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Epidemiological characteristics and molecular basis of fluoroquinolone-resistant *Neisseria gonorrhoeae* strains isolated in Korea and nearby countries

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***Objectives*: This study was performed to examine the cause of the increase in quinolone-resistant *Neisseria gonorrhoeae* (QRNG) observed in Korea.**

***Methods*: The antimicrobial susceptibilities of 190 isolates of gonococci from Korea in 2000 were examined by NCCLS methods, and subsets of these isolates underwent mutation analysis of the quinolone resistance-determining regions (QRDRs) of *gyrA* and *parC*. Molecular epidemiological characterization of 25 Korean isolates and 54 isolates from overseas was performed by pulsed-field gel electrophoresis (PFGE) and the results compared.**

***Results*: Most (172, 90.5%) of the 190 gonococci tested displayed reduced susceptibility to ciprofloxacin. All strains with high-level ciprofloxacin resistance (ciprofloxacin MIC ≥ 4 mg/L) contained a double amino acid alteration at the 91 and 95 positions in the QRDR of GyrA and a single alteration in ParC. PFGE types of high-level QRNG in Korea were mostly different from those of other nearby countries.**

***Conclusions*: These results suggest that the observed increase in ciprofloxacin-resistant isolates is due to the mutation and spread of Korean multiclonal isolates rather than importation from overseas.**

Keywords: *N. gonorrhoeae*, pulsed-field gel electrophoresis, ciprofloxacin, resistance, QRDRs

Introduction

Neisseria gonorrhoeae is the causative agent of gonorrhoea, one of the most commonly reported sexually transmitted diseases worldwide. The emergence of resistance to antimicrobial agents in gonococci has complicated its treatment and control.[1] However, the prevalence of antimicrobial resistance varies greatly between countries.[2]

In recent years, a decrease in the proportion of penicillinase-producing *N. gonorrhoeae* (PPNG) and a rapid increase in quinolone-resistant *N. gonorrhoeae* (QRNG) have been reported to be related in some countries.[3–5] However, in Korea, the proportion of PPNG has remained high (84%), and strains with reduced susceptibility to ciprofloxacin increased rapidly from 9% in 1992 to 84% in 1999.[6,7] This phenomenon may be caused by the spread of strains in which the *gyrA* and *parC* mutations arose locally or by the importation of QRNG from overseas followed by sustained domestic transmission of resistant subtypes. In either case, the QRNG may be of multiple subtypes or else represent the expansion of limited numbers of subtypes.

The aims of this study were to examine the cause of the increase in QRNG observed in Korea by testing antimicrobial susceptibility, analysing the mutations of the quinolone resistance-determining regions (QRDRs) of *gyrA* and *parC* of fluoroquinolone-non-susceptible isolates, and comparing the molecular epidemiological characteristics of QRNG in Korea with those of other nearby countries.

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Materials and methods

N. gonorrhoeae isolates

One hundred and ninety gonococci were isolated from Korean patients or prostitutes attending sexually transmitted diseases clinics in Seoul in 2000 using modified Thayer-Martin agar (BBL, Becton Dickinson, Cockeysville, MD, USA). Species identification was based on conventional culture and biochemical characteristics.[8] The isolates were kept frozen at −70°C until used.

For comparison purposes, 54 random gonococcal isolates from patients infected in nearby countries, namely, Australia, China, Mongolia, the Philippines, India, Malaysia, Singapore, Thailand, Vanuatu and Vietnam from 1995 to 2000, were obtained from the regional World Health Organization (WHO) reference laboratory in Sydney, Australia.

Antimicrobial susceptibility testing

The medium used for susceptibility testing was GC II agar base supplemented with 1% IsoVitaleX (Becton Dickinson). All 190 Korean isolates were initially examined by the disc diffusion testing methods of the NCCLS[9] using a 5 μg ciprofloxacin disc (Becton Dickinson).

Forty-nine Korean gonococcal strains isolated consecutively between April and June 2000, and the 54 gonococci from nearby countries were examined for susceptibility by the NCCLS agar dilution method.[10] Seventeen of 20 isolates with no ciprofloxacin inhibition zone among 190 isolates were tested by the agar dilution test to determine the level of ciprofloxacin resistance. Ciprofloxacin powder was supplied by Bayer Korea (Seoul, Korea). Inocula of $\sim 10^4$ cfu were applied using a Steers replicator (Craft Machine, Chester, PA, USA). Plates were incubated in a 5% CO_2 incubator at 35°C for 24 h after which the results were read. Strains with high-level ciprofloxacin resistance were defined as those for which the MIC was ≥4 mg/L.[11] *N. gonorrhoeae* ATCC 49226 was used as a control strain. During 2000 and 2001, our laboratory accurately categorized those QRNG included in an external quality assurance programme conducted by the WHO Western Pacific Region Gonococcal Antimicrobial Surveillance Programme (GASP).[12]

