Development of Resistance to Nalidixic Acid and the Fluoroquinolones after the Introduction of Norfloxacin and Ofloxacin

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Since 1975, the group Resistenz of the Paul-Ehrlich-Gesellschaft für Chemotherapie has monitored the development of resistance in isolates of members of the family *Enterobacteriaceae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Enterococcus faecalis* in the Federal Republic of Germany, West Berlin, Austria, and Switzerland. Despite a marked increase in the use of 4-quinolones, there was no increase in the percentages of nalidixic acid-resistant strains of *Enterobacteriaceae* between 1975 and 1986. However, different bacterial species showed considerable variation, and there were also considerable differences in the percentages of nalidixic acid-resistant strains of *Enterobacteriaceae* from different centers. The frequency of resistance to fluoroquinolones was unchanged from 1983 to 1986 and was less than 4% in all species except *P. aeruginosa*. In this species, there was an increase from about 3 to 10% from 1983 to 1986 for strains for which the MIC was fourfold above the mode MIC of ciprofloxacin, enoxacin, and ofloxacin.

Nalidixic acid and similar antimicrobial agents have been available for more than 20 years, primarily for the treatment of urinary tract infections caused by gram-negative enteric bacteria. The main limitation of their use has been the restricted antibacterial spectrum and the ease with which bacterial resistance develops, even during treatment (15). In the past several years, a number of structural analogs of nalidixic acid—the fluoroquinolones—have been developed: ciprofloxacin (21), enoxacin (3), norfloxacin (9), and ofloxacin (17); these show a broad antibacterial spectrum with high intrinsic activity. Between 1984 and 1986, when the first two fluoroquinolones, norfloxacin and ofloxacin, were launched in the Federal Republic of Germany, there was a marked increase in the use of these drugs, about 1.7 and 2.0 million prescriptions of norfloxacin and ofloxacin, respectively, during the first 18 months (18). To evaluate the influence of this drug usage on the prevalence of 4-quinolone resistance, we analyzed the susceptibility data from the group Resistenz of the Paul-Ehrlich-Gesellschaft für Chemotherapie, a cooperative venture between institutes for medical microbiology that have surveyed antibiotic susceptibility of clinical isolates in the Federal Republic of Germany, West Berlin, Austria, and Switzerland since 1975.

Antimicrobial agents. The following 4-quinolones were kindly provided by the manufacturers: nalidixic acid (Winthrop, Neu-Isenburg, Federal Republic of Germany), ciprofloxacin (Bayer, Leverkusen, Federal Republic of Germany), enoxacin (Parke-Davis, Freiburg, Federal Republic of Germany), norfloxacin (Merck Sharp & Dohme, Munich, Federal Republic of Germany), and ofloxacin (Hoechst, Frankfurt, Federal Republic of Germany). Other antimicrobial agents were purchased from their manufacturers.

Bacterial strains. In each of the 20 to 30 laboratories, about 100 to 200 fresh clinical isolates were randomly collected over a period of 1 week, usually once or twice a year (the

first week of May and November). Isolates were identified by uniform standard methods for species. Isolates of members of the family *Enterobacteriaceae*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* regarded as causative pathogens were studied. Repeat isolates of the same biotype from one patient were excluded. From rarely isolated species each isolate was included, and from less rare species every second isolate was included. In total, 24,679 strains were under investigation: 9,944 *Escherichia coli*, 3,005 *Proteus mirabilis*, 2,645 *Klebsiella* spp., 1,575 *Enterobacter* spp., 1,535 *Salmonella* spp., 1,311 *S. aureus*, 987 *E. faecalis*, 934 *P. aeruginosa*, 853 other *Proteus* spp., 440 *Serratia* spp., and 438 *Citrobacter* spp.

Susceptibility testing. To obtain comparable results, all the participating institutes used the same standardized method. From 1975 to 1981, we used the ICS agar diffusion test (7) with Mueller-Hinton agar as a nutrient medium. In 1982, we changed to the microdilution method according to recommendations by the National Committee for Clinical Labora-

FIG. 1. Development of nalidixic acid resistance in members of the *Enterobacteriaceae* from 1975 to 1986. NOR, Introduction of norfloxacin; OFX, introduction of ofloxacin. Criteria for nalidixic acid resistance: ≤ 13 mm in the agar diffusion test; ≥ 32 $\mu\text{g/ml}$ in the microdilution test.

