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Drift in Susceptibility of *Neisseria gonorrhoeae* to Ciprofloxacin and Emergence of Therapeutic Failure

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Ciprofloxacin, 500 mg, was introduced as the first-line therapy for gonorrhea at St. Mary's Hospital, London, in 1989, when a surveillance program was initiated to detect the emergence of resistance. Isolates of *Neisseria gonorrhoeae* from consecutive patients attending the Jefferiss Wing, Genitourinary Medicine Clinic at St. Mary's Hospital, between 1989 and 1997 have been tested for susceptibility to ciprofloxacin by using an agar dilution breakpoint technique. Isolates considered potentially resistant (MIC, >0.12 μg/ml) were further characterized by determination of the MICs of ciprofloxacin, nalidixic acid, and penicillin, auxotyped and serotyped, and screened for mutations in the DNA gyrase gene, *gyrA*, and the topoisomerase IV gene, *parC*. A total of 4,875 isolates were tested. While the majority of isolates were highly susceptible (MIC, ≤0.008 μg of ciprofloxacin/ml), there was a drift toward reduced susceptibility in *N. gonorrhoeae* isolated between 1993 and 1996 ($P < 0.001$). In 1997 this drift was reduced but remained above pre-1993 levels. Isolates from 18 patients were classed as potentially resistant (MIC, >0.12 μg/ml); all of these belonged to serogroup B, and NR/IB-1 was the most common auxotype/serovar class. The infections in 14 of the 18 patients were known to be acquired abroad, and 5 were known to result in therapeutic failure. The surveillance program has established that ciprofloxacin is still a highly effective antibiotic against *N. gonorrhoeae* in this population. However, it has identified a drift in susceptibility which may have resulted from increased usage of ciprofloxacin. High-level resistance has now emerged, although treatment failure is still uncommon.

Ciprofloxacin is a fluoroquinolone that is highly effective against *Neisseria gonorrhoeae*. The spread of isolates of *N. gonorrhoeae* exhibiting chromosomal and/or plasmid-mediated resistance to penicillin in the last decade has resulted in increased usage of ciprofloxacin for the therapy of gonorrhea. Ciprofloxacin has the advantage of being administered as a single oral dose of either 250 or 500 mg (11). The relationship between dosage, MIC of ciprofloxacin for the infecting isolate, and therapeutic failure was unknown when ciprofloxacin was introduced as the first-line therapy for gonorrhea. Therapeutic failure was undocumented, and resistance mechanisms, attributed to mutations in DNA gyrase genes and reduced permeability of the cell membrane in other organisms, were unknown for *N. gonorrhoeae*. Subsequently, low-level resistance was reported for *N. gonorrhoeae* isolates for which MICs were 0.06 to 0.5 mg/liter (8, 14, 18, 21–23, 25, 27, 29, 30), followed by reports of high-level resistance in isolates for which MICs were >1 mg/liter (3, 4, 6, 8, 22, 28–30). Mutations in the DNA gyrase gene, *gyrA*, have been found in these isolates (6, 7, 9, 26), and high-level resistance has been associated with additional mutations in the topoisomerase gene, *parC*, in some isolates (8, 10).

Ciprofloxacin as a single 500-mg dose was introduced as the first-line herapy for gonorrhea at St. Mary's Hospital in 1989. At the same time we initiated a surveillance program to monitor the susceptibility of all isolates to ciprofloxacin and to detect the emergence of resistance and hence the efficacy of ciprofloxacin as a first-line therapy. We report the results of 9 years of surveillance.

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MATERIALS AND METHODS

Collection of isolates. Isolates of *N. gonorrhoeae* from consecutive patients attending the Jefferiss Wing, Genitourinary Medicine Clinic at St. Mary's Hospital, London, United Kingdom, between January 1989 and December 1997 have been monitored for susceptibility to ciprofloxacin. *N. gonorrhoeae* was isolated and identified as described previously (31). All isolates were screened for penicillinase production by using the chromogenic cephalosporin Nitrocefin (Unipath, Basingstoke, United Kingdom) and for high-level tetracycline resistance by using growth on GC agar supplemented with 10 μg of tetracycline/ml. All isolates were purified by subculture onto GC agar base (Difco Laboratories, East Molesey, United Kingdom) supplemented with 1% IsoVitaleX but without antibiotics (GC medium) and were stored in 15% glycerol broth in the vapor phase of liquid nitrogen (−132°C).

