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Molecular Characterization of Quinolone-Resistant *Neisseria gonorrhoeae* Isolates from Brazil▽

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Despite the rapid spread of antibiotic resistance among gonococci worldwide, limited reports are available from Brazilian locations. In the present study, 25 quinolone-resistant *Neisseria gonorrhoeae* (QRNG) strains isolated in Rio de Janeiro, Brazil, were characterized by phenotypic and molecular methods, including analysis of mutations in the *gyrA* and *parC* genes. They represented 16.5% of the *N. gonorrhoeae* isolates obtained during a survey performed from 2006 to 2010. A trend for increasing resistance to ciprofloxacin was observed in the period investigated. The most prevalent pattern of mutation observed among QRNG isolates, Ser-91 to Phe and Asp-95 to Gly in *gyrA* and Ser-87 to Arg in *parC*, was detected in 40% of the isolates exhibiting MICs ranging from 4 to >32 μg/ml. Rare types of mutations were found in the *gyrA* gene (Gln-102 to His [12%] and Asp-95 to Tyr [4%]) and in the *parC* gene (Ser-88 to Thr [4%]). The genetic relationship of the QRNG isolates, evaluated by pulsed-field gel electrophoresis, suggested that the increase in the frequencies of the QRNG isolates in Rio de Janeiro, Brazil, may have arisen as a result of simultaneous spread of two clonal groups. The results also indicate that fluoroquinolones may no longer be used as first line antibiotics for the treatment of gonorrhea in Rio de Janeiro, and that programs for antimicrobial susceptibility surveillance of *N. gonorrhoeae* should also be implemented in other regions of Brazil.

Gonorrhea is among the most prevalent sexually transmitted diseases (STDs) throughout the world. The causative agent, *Neisseria gonorrhoeae*, predominantly infects human urogenital tract, causing cervicitis, urethritis, and rectal infections. In 2003, the incidence of gonococcal infections in Brazil was estimated in 1,54 million of cases, being 657,139 cases in the Southeast region and more than 130,000 cases in Rio de Janeiro state (5). Recently, two studies conducted among men attending STD clinics (1) and among pregnant women (15) in six Brazilian cities revealed prevalences of infection by *Neisseria gonorrhoeae* of 18.4 and 1.5%, respectively.

Since the emergence and increasing occurrence of plasmid-mediated high-level or chromosomally mediated low-level resistance to penicillin and/or tetracycline, fluoroquinolones (ciprofloxacin or ofloxacin) or cephalosporins (ceftriaxone and cefixime) constitute the therapeutic regimen recommended by the World Health Organization for treatment of gonococcal infections worldwide (25).

However, the emergence of quinolone-resistant *N. gonorrhoeae* (QRNG) isolates has generated significant concern in several countries (13, 26, 31). Mechanisms of quinolone resistance in *N. gonorrhoeae* isolates include point mutations at the "quinolone resistance-determining regions" (QRDRs) of the *gyrA* and *parC* genes which code for the enzymes DNA gyrase and topoisomerase IV, respectively, that were the target of this drug, conferring high-level quinolone resistance (13).

In Brazil, reports on the antimicrobial susceptibility of *N. gonorrhoeae* have been sporadically available, and only a few isolates with decreased susceptibility to ciprofloxacin were described (2, 11, 12). In 2007, we detected the occurrence of resistance to ciprofloxacin in 7.7% (4/52) of the *N. gonorrhoeae* isolates recovered from male urethral specimens or vaginal specimens in Rio de Janeiro, Brazil (A. A. Uehara and S. E. L. Fracalanzza, unpublished data). Simultaneously, a resistance rate of 3.1% was found in strains isolated from patients attending the STD Service of the University of São Paulo teaching hospital, in São Paulo, Brazil (3).

The aim of the present study was to characterize ciprofloxacin-resistant *N. gonorrhoeae* isolates recovered in Rio de Janeiro, Brazil, from 2006 to 2010, by using phenotypic and molecular methods.

