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Characterization of *Neisseria gonorrhoeae* Strains with Decreased Susceptibility to Fluoroquinolones Isolated in Greece from 1996 to 1999

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Of the 331 *Neisseria gonorrhoeae* strains isolated in Greece from 1996 to 1999, 39 (11.8%) exhibited decreased susceptibility to quinolones due to *gyrA* and *parC* mutations. Conventional typing and pulsed-field gel electrophoresis showed that 34 of these isolates were clonally related. Epidemiological data indicated that the epidemic clone was sustained in a group of high-frequency transmitters.

Fluoroquinolones are highly active in vitro against *Neisseria gonorrhoeae* and are effective in treating gonococcal infections (3, 4). However, resistance to these antimicrobial agents, associated in some cases with treatment failures, has been increasingly reported (9, 17). Gonococcal resistance to fluoroquinolones involves a number of chromosomally mediated mechanisms, among which mutations resulting in amino acid changes in the A subunit of DNA gyrase and the *parC*-encoded region of topoisomerase IV seem to be the most important (1). In Greece, fluoroquinolones were introduced in 1985 but have not yet been used as first-choice drugs for gonorrhea therapy in state sexually transmitted disease clinics. However, they are being extensively prescribed in private practice. The first quinolone-resistant *N. gonorrhoeae* (QRNG) strain was isolated in Greece in 1990 from a patient infected in the Philippines (20). QRNG was not isolated again before 1996, when strains with reduced susceptibility began to appear with increasing frequency. In this study, we present data on fluoroquinolone susceptibility of gonococcal isolates collected from the Greek National Reference Center for *Neisseria gonorrhoeae* during the years 1996 to 1999 and on the characterization of the QRNG strains.

A total of 331 nonreplicate *N. gonorrhoeae* isolates were examined. They were derived consecutively from cases of male gonococcal urethritis seen in two major sexually transmitted disease clinics of Athens and Thessaloniki, Greece, and represented 72% of all gonorrhea cases reported to the Reference Center during the study period. Antimicrobial susceptibilities of the isolates were determined on GC agar supplemented with Vitox (Oxoid), by using Etest (AB Biodisk) according to the instructions of the manufacturer and with the *N. gonorrhoeae* strains WHO A to WHO D as quality control standards. For susceptibility categorization, the breakpoints set by the National Committee for Clinical Laboratory Standards (16) and those recently proposed for fluoroquinolones (10, 12) were followed.

Characterization of the QRNG isolates by auxotype, serovar, and plasmid content was performed as described previously (20). The Phadebact GC serovar test (Boule Diagnostics) and the Genetic System (Syva) panels of monoclonal antibodies for serotyping gonococci were used. Phenotypically identical isolates were further typed by pulsed-field gel electrophoresis (PFGE) of their genomic DNA after digestion with *Spe*I endonuclease (New England BioLabs). PFGE was performed as described previously (21), using the CHEF-DR III apparatus (Bio-Rad) and a lambda ladder PFG marker (New England BioLabs).

Mutations in the *gyrA* and *parC* genes of the QRNG strains were identified by PCR and direct sequencing of amplified products that included the quinolone resistance-determining regions (QRDR) of these genes. The primers used were GA1 (5′-AGCTATCTCGACTACGCC-3′) and GA2 (5′-CCGAAA CTGTCTTGCAGC-3′), which amplify a 937-bp segment corresponding to amino acids 28 to 340 of GyrA (GenBank accession no. UO8817), and PAR1 (5′-TCTCGAATACGCCAT GAGCG-3′) and PAR2 (5′-ATTGTGCGACGGAATCTCG G-3′), which amplify a 495-bp segment corresponding to amino acids 26 to 190 of *N. gonorrhoeae* ParC (GenBank UO8907). Chromosomal DNA for PCR was extracted as described previously (15), and PCR assays were performed in 100-μl reaction mixtures containing 50 pmol of each of the two primers, a 200 μM concentration of each deoxynucleoside triphosphate, 2.5 U of *Taq* DNA polymerase (MBI Fermentas), and 100 ng of template DNA. Thirty cycles were performed for each reaction, with each cycle consisting of 60 s at 94°C, 30 s at 60°C, and 60 s at 72°C. PCR products were prepared for sequencing with a PCR product presequencing kit (United States Biochemicals) and directly sequenced by the dideoxy chain termination method, using a Sequenase DNA sequencing kit (version 2.0; United States Biochemicals).