Susceptibility testing. Isolates were tested for susceptibility to ciprofloxacin by an agar dilution breakpoint technique described previously (16) by using ciprofloxacin (Bayer UK, Newbury, United Kingdom) at three concentrations, 0.008, 0.03, and 0.12 μg/ml. Isolates that grew with all three concentrations of ciprofloxacin tested (predicted MIC, >0.12 μg/ml) were considered potentially resistant, and resistance was confirmed by determining the full range of concentrations of ciprofloxacin for these isolates (range, 0.008 to 32 μg/ml); in addition, they were tested for susceptibility to nalidixic acid (range, 4 to 512 μg/ml; Sigma Chemical Co., Poole, United Kingdom) and penicillin (range, 0.015 to 4 μg/ml; Adatabs; Mast Laboratories, Bootle, United Kingdom). The method used was similar to the breakpoint technique, with the exception that the inoculum was 10^4 CFU. All susceptibility testing was controlled by using World Health Organization strains A through E and a strain known to show reduced susceptibility to ciprofloxacin, 81-10, kindly given by I. Phillips, St. Thomas' Hospital, London, United Kingdom. In addition, a subset of 24 isolates, including 4 susceptible isolates, 14 potentially resistant isolates, and the 6 control strains described above, was tested by the method recommended by the National Committee for Clinical Laboratory Standards (NCCLS) (24). The NCCLS method uses GC agar base with a defined supplement, and the results are read after 24 h of incubation; in contrast, the method described above uses Diagnostic Sensitivity Test Agar (Unipath, Basingstoke, United Kingdom) supplemented with 1% IsoVitaleX and 5% lysed horse blood, and the results are read after 48 h of incubation.

Characterization of resistant isolates. Isolates exhibiting reduced susceptibility to ciprofloxacin were further characterized by auxotyping and serotyping as described previously (31).

Determination of changes in *gyrA*. The DNA sequence of the quinolone resistance-determining region (QRDR) of the *gyrA* gene was determined for all the isolates showing reduced susceptibility to ciprofloxacin. Four clinical isolates known by MIC determination to be susceptible to ciprofloxacin, as well as the isogenic mutants of one (FA19) of these isolates, FA19E and FA19G (2), were used as controls. Two primers (CAI1, 5′-^{1}ATGACCGACGCAACCATCCG^{20}-3′, and CAI2, 5′-^{1020}GCCGAAACTGTCTTGCAGCG^{1001}-3′) based on the sequence described by Belland et al. (2) were used to amplify a 1-kb region of the *gyrA* gene. The reaction was performed in 50-μl volumes, and the reaction mixture contained DNA (5 μl of whole-cell suspension of an overnight growth on GC agar adjusted to approximately 10^8 CFU/ml or 100 ng of chromosomal DNA), 200 μM each deoxynucleoside triphosphate (dATP, dGTP, dCTP, dTTP; Boehringer Mannheim, Lewes, United Kingdom), 100 pmol of each primer, and 2.5 U of *Taq* polymerase in the manufacturer's buffer with 1.5 mM $MgCl_2$. After an initial denaturation step of 94°C for 5 min, 30 cycles of 60 s at 94°C, 60 s at 60°C, and 60 s at 72°C were followed by an extension step of 10 min at 72°C. The PCR product was checked by electrophoresis on 1% agarose (IBI Limited, Cambridge, United Kingdom) gel and purified by using Geneclean (Anachem, Luton, United Kingdom) as described by the manufacturers. Two internal primers (CAI3, 5′-^{155}TGCACCGGCGCGTACTGTA^{173}-3′, and CAI4, 5′-^{330}CAGC ACATAACGCATAGC^{313}-3′) were used to determine the sequence of a 176-bp region either by cycle sequencing performed with the Toyobo Sequencing kit (Cambridge Bioscience, Cambridge, United Kingdom) and subsequent polyacrylamide gel electrophoresis and autoradiography or by automated sequencing with an ABI 310 Genetic Analyzer and a fluorescent dye terminator cycle sequencer (Perkin-Elmer Applied Biosystems, Warrington, United Kingdom). In addition, the sequence of the QRDR of *parC* was determined for all isolates for which the MIC of ciprofloxacin was 16 μg/ml and for the controls, FA19, FA19E, and FA19G, in a similar manner, by using the primers CAI5 (5′-^{1}ATGAATAC GCAACCGCACGC^{20}-3′) and CAI6 (5′-^{1020}GAAGGTATCGGTATCGATCG ^{1001}-3′) to amplify a 1-kb region and two internal primers, CAI7 (5′-^{161}TTGCC ATGCGCGATATGGGT^{180}-3′) and CAI8 (5′-^{420}GGACAACAGCAATTCCG AAA^{401}-3′), to determine the sequence of a 260-bp region under the conditions described above.