MATERIALS AND METHODS

Collection and identification of isolates. A total of 25 ciprofloxacin-resistant *N. gonorrhoeae* strains (19 presenting full resistance [MIC ≥ 1 μg/ml] and six intermediate [MIC = 0.125 to 0.75 μg/ml]) were included in the present study. They were identified among 152 *N. gonorrhoeae* isolates collected from patients with acute gonorrhea at sexually transmitted diseases clinics and microbiology diagnostic laboratories in Rio de Janeiro, Brazil, from 2006 to 2010. Conventional procedures were used for isolation and identification (16).

Antimicrobial susceptibility testing. Susceptibility to azithromycin, ceftriaxone, ciprofloxacin, penicillin, and tetracycline was tested by the disk diffusion according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (7). In addition, MICs for ciprofloxacin, penicillin, and tetracycline were determined by the Etest method according to the manufacturer's instructions (AB

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TABLE 1. Characterization of 25 QRNG isolates in Rio de Janeiro, Brazil, from 2006 to 2010 in relation to the *gyrA* and *parC* mutation patterns, the presence of TetM and β-lactamase plasmids, and the profiles of resistance to penicillin, tetracycline, and azithromycin

Mutation pattern	Isolate	Yr	CIP[a] MIC (μg/ml)	Mutation change[b]								TetM/β-lactamase plasmid[c]	Resistance profile[d]
				gyrA gene			*parC* gene						
				Ser-91	Asp-95	Gln-102	Asp-86	Ser-87	Ser-88	Glu-91	Aln-92		
P1	4825	2006	>32	Phe	Gly			Arg				–	CMRPT
	4957	2006	>32	Phe	Gly			Arg				–	CMRPT
	5907	2008	>32	Phe	Gly			Arg				–	IRP
	6070	2008	8	Phe	Gly			Arg				–	AZRNG/IRPT
	6206	2008	8	Phe	Gly			Arg				–	IRPT
	6207	2008	24	Phe	Gly			Arg				–	
	6334	2008	>32	Phe	Gly			Arg				–	IRP
	7593	2009	8	Phe	Gly			Arg				–	IRP
	7712	2009	4	Phe	Gly			Arg				–	CMRP/IRT
	8286	2009	8	Phe	Gly			Arg				–	IRP
P2	5026	2006	>32	Phe	Gly	His		Arg				–	CMRPT
	7711	2009	>32	Phe	Gly	His		Arg				–	IRP
	7784	2009	8	Phe	Gly	His		Arg				–	CMRPT
P3	5852	2007	>32	Phe	Gly		Asn	Arg				–	AZRNG/IRP
P4	6205	2008	16	Phe	Gly			Arg			Gly	–	CMRPT
P5	7029	2008	6	Phe	Gly					Lys	Gly	–	CMRPT
P6	8600	2010	0.125	Phe	Aln							–/African	PPNG/IRT
P7	6192	2008	0.5	Phe	Aln			Arg				American/African	PPNG/TRNG
P8	6847	2008	0.75	Phe	Aln			Arg	Thr			—	IRT
P9	5728	2007	3	Phe	Aln			Asn		Gln		American/African	PPNG/TRNG
P10	5116	2006	3	Phe	Gly							–	IRPT
P11	7592	2009	1.5		Tyr							–	
	6324	2008	0.75									Dutch/–	TRNG/IRP
	6333	2008	0.25									Dutch/–	TRNG/IRP
	7606	2009	0.25									–	IRP

[a] CIP, ciprofloxacin.
[b] Amino acids: Aln, alanine; Arg, arginine; Asn, asparagine; Asp, aspartic acid; Gln, glutamine; Glu, glutamic acid; Gly, glycine; His, histidine; Lys, lysine; Phe, phenylalanine; Ser, serine; Thr, threonine; Tyr, tyrosine.
[c] TetM plasmid types/β-lactamase plasmid types. –, Not known or not applicable.
[d] PPNG, penicillinase-producing *N. gonorrhoeae*; TRNG, plasmid-mediated tetracycline resistance; CMRP, chromosomally mediated resistance to penicillin; CMRT, chromosomally mediated resistance to tetracycline; AZRNG, azithromycin-resistant *N. gonorrhoeae*; IRP/IRT/IRPT, intermediate to penicillin, tetracycline, or both.