For 39 (11.8%) of the 331 gonococcal isolates obtained from 1996 to 1999, nalidixic acid MICs were >256 μg/ml. The 39 strains also exhibited decreased susceptibility to one or more fluorinated quinolones and, therefore, were considered to be QRNG. Only one strain exhibited high-level fluoroquinolone resistance. The MICs of five fluoroquinolones tested against QRNG strains are presented in Table 1. Trovafloxacin was the most active compound, followed by ciprofloxacin, ofloxacin, norfloxacin, and pefloxacin.

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TABLE 1. In vitro susceptibilities of 39 QRNG isolates to five fluoroquinolones

Antibiotic (breakpoints [μg/ml])	MIC (μg/ml)[a]			No. of strains categorized as[b]:		
	Range[c]	50%	90%	R	I	S
Trovafloxacin (R ≥ 0.5, S ≤ 0.125)[d]	0.016–0.25 (4)	0.016	0.094	1	2	36
Ciprofloxacin (R ≥ 1.0, S ≤ 0.06)[e,f]	0.047–0.5 (16)	0.094	0.125	1	26	12
Ofloxacin (R ≥ 2.0, S ≤ 0.25)[e,f]	0.094–0.75 (>32)	0.19	0.5	1	2	36
Norfloxacin (R ≥ 1.0, S ≤ 0.25)[f]	0.38–1.5 (48)	0.5	0.75	6	33	0
Pefloxacin[g]	0.5–2 (64)	0.5	1.5			

[a] 50% and 90%, MICs at which 50 and 90% of isolates are inhibited, respectively.
[b] R, resistant; I, intermediate; S, susceptible. For susceptibility categorization according to the breakpoints used, Etest MIC values lying between twofold dilutions were rounded to the nearest higher dilution.
[c] Value in parentheses is the MIC for a unique strain exhibiting high-level fluoroquinolone resistance.
[d] According to reference 10.
[e] According to reference 16.
[f] According to reference 12.
[g] Interpretive criteria (breakpoints) not available.

In a phenotypic analysis, 34 of the QRNG strains formed a homogeneous cluster of isolates with identical characteristics. The remaining five strains were diverse, displaying different serovar-auxotype combinations and various plasmid and antibiotic susceptibility profiles (Table 2). The 34 phenotypically identical isolates had no nutritional requirements, were serotyped as Arst/IA-6, and shared a very unusual plasmid profile: they lacked the cryptic plasmid and harbored only the conjugative gonococcal plasmid (Arst/conj$^+$cr$^-$ type). Moreover, the isolates exhibited a quite uniform antibiotic susceptibility pattern, being moderately susceptible to fluoroquinolones and highly susceptible to penicillin G and cefotaxime, and most of them were also susceptible to tetracycline, erythromycin, and chloramphenicol. In PFGE, the Arst/conj$^+$cr$^-$ isolates displayed identical DNA fingerprints, suggesting that they were clonally related (data not shown).

The Arst/conj$^+$cr$^-$ type strains were continuously isolated during the study period, with a peak frequency in 1998. All of these isolates were from patients infected in Greece, in either the Athens or the Thessaloniki area. Twelve (35%) of these isolates were derived from homosexuals or bisexuals, who represented 20% of all 59 patients reporting these sexual orientations during the study period (chi-square test, $P < 0.05$). The five phenotypically diverse QRNG strains were isolated sporadically, with four isolates from patients who stated that they had acquired the infection while abroad. The strain with high-level fluoroquinolone resistance was acquired in Sweden in 1997.

The nucleotide sequences of the QRDR of *gyrA* and *parC* from all six distinct QRNG strains, including a representative isolate of the Arst/conj$^+$cr$^-$ clone (QA7), showed various changes compared with the wild-type sequences. The deduced alterations in the amino acid sequences of the respective proteins are shown in Table 3. The strains with decreased susceptibility to fluoroquinolones carried single amino acid substitutions in GyrA, while the strain with high-level resistance possessed a double substitution in GyrA and a single substitution in ParC. In all cases, the alterations in the gyrase protein included the replacement of either serine-91 or aspartic acid-95, or both residues, by various amino acids. The most frequent replacement among the distinct strains was the substitution of phenylalanine for serine-91, which was also present in the GyrA of the highly resistant strain. The Arst/conj$^+$cr$^-$ strain QA7, however, had tyrosine at this position.