Sequencing the QRDRs of gyrA and parC

Twenty-nine Korean gonococcal isolates with various ciprofloxacin disc susceptibility patterns were randomly selected, and the ciprofloxacin MICs for these isolates were also determined by the agar dilution method. The QRDRs of these isolates were investigated by sequencing and these results were correlated with the MICs of ciprofloxacin. The QRDR sequences of the *gyrA* and *parC* genes were amplified using PCR primers as reported by Tanaka *et al.*[3] PCR was carried out with 1 μL of heat-extracted template DNA, 10 pmol of each primer and PreMix (Bioneer, Cheongwon, Korea) containing 1 U of *Taq* DNA polymerase in a total volume of 20 μL. A thermal cycler (Eppendorf, Hamburg, Germany) was used under the following conditions: 35 cycles of 93°C for 30 s, 58°C for 30 s and 72°C for 1 min. The PCR products were extracted using a DNA extraction kit (Qiagen, Hilden, Germany), and their nucleotide sequences were determined by the dideoxynucleotide-chain termination method with ABI PRISM BigDye Terminator Sequencing Kits (Perkin-Elmer, Foster City, CA, USA) and using an automatic DNA sequencer (ABI 3700; Perkin-Elmer).

Epidemiological study

Pulsed-field gel electrophoresis (PFGE) was performed on a total of 79 strains for which the ciprofloxacin MICs were determined by agar dilution methods, namely, 25 of the 29 randomly selected isolates from Korea, and the 54 gonococci from overseas, as described previously.[7] Briefly, the plugs were prepared by using saline EDTA solution-suspended cells cultured overnight. The genomic DNA was digested with *Nhe* I (Takara, Tokyo, Japan) for 18 h at 35°C. The CHEF DR II instrument (Bio-Rad, Hercules, CA, USA) was used to separate the fragments, with switch times of 0.5 s (initial) and 54 s (final), and a running time of 20 h at 6 V/cm. PFGE banding pattern similarities were determined using the Dice coefficients and unweighted pair group method using arithmetic averages (UPGMA) clustering method using Molecular Analyst Fingerprinting Software (v. 1.12; Bio-Rad). A tolerance in the band positions of 0.5% was used. An 80% similarity threshold was used to divide the outputs from the dendrogram into clusters.

Results

Antimicrobial susceptibility

Ciprofloxacin disc susceptibilities of the 190 isolates were: 18 (9.5%) susceptible, 122 (64.2%) intermediate and 50 (26.3%) resistant. For the 17 isolates with no ciprofloxacin inhibition zone, ciprofloxacin MICs were ≥16 mg/L (data not shown). The susceptibilities of the 49 Korean and the 54 non-Korean isolates, as determined by agar dilution methods, are shown in Table 1. The MIC range of ciprofloxacin for *N. gonorrhoeae* was ≤0.015–32 mg/L, and the geometric mean ciprofloxacin MIC was 0.396 mg/L. The ciprofloxacin susceptibility categories of the 54 overseas isolates were: eight susceptible, 12 intermediate and 34 resistant. Among these isolates, 22 (40.7%) were high-level ciprofloxacin resistant with MICs of ≥4 mg/L.

Table 1. Ciprofloxacin susceptibility of 49 Korean and 54 non-Korean *N. gonorrhoeae* isolates tested by the agar dilution method

Source of isolates (no. of isolates tested)	No. of isolates with MIC (mg/L)												MIC (mg/L)			Percentage of isolates		
	≤0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	50%	90%	geometric mean	S	I	R
Korea (49)	4			4	6	32						3	0.5	0.5	0.396	8	86	6
Nearby countries (54)	2	2	4	3	3	6	6	6	12	5	5		2	16	1.071	19	18	63

S, susceptible; I, intermediate; R, resistant.

Table 2. Amino acid substitutions within GyrA and ParC in 29 randomly selected Korean isolates of *N. gonorrhoeae*[a]

		GyrA		ParC	
Ciprofloxacin MIC (mg/L)	No.	Ser-91	Asp-95	Gly-85	Ser-87
16–64	16	Phe	Gly	–	Arg
	1	Phe	Asn	Cys	–
2	1	Phe	Gly	–	–
0.25–0.5	5	Phe	–	–	–
	1	Phe	Gly	–	–
0.12	1	–	Gly	–	–
	1	–	Asn	–	–
≤0.008	3	–	–	–	–

A dash indicates sequence homology.
[a]The numbering of the amino acids of GyrA and ParC proteins is from GenBank accession numbers U08817 and U08907, respectively.