Biodisk. Solna, Sweden). Production of β-lactamase was assayed by the chromogenic cephalosporin method (Cefinase; BBL Microbiology Systems, Cockeysville, MD). The gonococcal reference strain ATCC 49226 was used as a control. The breakpoints used for categorization of susceptibility, intermediate, and resistance, according to the CLSI, were, respectively: ≤0.06, 0.125 to 0.75, and ≥ 1 μg/ml for ciprofloxacin; ≤0.06, 0.125 to 1, and ≥2 μg/ml for penicillin; and ≤ 0.25, 0.5 to 1, and ≥2 μg/ml for tetracycline. Isolates with MICs of ≥16 μg/ml for tetracycline were presumed to contain TetM plasmids. Penicillinase-producing *N. gonorrhoeae* (PPNG) was defined by positive results on the β-lactamase test. Resistant strains that lacked these criteria for plasmid-mediated resistance were considered chromosomally resistant (6).

Analysis of β-lactamase plasmids. The DNA plasmids of PPNG strains were analyzed by PCR as previously described (9).

Analysis of the TetM determinants. The TetM DNA plasmids were analyzed by PCR according to previous recommendations (32).

Determination of mutations in the nucleotide sequences of the *gyrA* and *parC* genes. PCR and direct DNA sequencing were performed to identify mutations in the *gyrA* and *parC* genes of the QRNG isolates. Chromosomal DNA was obtained and purified by using a Qiagen DNeasy tissue kit (Qiagen; Hilden, Germany) and used as a template for PCR. The amplification of the QRDRs within the *gyrA* and *parC* genes was performed as previously described (4, 24). Purified PCR amplicons of *gyrA* (279 bp) and *parC* (255 bp) genes of the resistant strains, as well as of the control strain, were directly sequenced using PCR primers. The nucleotide sequences were dideoxy cycle sequenced with fluorescent terminators (BigDye; Applied Biosystems, Foster City, CA) on an Applied Biosystems ABI Prism 3730 automated DNA sequencer. A similarity search for the deduced amino acid sequences was conducted using BLAST. Gene translation and frame determination, was performed with Transeq from the Emboss package (http://www.ebi.ac.uk/Tools/emboss/transeq/index.html). The alignments of translated peptides with reference sequences (U08817 for *gyrA* and U08907 for *parC*) for the determination of mutation positions were performed using the BioEdit sequence alignment editor (14).

Analysis of genetic diversity by PFGE. The isolates were analyzed by pulsed-field gel electrophoresis (PFGE), using a procedure based on previously described recommendations (20, 27). Briefly, nucleic acid was isolated from bacterial culture. Subsequently, each DNA preparation was digested with NheI (New England Biolabs, Ipswich, MA) overnight at 37°C in 100 μl of restriction endonuclease buffer containing 15 U of the enzyme. The CHEF DR II instrument (Bio-Rad Laboratories, Hercules, CA) was used to separate the fragments, with switch times of 1 s (initial) and 20 s (final), and a running time of 18 h at 6 V/cm. PFGE banding pattern similarities were determined using the Dice coefficients and the UPGMA (unweighted pair group method using arithmetic averages) clustering method by using the Molecular Analyst Fingerprinting Software package, version 1.12, of the Image Analysis System (Bio-Rad). A tolerance in the band positions of 1.5% was used. An 80% similarity threshold was used to divide the outputs from the dendrogram into clusters.

RESULTS

The characteristics of the 25 QRNG isolates included in the present study are presented in the Table 1 and Fig. 1. They represented 16.5% (25/152) of *N. gonorrhoeae* isolates obtained during a survey performed from 2006 to 2010. The frequencies of isolation of QRNG in relation of the total number of *N. gonorrhoeae* isolates recovered per year were as follows: 6.2% (4/65) in 2006, 6.3% (2/32) in 2007, 33.3% (11/33) in 2008, 36.8% (7/19) in 2009, and 33.3% (1/3) in 2010.