In this study we have shown that quinolone resistance has been established among *N. gonorrhoeae* strains currently circulating in Greece. The most salient point was the detection of an epidemic caused by clonal spread of a single strain. Apart from extensive phenotypic and genotypic similarities and the rarity of their plasmid profile, which are all indications of clonality, the Arst/conj$^+$cr$^-$ isolates were epidemiologically related, based on time distribution, the exclusively domestic acquisition in the two main urban areas of Greece, and a significantly higher prevalence in a subgroup of the population, namely, men who were having sex with men (5, 19). Although we could not trace the original source of this clonal type, its persistence in the community over 4 years could be explained by the health and sexual behavior within this group of high-frequency transmitters. The bisexual orientation reported by some of the patients may explain the spread of this strain among heterosexuals also, illustrating a communication between transmission networks.

TABLE 2. Phenotypic characteristics and geographic origins of 39 QRNG strains isolated during the period 1996 to 1999[a]

Strain(s)	GC/GS serovar, auxotype[b]	Plasmid sizes (MDa)[c]	Etest MIC (μg/ml)							Location and time of acquisition of infection
			CIP	PEN	CTX	TET	ERY	CM	SP	
QA1–QA34	Arst/IA-6, NR	24.5	0.047–0.125	≤0.016	≤0.008	0.047–0.25	0.064–0.38	0.125–0.5	8–16	Greece, 1996–1999
QB	Bopt/IB-26, P$^-$	2.6	16	2	0.094	4	3	6	16	Sweden, 1997
QC	Bropyst/IB-1, NR	24.5, 2.6	0.125	1	0.032	1	1.5	3	16	England, 1998
QD	Arst/IA-17, NR	25.2, 2.6	0.125	0.25	0.016	24	1	1	12	Greece, 1998
QE	Bopst/IB-1, P$^-$	24.5, 2.6, 3.2	0.25	>256	0.064	1.5	1.5	3	16	Thailand, 1998
QF	Arst/IA-4, NR	25.2, 2.6, 3.2	0.75	32	0.012	32	0.38	0.75	16	Thailand, 1999

[a] Abbreviations: NR, no nutritional requirements; P$^-$, requiring proline for growth; CIP, ciprofloxacin; PEN, penicillin G; CTX, cefotaxime; TET, tetracycline; ERY, erythromycin; CM, chloramphenicol; SP, spectinomycin.
[b] GC, serotype from Phadebact GC test; GS, serotype from Genetic System test.
[c] Plasmids of 25.2 and 3.2 MDa conferred resistance to tetracycline and penicillin, respectively.

TABLE 3. Quinolone susceptibilities and alterations in the QRDR of gyrase A and topoisomerase IV in six distinct QRNG strains[a]

Strain	PEN/TC resistance type	Etest MIC (μg/ml)						Amino acid (codon) change	
		NAL	PRF	NOR	OFL	CIP	TVX	GyrA	ParC
QA7[b]		>256	0.5	0.5	0.19	0.094	0.047	Ser-91 (TCC)→Tyr (TAC)	
QB	CMPR, CMTR	>256	64	48	32	16	4	Ser-91 (TCC)→Phe (TTC) Asp-95 (GAC)→Gly (GGC)	Asp-86 (GAC)→Asn (AAC)
QC		>256	0.75	0.38	0.19	0.125	0.064	Asp-95 (GAC)→Asn (AAC)	
QD	TRNG	>256	1.5	0.75	0.25	0.125	0.25	Ser-91 (TCC)→Phe (TTC)	
QE	PPNG, CMTR	>256	1.5	1	0.5	0.25	0.094	Asp-95 (GAC)→Asn (AAC)	
QF	PPNG, TRNG	>256	2	1.5	0.75	0.38	0.125	Ser-91 (TCC)→Phe (TTC)	

[a] Abbreviations: PEN, penicillin; TC, tetracycline; NAL, nalidixic acid; PRF, pefloxacin; NOR, norfloxacin; OFL, ofloxacin; CIP, ciprofloxacin; TVX, trovafloxacin; CMPR and CMTR, chromosomally resistant to penicillin and tetracycline, respectively; TRNG and PPNG, carrying plasmid for resistance to tetracycline and penicillinase production, respectively.

[b] Representative of the Arst/conj$^+$ cr$^-$ clone.

In a 15-year period of continuous surveillance, this is the first time we have documented a sustained outbreak of an *N. gonorrhoeae* strain in Greece. The epidemic described here, as well as five cases of endemic transmission that occurred during the last decade in London, United Kingdom (7), Cleveland, Ohio (6, 11, 13), Seattle, Wash. (2), Ontario, Canada (8), and Sydney, Australia (18), all were due to strains with decreased susceptibility to fluoroquinolones. This may reflect changes in the epidemiology of gonorrhea (e.g., a trend towards core groups characterized by higher transmissibility and a longer duration of infectiousness) and extensive use of quinolones in the treatment of community-acquired infections (11, 14).

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