Genetic alterations in the QRDRs

The ciprofloxacin MICs for 29 randomly selected Korean isolates are shown in Table 2 together with details of the QRDR alterations present. All 17 of the high-level ciprofloxacin-resistant Korean isolates contained three amino acid substitutions in the GyrA and ParC proteins. Of these, the most common substitutions (in 16 of 17 strains) were serine to phenylalanine at position 91 and aspartic acid to glycine at position 95 in GyrA, and serine to arginine at position 87 in ParC. The isolates for which ciprofloxacin MICs were 0.12–2 mg/L contained one or two amino acid substitutions in GyrA alone. No alterations in GyrA and ParC were detected in the three susceptible strains.

Molecular types of the Korean and overseas strains

Among the 25 Korean isolates examined, 13 PFGE types were observed: eight strains (32.0%) were E type and four (16.0%) α type (Figure 1). In contrast, 33 PFGE types were observed among the 54 overseas isolates: five strains (9.3%) were Q type and four (7.4%) showed β and γ types.

The 13 Korean isolates with high-level ciprofloxacin resistance were of the E1 (four strains), E2 (four), α (four) or W2 (one) PFGE type, while the 22 overseas isolates belonged to β type (four strains), K type (two), ε type (two), κ2 type (two) and several other types (12). One ciprofloxacin-susceptible Korean isolate was of the W1 type, which resembled the W2 type of a high-level ciprofloxacin-resistant strain. K, κ2 and ν types were shown by four ciprofloxacin-susceptible Korean isolates and five ciprofloxacin-susceptible or -resistant overseas isolates.

Discussion

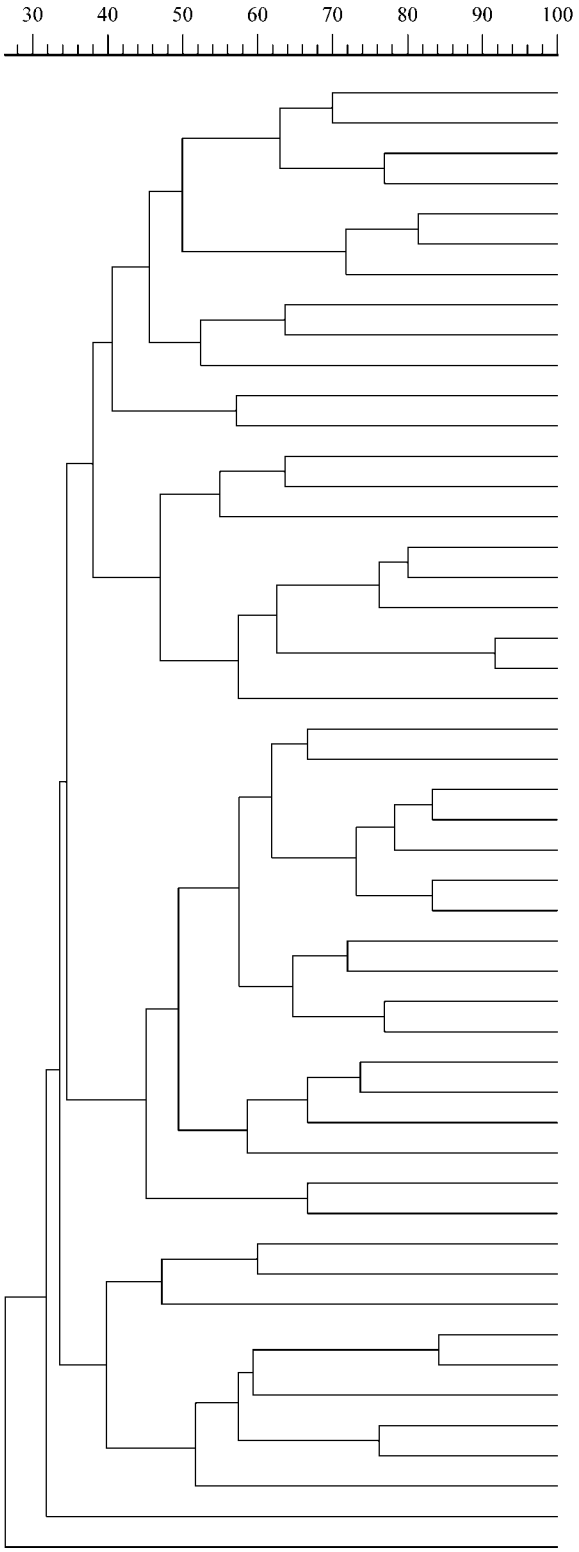
N. gonorrhoeae strains with high-level ciprofloxacin resistance (MICs of ≥4 mg/L) have been reported in several countries.[11,13] In our previous studies,[6,7] all isolates were inhibited by ≤1 mg/L of ciprofloxacin. In the present study, 20 of 190 isolates in 2000 showed no ciprofloxacin inhibition zone. These isolates were tested by agar dilution test and showed that they were highly resistant to ciprofloxacin, with MICs of ≥16 mg/L (Tables 1 and 2). Also, the MIC_{50} for strains isolated in 2000 was 0.5 mg/L, which was 4- to 32-fold higher than those found in our previous studies in 1993 and 1996.[6]