Statistical analysis. Differences in the numbers of isolates with various levels of susceptibility (full susceptibility, reduced susceptibility, or resistance) were determined by the chi-square test.

RESULTS

Surveillance of susceptibility to ciprofloxacin. A total of 4,962 isolates were stored between January 1989 and December 1997, of which 4,875 (98%) were successfully retrieved and tested for susceptibility to ciprofloxacin. This collection contains >95% of possible isolates from each year. The distribution of isolates into those that were fully susceptible (predicted MIC, ≤0.008 μg/ml), those with reduced susceptibility (predicted MIC, 0.015 to 0.12 μg/ml), and those resistant (predicted MIC, >0.12 μg/ml) to ciprofloxacin remained stable prior to 1993, but between 1993 and 1996 there was a significant decrease in the number of susceptible isolates (93.9 versus 76.1%) and a concomitant increase in the number of isolates exhibiting reduced susceptibility (5.3 versus 23.3%; $P < 0.001$) (Table 1). In 1997 the number of isolates exhibiting reduced susceptibility fell to 14.7%, but this level still remains higher than that between 1989 and 1993. The number of potentially resistant isolates remained low until 1996, <1% of the total tested in each year, but rose in 1997 to 1.5% of isolates tested.

Characteristics of resistant isolates. For 22 isolates from 18 patients, MICs of ciprofloxacin were >0.12 μg/ml (Table 2). Repeat isolates were available for four patients, and in none of these cases did the phenotype or the MIC differ from that for the initial isolate; hence, all further studies were performed on a single isolate from each patient. Of the 18 isolates, the MICs of ciprofloxacin were 0.25 to 0.5 μg/ml for 14 isolates and 16 μg/ml for 4 isolates. Fourteen of these isolates, together with 4 susceptible isolates, were retested by methods recommended by the NCCLS, and the MICs were identical (for 15 of 18 isolates) or differed by a single concentration (for 3 of 15 isolates).

Seven of the resistant isolates were penicillinase producers, and the remaining 11 isolates exhibited decreased susceptibility to penicillin (MIC, 0.12 to 0.5 μg/ml) or were chromosomally resistant (MIC, ≥1.0 μg/ml) (Table 2). None of the isolates exhibited plasmid-mediated high-level resistance to tetracycline. The isolates belonged to serogroup B and to various auxotype/serovar (A/S) classes, NR/IB-1 being the most common (six isolates). In the 14 instances where information was available, the infections were all acquired abroad (Table 2). Treatment failure occurred for five patients. For four patients, *N. gonorrhoeae* of the same A/S class was isolated on return to the clinic; for three of these isolates, the MICs of ciprofloxacin were 0.25 or 0.5 μg/ml, and for one, the MIC was 16.0 μg/ml. One patient returned to the clinic 9 days after receiving ciprofloxacin treatment, and intracellular gram-negative diplococci were seen on the Gram stain of the urethral discharge. Culture was not performed on this occasion, but it was still considered a treatment failure. The MIC of ciprofloxacin for the initial isolate taken from this patient was 16 μg/ml.

TABLE 1. Susceptibilities of clinical isolates of *N. gonorrhoeae* between 1989 and 1997

Yr	Total no. of isolates	Total no. tested	No. (%) of isolates for which the following ciprofloxacin MIC is predicted:		
			≤0.008 μg/ml	0.015–0.12 μg/ml	>0.12 μg/ml[a]
1989	678	654	616 (94.2)	36 (5.5)	2 (0.3)
1990	928	890	840 (94.4)	49 (5.5)	1 (0.1)
1991	637	630	611 (97.0)	17 (2.7)	2 (0.3)
1992	479	479	451 (94.2)	28 (5.8)	0
1993	361	361	339 (93.9)	19 (5.3)	3 (0.8)
1994	401	401	361 (90.0)	40 (10.0)	0
1995	430	428	348 (81.3)	77 (18.0)	3 (0.7)
1996	486	481	366 (76.1)	112 (23.3)	3 (0.6)
1997	562	551	462 (83.8)	81 (14.7)	8 (1.5)

[a] Data include four repeat isolates from treatment failures.