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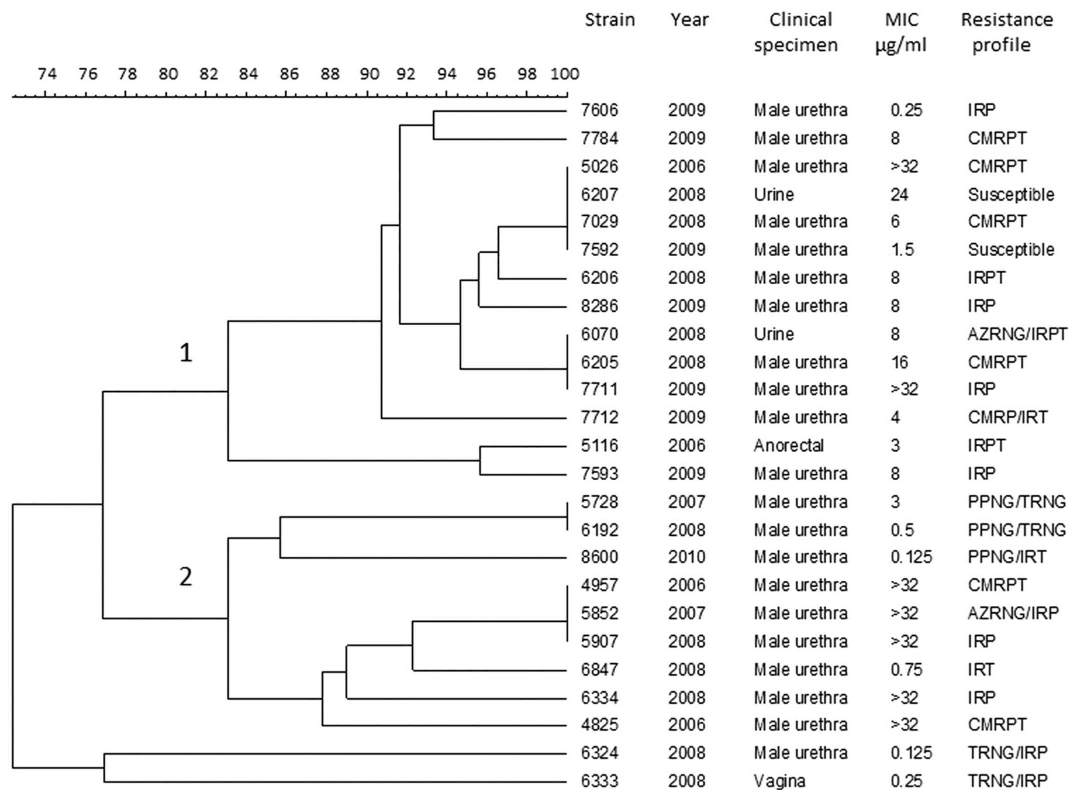

FIG. 1. Dendrogram based on NheI PFGE patterns of the genomic DNA of 25 ciprofloxacin-resistant *N. gonorrhoeae* isolates from Rio de Janeiro, Brazil. Abbreviations: PPNG, penicillinase-producing *N. gonorrhoeae*; TRNG, plasmid-mediated tetracycline resistance; CMRP, chromosomally mediated resistance to penicillin; CMRT, chromosomally mediated resistance to tetracycline; AZRNG, azithromycin-resistant *N. gonorrhoeae*; IRP/IRT/IRPT, intermediate to penicillin, tetracycline, or both.

In addition, the profiles of resistance to penicillin and tetracycline of the 25 QRNG isolates were determined. Most of them (32%) had chromosomal resistance to either penicillin and tetracycline, three (12%) isolates harbored a beta-lactamase plasmid (Africa type), and four (16%) isolates exhibited plasmid-mediated tetracycline resistance (two had the American type of plasmids, and two had the Dutch type) (Table 1).