Generally, *gyrA* mutations play an important role in the development of fluoroquinolone resistance in gonococci, and simultaneous *parC* mutations play a complementary role in increasing the level of resistance.[14,15] Shultz *et al.*[11] also reported that the sequence and nature of the QRDR changes were found to correlate with MICs of ciprofloxacin, and some *parC* alterations do not have a significant effect on MIC. In this study, we found that all strains with high-level ciprofloxacin-resistance contained a double amino acid alteration at the 91 and 95 positions in the QRDR of GyrA and a single alteration in ParC. These findings concur with those of other investigators.[14,15] However, the amino acid substitutions in Korean isolates with high-level ciprofloxacin resistance were of a similar configuration in 16 of 17 instances, suggesting that 'clonal' expansion of a QRNG may have been involved. The amino acid substitutions in the predominant QRDR change seen differed from those reported by some other investigators in the region,[3,13,15] although they were also observed in a QRNG isolated from an infection acquired in China.[11]

One of the aims of this study was to determine whether the increase in high-level ciprofloxacin-resistant *N. gonorrhoeae* observed in Korea was caused by the importation of resistant strains from overseas, or by the spread of resistant variants, perhaps selected by antibiotic pressures in Korea.

N. gonorrhoeae strains are genetically diverse due to the frequent transfer and recombination of genetic elements between strains.[16] Su & Lind[15] reported that a common epidemic 'clone' might be widely spread in the Philippines, Hong Kong, Romania, New Guinea and Denmark, but the study was based on examination of small numbers of ciprofloxacin-resistant isolates. In contrast, Trees *et al.*[13] reported that ciprofloxacin-resistant isolates in Bangkok were not from a single 'clonal' source, but of diverse subtypes from multiple cases of importation or local emergence.

Among the 25 Korean isolates examined, 13 PFGE types were found (Figure 1). Of these, 13 isolates were high-level ciprofloxacin resistant and all had the same QRDR changes. Eight of the 13 high-level ciprofloxacin-resistant strains were of the E type, indicating a significant degree of strain relatedness. However, the other five high-level resistant strains with the same QRDR alterations were from unrelated PFGE types (four α and one W2). One high-level ciprofloxacin-resistant W2-type Korean isolate was closely related to a ciprofloxacin-susceptible W1-type Korean isolate. The PFGE patterns of the high-level ciprofloxacin-resistant strains from Korea differed from those of isolates in other nearby countries. It is tempting to suggest from these results that the observed increase in high-level ciprofloxacin-resistant strains in Korea may have arisen as a result of the spread of multiclonal resistant variants of domestic strains. In this circumstance, the predominant QRDR change observed could have arisen locally and spread amongst already circulating strains. This phenomenon has been observed in a study of a closed population in Mongolia.[17] Among the isolates with K, κ2 and ν types in this study, all Korean isolates were ciprofloxacin-susceptible, while some non-Korean isolates were ciprofloxacin-resistant, suggesting that the resistance was not introduced from overseas.



PFGE Pattern	No. of isolates from: Korea	Nearby Countries
A	0	1
B	1	0
C	0	1
D	0	2
E1	**4**	0
E2	**4**	0
F	0	1
G	0	2
H	0	1
I	0	1
J	0	**1**
K	2	**2**
L	1	0
M	1	0
N	1	0
O1	0	2
O2	0	1
P	0	1
Q1	0	4
Q2	0	1
R	0	1
S	1	0
T	1	0
U1	0	**1**
U2	0	**1**
V	0	**1**
W1	1	0
W2	**1**	0
X	0	1
Y	0	**1**
Z	0	**1**
α	**4**	0
β	0	**4**
χ	0	**1**
δ	0	**1**
ε	0	**2**
ϕ	0	1
γ	0	**1**
η	0	1
ι	0	**1**
φ	0	**1**
κ1	0	**1**
κ2	1	**2**
λ	0	4
μ	1	0
ν	1	1
ο	0	3
π	0	1
θ	0	1

Figure 1. Dendrogram of PFGE band pattern of strains from Korea and nearby countries. The band differences of *Nhe* I-digested genomic DNAs of 79 strains of *N. gonorrhoeae* (25 isolated in Korea and 54 in other countries) were analysed using the Dice coefficients and unweighted pair group method using arithmetic averages (UPGMA) clustering method using Molecular Analyst Fingerprinting Software. Bold type indicates strains with high-level ciprofloxacin resistance (MIC ≥ 4 mg/L).

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