	⩽ 0·001	0·063	0·004	0·25	8·0	29
WHO-A	0·004	0·004	⩽ 0·001	0·008	⩽ 0·001	0·25	32·0	22
WHO-B	0·031	0·016	0·016	0·25	0·004	0·125	8·0	87
WHO-D	0·063	0·063	0·004	1·0	0·031	2·0	8·0	48

*NFLX, norfloxacin; OFLX, ofloxacin; CPFX, ciprofloxacin; PCG, penicillinG; CTRX, ceftriaxsone; TC, tetracycline; SPCM, spectinomycin.
†Data from reference 16.

Table 2 Analysis of GyrA mutation in clinical isolates of N gonorrhoeae

Strain	MIC of NFLX* (μg/ml)	GyrA mutation		
		Codon	Nucleotide changed	Amino acid changed
A-10	8·0	91	TCC → TTC	Ser → Phe
A-22	4·0	91	TCC → TTC	Ser → Phe
A-55	1·0	91	TCC → TTC	Ser → Phe
A-161	2·0	91	TCC → TTC	Ser → Phe
A-37	0·063		None	None
A-69	0·004		None	None
WHO-A	0·004		None	None
WHO-B	0·031		None	None
WHO-D	0·063		None	None

*NFLX, norfloxacin.

lates, the strains A-10 and A-161 did not accumulate norfloxacin after 20 minutes, whereas the strains A-22 and A-55 accumulated norfloxacin as well as norfloxacin-susceptible strains (table 1).

Nucleotide and amino acid changes within the quinolone resistance-determining region are shown in table 2. A single base change (serine codon TCC changed to phenylalanine codon TTC), which resulted in an amino acid change in GyrA at position 91, was identified in all of the four gonococcal isolates (A-10, A-22, A-55, and A-161) resistant to norfloxacin, while in norfloxacin-susceptible strains (A-37, A-69, WHO-A, WHO-B, and WHO-D), no mutation within the quinolone resistance-determining region in GyrA was detected.

Discussion

Belland *et al*[17] have recently generated a series of ciprofloxacin-resistant mutants by passaging *N gonorrhoeae* in the presence of increasing concentrations of the antibiotic and examined whether mutations occurred in DNA gyrase genes. Their results demonstrated that the serine-91 (serine-91 in gonococcal *GyrA* corresponds to serine-83 in *E coli*) to phenylalanine substitution in GyrA may be an essential mutation in the laboratory variants of gonococci showing ciprofloxacin resistance.

We performed sequencing the quinolone resistance-determining region within *GyrA* gene in clinical isolates of *N gonorrhoeae* resistant to norfloxacin. All four norfloxacin-resistant isolates (A-10, A-22, A-55, and A-161) for which the MICs of norfloxacin ranged from 1·0 to 8·0 μg/ml had the same amino acid substitution at serine-91 to phenylalanine identified in the laboratory variants resistant to ciprofloxacin by Belland *et al*, while no mutation in the quinolone resistance-determining region within GyrA gene was detected in five norfloxacin-susceptible strains for which the MIC of norfloxacin ranged from 0·004 to 0·063 μg/ml. Our results and Belland's indicate that the serine to phenylalanine substitution at position 91 may be an essential mutation in both laboratory variants and clinical isolates of *N gonorrhoeae* resistant to fluoroquinolones. More recently, another study has also reported the same mutation of serine to phenylalanine in neisserial GyrA at the position corresponding to serine-83 in *E coli* in clinical isolates of *N gonorrhoeae*.[20]

Our previous study[16] demonstrated that the norfloxacin-resistant strains A-10 and A-161 carrying a mutation in their GyrA did not accumulate norfloxacin, whereas the other norfloxacin-resistant strains A-22 and A-55 accumulated norfloxacin as well as norfloxacin-susceptible strains. It is therefore suggested that both alteration in drug permeability and change in GyrA may relate to fluoroquinolone resistance in the strains A-10 and A-161.

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