In order to determine the mechanisms associated with quinolone resistance in the 25 QRNG isolates, we analyzed the mutation patterns in the *gyrA* and *parC* genes. Eleven mutation patterns were identified: eight were only found among fully resistant isolates, and three were observed among the intermediate isolates (Table). The predominant pattern (P1) was present in 10 (40%) of the QRNG isolates and comprised double mutations at codons 91 (Ser-91 to Phe) and 95 (Asp-95 to Gly) in the *gyrA* gene and a single mutation in the *parC* gene at codon 87 (Ser-87 to Arg). All 10 isolates possessing the P1 pattern exhibited high-level resistance to ciprofloxacin (MIC ≥ 4 μg/ml); for 5 the MICs were ≥24 μg/ml. The second most common mutation pattern was P2, found in three isolates. P2 pattern was characterized by the presence of three mutations in the QRDR of *gyrA* at codons 91 (Ser-91 to Phe), 95 (Asp-95 to Gly), and 102 (Gln-102 to His) and a single mutation in the *parC* gene at codon 87 (Ser-87 to Arg). The other nine mutation patterns were identified in a single isolate each. Patterns P6, P10, and P11 did not show mutations in *parC*, but P11 showed a single mutation at codon 95 (Asp-95 to Tyr). Three of the six isolates that were intermediate by susceptibility testing to ciprofloxacin did not show any mutation in *gyrA* and *parC*, whereas the other three isolates had double mutations at codons 91 (Ser-91 to Phe) and 95 (Asp-95 to Ala) in *gyrA* and none, one (Ser-87 to Arg), or two (Ser-87 to Arg; Ser-88 to Thr) mutations in *parC*.

Genomic DNAs of the 25 QRNG isolates were examined by PFGE after treatment with the endonuclease NheI. The application of the Dice coefficient and an 80% similarity threshold allowed the identification of 17 profiles that were grouped in two clonal groups (CG-1 and CG-2) encompassing 23 isolates. The two remaining isolates showed unique profiles not related to each other (Fig. 1). In CG-1, 11 of the 14 isolates were resistant to high levels of ciprofloxacin (MIC ≥ 4 μg/ml), whereas 5 of the 9 isolates in CG-2 showed MICs of >32 μg/ml for ciprofloxacin. All of the isolates expressing plasmid-mediated resistance to penicillin or tetracycline were grouped in CG-2. P1 was the most common mutation pattern (10/25) found among isolates included in both clonal groups. Although showing similar restriction profiles, some isolates in both CG expressed different mutation patterns in their *gyrA* and *parC* genes and also showed a variety of additional resistance profiles.

DISCUSSION

The emergence and dissemination of resistance to antimicrobial agents among *N. gonorrhoeae* isolates have limited the variety of drugs that can be used for the treatment of gono-

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coccal infections. Ciprofloxacin has been one of the recommended first-line therapies for these infections worldwide, including in Brazil. However, a number of studies have revealed an increasing frequency of ciprofloxacin-resistant *N. gonorrhoeae* isolates in different geographic regions (13, 26, 31). In Brazil, information about epidemiologic and antimicrobial susceptibility aspects of this pathogen is still scarce (2, 3, 10, 11).

Decreased susceptibility to ciprofloxacin has been described in previous studies with *N. gonorrhoeae* isolates recovered in different Brazilian regions, with rates ranging from 1.7% (2) to 5.5% (12) to 9.7% (11). However, in 2007, full resistance to ciprofloxacin was found among isolates in two major Brazilian cities: 7.7% among isolates from Rio de Janeiro (A. A. Uehara and S. E. L. Fracalanzza, unpublished data) and 3.1% among isolates from São Paulo (3).

The identification of ciprofloxacin-resistant *N. gonorrhoeae* isolates in association with the characterization of QRDR mutations in the *gyrA* and *parC* genes, which are significantly associated with ciprofloxacin resistance (4, 8, 24), should be used to monitor the evolution of resistant genotypes patterns and the gonococcal transmission networks.

A correlation between levels of ciprofloxacin resistance and the location and number of mutations in the *gyrA* and *parC* genes has been observed in different studies (21, 30, 35), although it has not been found in others (19, 29).

Our data revealed that almost all (18/19) of the full ciprofloxacin-resistant isolates had two or three *gyrA* mutations and, of these, 17 isolates had at least one mutation in *parC*. These results are consistent with previous findings indicating that *gyrA* (Ser-91 to Phe; Asp-95 to Gly) and *parC* (Ser-87 to Arg) mutations are related to higher MIC levels (13, 22, 35). In addition, as previously reported (23), we found the same pattern of mutation in *parC* gene in association with different levels of ciprofloxacin-resistance (0.5 to 32 μg/ml), suggesting that the number of mutations in the *parC* gene did not correlate with the level of ciprofloxacin resistance.