Changes in *gyrA* and *parC*. Determination of the QRDR of *gyrA* in 17 of these 18 isolates and in known sensitive (strains FA19, 850, 895, 930, and 994) and resistant (81-10, FA19E, and FA19G) controls showed six patterns (Table 3). Isolates that were fully susceptible to ciprofloxacin (MIC, ≤0.03 μg/ml) showed no changes from the published sequence of FA19, a wild-type genetically characterized strain (Table 3). Isolates for which MICs were 0.25 to 0.5 μg/ml showed either a change from serine to phenylalanine or tyrosine at position 91 (corresponding to S-83 in *Escherichia coli*) or a change from aspartic acid to asparagine at position 95 (corresponding to D-87 in *E. coli*). In contrast, the laboratory mutants FA19E and FA19G (2), for which the MICs are 4 and 16 μg/ml, respectively, showed changes at both positions 91 and 95. The clinical isolates for which the MIC of ciprofloxacin was 16 μg/ml also showed two changes, but in all four isolates the change at position 95 was from aspartic acid to glycine rather than to asparagine (Table 3). Determination of the QRDR of the *parC* gene in the four isolates exhibiting high-level resistance showed a single change in the DNA sequence from GAC to GAG, but this did not result in an amino acid change at position 88. The susceptible control showed the expected Ser-88 and Glu-91, FA19E showed the Ser-88-to-Pro mutation, and FA19G showed both the Ser-88-to-Pro and the Glu-91-to-Lys mutation, as expected (2).

DISCUSSION

The surveillance of isolates from consecutive patients attending this clinic since January 1989 has shown that (i) resistance to ciprofloxacin resulting in therapeutic failure has emerged and (ii) a drift in susceptibility has occurred over time. Cipro-

TABLE 2. Characteristics of isolates of *N. gonorrhoeae* exhibiting reduced susceptibility to ciprofloxacin

Isolate no.[a]	Yr of isolation	Sex of patient[b]	Site[c]	MIC[d] (μg/ml) of:		Penicillinase production	A/S class[e]	Country of infection	Treatment failure
				CIP	PEN				
990	1989	M	U	0.25	>4.0	+	NR/IB-1	Philippines	NK[f]
1075	1989	M	U	0.5	0.5	−	Pro/IB-1	NK	NK
1390*	1989	M	U	0.25	>4.0	+	NR/IB-22	Thailand	Yes
2678	1991	M	R	0.5	>4.0	+	Pro/IB-8	Thailand	NK
2887	1991	M	U	0.25	>4.0	+	Pro/IB-14	Australia	No
3633	1993	F	U	0.5	1.0	−	Pro/IB-4	Japan	No
3761	1993	M	U	>0.12	>4.0	+	NR/IB-1	NK	NK
3767	1993	M	U	0.25	0.5	−	NR/IB-1	India	NK
4300	1995	M	U	16.0	>4.0	+	Pro/IB-8	Philippines	Yes
4482*	1995	M	U	0.5	0.5	−	Pro/IB-26	Grand Canary	Yes
4688*	1996	M	U	0.5	0.5	−	NR/IB-1	Thailand	Yes
5218	1997	M	U	1.0	0.25	−	Pro/IB-7	India	No
5259	1997	M	U	16.0	0.25	−	Pro/IB-18	Thailand	NK
5297	1997	F	UC	16.0	>4.0	+	NR/IB-1	Austria	NK
5515	1997	M	R	0.5	0.5	−	NR/IB-7	NK	NK
5532	1997	M	U	0.5	1.0	−	Pro/IB-1	Sri Lanka	NK
5621*	1997	F	U	16.0	0.25	−	NR/IB-1	Thailand	Yes
5704	1997	M	U	0.5	1.0	−	Pro/IB-1	NK	NK

[a] *, two consecutive isolates were taken from each of these patients.
[b] M, male; F, female.
[c] U, urethra; R, rectum; UC, urethra and cervix.
[d] The MIC of nalidixic acid was ≥256 μg/ml for all isolates. CIP, ciprofloxacin; PEN, penicillin.
[e] Pro, proline requiring; NR, nonrequiring.
[f] NK, not known.

floxacin is a highly active antibiotic which has now replaced penicillin as the antibiotic most commonly used for gonorrhoea in the United Kingdom (12). At the Jefferiss Wing, a single 500-mg dose of ciprofloxacin has been in use as the first-line therapy for more than 9 years. Therapeutic failure has been uncommon during this period and has occurred in only five patients, to our knowledge. For three patients, the MIC for the infecting isolate was 0.25 or 0.5 μg/ml. Two additional patients were infected with isolates for which the MIC was 0.5 μg/ml, and their treatment was not known to result in failure. Therapy for patients with two of the highly resistant isolates failed, and the result of therapy was unknown for the remaining two patients. Such high-level resistance has been documented most commonly in the Far East, the Philippines, Hong Kong, and Japan (1, 20, 22, 29) or in cases of infection acquired in these countries. We have also found that all of the potentially resistant isolates we have seen, for which we have demographic data, were acquired abroad.