In the present study, the most common patterns of mutation, found in 40% of QRNG isolates, were the Ser-91-to-Phe and Asp-95-to-Gly substitutions in the *gyrA* gene, associated with a Ser-87-to-Arg substitution in the *parC* gene (MIC $\geq$ 4 μg/ml). Our results are in accordance with previous reports (13, 17, 18, 22). Four isolates, including three that were intermediate and one resistant to ciprofloxacin, showed an Asp-95-to-Ala mutation in the *gyrA* gene. In a previous study such a characteristic was shown to be predominant among resistant isolates (22).

A new mutation at codon 102 (Gln-102 to His) in the *gyrA* gene was found to account for 2% of the QRNG tested in China (30). Interestingly, in our study, three (12%) QRNG exhibited the same mutation at codon 102 associated with two additional mutations in *gyrA* (Ser-91 to Phe and Asp-95 to Gly), and all three isolates were highly resistant to ciprofloxacin with MICs of $\geq$8 μg/ml. However, more data are necessary to establish the existence of any correlation among this type of mutation and the level of ciprofloxacin resistance. A new mutation pattern was observed in one fully resistant isolate (isolate 7592, MIC = 1.5 μg/ml) that showed only a single alteration in the *gyrA* gene at codon 95 (Asp-95 to Tyr). This alteration was described in a previous study (22), but the isolate identified had two other mutations associated with the *gyrA* gene.

In the present study, no correlation was detected among the number and type of mutations in *parC* and the level of ciprofloxacin resistance as previously suggested (19). Isolates that were intermediate to ciprofloxacin generally harbored a single or double mutation in *gyrA* and either a single or no mutation in the *parC* gene (13, 24). We detected three intermediate isolates with the same double mutations in *gyrA* gene (Ser-91 to Phe and Asp-95 to Ala), but two of them had double mutations in *parC* and one had no mutation. In contrast, no mutations in *gyrA* and *parC* genes were found in the other three isolates with intermediate to ciprofloxacin, suggesting the presence of other mechanisms of resistance.

The genetic diversity among ciprofloxacin-resistant strains has been previously studied. In Korea, 13 SpeI and 15 NheI restriction profiles were found among 25 and 61 QRNG isolates, respectively (33, 34), whereas in Canada, 39 NheI restriction profiles were identified among 68 QRNG isolates (19). Three clonal groups were identified among 26 QRNG isolates from Greece (28). In our study, 23 of 25 isolates were clustered in two clonal groups (CG-1 and CG-2), considering an 80% similarity threshold. This result suggests that transmission of QRNG within a limited geographic region may be clonal. The CG-1 and CG-2 showed different features. Most of the QRNG isolates in CG-1 showed MICs for ciprofloxacin of between 3 to 8 μg/ml, while five of the six strains in the CG-2 with full resistance to ciprofloxacin had MICs of $>$32 μg/ml. It is interesting that, with only one exception, all isolates expressing full resistance to ciprofloxacin did not show plasmid-mediated penicillin or tetracycline resistance. However, four of the six isolates intermediate to ciprofloxacin harbored plasmids associated with resistance to penicillin and or tetracycline.

In the present study, we demonstrate that isolates with different mutation patterns in their *gyrA* and *parC* genes and with a variety of additional resistance profiles may have similar restriction profiles when examined by PFGE, indicating that these resistance characteristics were not sufficient to genetically discriminate these isolates. A limitation of analysis described here is that these data were obtained from patients living in Rio de Janeiro and may not be applicable to other regions of Brazil.

The results of the present study indicate that both clinicians and microbiologists should be aware of the occurrence of ciprofloxacin resistance among *N. gonorrhoeae* in Rio de Janeiro so that these isolates can be promptly detected and appropriately treated and widespread dissemination avoided. Furthermore, changes in the recommended treatment of gonococcal diseases may be necessary in the area investigated. Follow-up studies with strains collected in this area and in other areas of this large country are needed in order to better trace the national scenario of antimicrobial resistance among *N. gonorrhoeae* isolates from Brazil.

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