A MIC of ≥1 μg/ml has been suggested as indicating resistance to ciprofloxacin (24). However, we have found that treatment failure occurred for patients infected with isolates for which MICs were 0.25 to 0.5 μg/ml, which has been classified as intermediate resistance. Methodologies are different in different countries and are known to affect the MIC obtained (32), but MICs for these isolates did not drift to further resistance when tested on GC agar. In our limited experience, we have found that therapeutic failure with ciprofloxacin is common for patients with isolates exhibiting both intermediate resistance and high-level resistance, even when a 500-mg dose is used for therapy. We, therefore, believe that it is important that isolates in both categories continue to be closely monitored in order to clarify the relationship between dosage, MIC, and therapeutic failure.

We have confirmed that the mutations resulting in resistance to ciprofloxacin in clinical isolates of *N. gonorrhoeae* are associated with changes in the QRDR of the *gyrA* gene ((7, 25, 26) and that double mutations are present in highly resistant isolates. Mutations in the QRDR of *parC* have also been associated with higher levels of resistance to quinolones in laboratory mutants (2) and in some clinical isolates (6, 8). The absence of *parC* mutations in gonococcal isolates with *gyrA* mutations in this study and in 71% of the isolates screened by Deguchi et al. (8) suggests that there may be additional mechanisms that combine with mutations in *gyrA* to result in high-level resistance. In *Staphylococcus aureus* (19) and *Streptococcus pneumoniae* (33), resistance to fluoroquinolones has been associated with efflux mechanisms. Resistance to hydrophobic agents in *N. gonorrhoeae* is known to be mediated by the *mtrRCDE* efflux system (15), and the acquisition of this operon in transformants has been shown to result in small decreases in susceptibility (13). The contribution of the efflux system to ciprofloxacin resistance, and the possibility of mutations in other regions of *gyrA* and *parC*, in clinical isolates of *N. gonorrhoeae* requires further investigation.

Detection of mutations in *gyrA* and *parC*, as indicators of resistance, by a molecular-based technique (9, 10) has been described elsewhere. While this may not be cost-effective for most clinical or public health laboratories, it does offer an alternative to susceptibility testing that is rapid and may overcome some of

TABLE 3. Changes in the QRDR of *gyrA* in clinical isolates of *N. gonorrhoeae*

Pattern	Amino acid residue[a] at position:		Strain(s)	Range of ciprofloxacin MICs (μg/ml)
	91	95		
1	Ser	Asp	FA19, 850, 895, 930, 994	0.008–0.03
2	Tyr	Asp	4688	0.5
3	Phe	Asp	81-10, 1075, 2678, 2887, 3633, 4482, 5218, 5532, 5704	0.25–1.0
4	Ser	Asn	990, 1390, 3767, 5515	0.25
5	Phe	Asn	FA19E, FA19G	4.0, 16.0
6	Phe	Gly	4300, 5259, 5297, 5621	16.0

[a] Ser, serine; Tyr, tyrosine; Phe, phenylalanine; Asp, aspartic acid; Asn, asparagine; Gly, glycine.

the technical difficulties. This approach may also be appropriate as an adjunct to molecular-based detection of gonorrhea (5), which does not provide a viable organism. Double mutation in the *gyrA* gene appears to be the best predictor of high-level quinolone resistance at present but does not address low-level resistance. If this approach is to be successful, all mechanisms that contribute to resistance need to be understood.

Ciprofloxacin remains a highly active antimicrobial agent for the treatment of gonorrhea at this London hospital. However, in our population there has been a drift in isolates toward reduced susceptibility, which is not presently affecting therapeutic success. This suggests that continual use of this agent is reducing the inherent susceptibility of the gonococcal population. A single stat dose of 500 mg is currently recommended, but it may be advisable to consider either a higher single dose, such as 750 mg, or a lower dose administered over several days in order to prevent the emergence of resistant isolates and retard the drift to reduced susceptibility. In the past year we have encountered an increase in the number of isolates exhibiting high-level resistance, but they have all been isolated from patients with imported infections and we have not seen any such isolates that have been acquired in the United Kingdom. There is increasing evidence that these quinolone-resistant isolates are most prevalent in the Western Pacific (17). Therefore, it seems prudent for clinicians to obtain information regarding recent travel for all patients presenting with symptoms of gonorrhea and to treat any patients who have had sexual contact in the Western Pacific with an alternative antibiotic such as ceftriaxone. The use of an effective antibiotic at the patient's initial visit, without waiting for the result of the culture and susceptibility testing, will prevent the further spread of quinolone-resistant *N. gonorrhoeae* into the local community. This surveillance program has achieved its objectives and will be continued in order to help prolong the useful life of ciprofloxacin for the treatment of gonorrhea.

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