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TABLE 1. Activity of 4-quinolones on strains isolated in November 1986

Organism	Agent	MIC ($\mu\text{g/ml}$)		
		Range	50%	90%
<i>Enterobacteriaceae</i> (<i>n</i> = 1,334)	Nalidixic acid	1– $\geq$ 512	4	8
	Enoxacin	0.031– $\geq$ 128	0.063	0.125
	Ofloxacin	0.016– $\geq$ 32	0.063	0.125
	Norfloxacin	$\leq$ 0.008– $\geq$ 32	0.031	0.031
	Ciprofloxacin	$\leq$ 0.008– $\geq$ 32	$\leq$ 0.008	0.016
<i>P. aeruginosa</i> (<i>n</i> = 286)	Nalidixic acid	16– $\geq$ 512	256	$\geq$ 512
	Enoxacin	$\leq$ 0.063–32	0.5	2
	Ofloxacin	0.125– $\geq$ 32	1	8
	Norfloxacin	0.031–16	0.25	1
	Ciprofloxacin	$\leq$ 0.008–4	0.063	0.5
<i>S. aureus</i> (<i>n</i> = 383)	Nalidixic acid	16– $\geq$ 512	64	128
	Enoxacin	0.125–32	0.5	1
	Ofloxacin	0.06– $\geq$ 32	0.25	0.5
	Norfloxacin	0.06– $\geq$ 32	0.5	1
	Ciprofloxacin	0.03– $\geq$ 32	0.25	0.5
<i>E. faecalis</i> (<i>n</i> = 288)	Nalidixic acid	256– $\geq$ 512	$\geq$ 512	$\geq$ 512
	Enoxacin	1– $\geq$ 64	4	8
	Ofloxacin	0.25–16	2	4
	Norfloxacin	0.25– $\geq$ 32	2	4
	Ciprofloxacin	0.125–8	0.5	1

tory Standards (11). Serial twofold dilutions of each antimicrobial agent were prepared in nonsupplemented Mueller-Hinton broth. The final inoculum was 10^5 CFU/ml. In 1982 and 1983, the commercially prepared microdilution system Micur-RST (Boehringer GmbH, Mannheim, Federal Republic of Germany) was used, and since 1984 the broth trays have been prepared by using the Dynatech MIC 2000 (Dynatech, Denkendorf, Federal Republic of Germany). The MIC was determined as the lowest concentration of an antimicrobial agent that inhibited visible growth of the test organism. To guarantee the comparability of the different methods and of the different laboratories, control strains were used every day: *E. coli* ATCC 29922, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 25923 (agar dilution test), *S. aureus* ATCC 29213 (microdilution test), and *E. faecalis* NCTC 775.

Data processing. Zone diameters and MICs were recorded and evaluated at the Hochschulrechenzentrum of the University of Bonn by the Statistical Analysis System (19).

Results. The most recent study was performed in November 1986. In Table 1, the MIC range, MIC for 50% of the strains tested (MIC₅₀), and MIC₉₀ are given. The width of the distribution curve for one species was usually five to six MIC steps.

TABLE 2. Mean incidence of resistance of various members of the *Enterobacteriaceae* to nalidixic acid (1975 to 1986)^a

Species	No. of strains	% Resistant
<i>Klebsiella</i> spp.	2,645	8.3
<i>Proteus</i> spp. (other)	853	6.5
<i>Serratia</i> spp.	440	5.8
<i>Enterobacter</i> spp.	1,575	5.3
<i>Proteus mirabilis</i>	3,005	5.1
<i>Citrobacter</i> spp.	438	4.8
<i>Escherichia coli</i>	9,544	1.9
<i>Salmonella</i> spp.	1,535	1.0

^a The breakpoints used were $\leq$ 13 mm (agar diffusion test) and $\geq$ 32 $\mu\text{g/ml}$ (MIC determination).

Using breakpoints of $\leq$ 13 mm (agar diffusion test) and $\geq$ 32 $\mu\text{g/ml}$ (MIC determination), resistance of members of the *Enterobacteriaceae* to nalidixic acid was 2.3% for 1986 or an average of 3.7% from 1975 to 1986 (Fig. 1). We could not find any statistically significant differences in the data between 1975 and 1986. Even the introduction of norfloxacin and ofloxacin does not seem to have had an influence (Fig. 1). Within this group of bacteria, however, there were great differences from one species to another, ranging from *Salmonella* spp. with 1% to *Klebsiella* spp. with 8.3% resistant strains (Table 2). When comparing the number of resistant strains isolated in different centers, it became evident that there were striking differences in resistance (Table 3). The high percentage of nalidixic acid resistance in strains from Innsbruck is mainly due to the 30.8% *Klebsiella pneumoniae* strains which were resistant not only to nalidixic acid but also to unrelated drugs (data not shown). Thus, specific hospital epidemiology contributes markedly to the overall resistance.

Although the percentage of resistant members of the *Enterobacteriaceae* did not show a significant difference, changes can be seen when comparing the data from 1983 with those from 1986 for the new fluorinated quinolones. We

TABLE 3. Percentage of nalidixic acid-resistant strains of the *Enterobacteriaceae* from different centers in 1984

Center	No. of strains	% Resistant
Hannover	104	0
Tübingen	115	0
Augsburg	152	0.7
Kiel	110	1.8
Oldenburg	107	1.9
Karlsruhe	108	2.8
Bonn	123	4.9
Lübeck	102	4.9
Zurich	102	6.9
Innsbruck	132	12.1

TABLE 4. Percentage of strains of the *Enterobacteriaceae*, *S. aureus*, and *E. faecalis* resistant to 4-quinolones in 1983 and 1986

Antibacterial agent	Yr	<i>Enterobacteriaceae</i>			<i>P. aeruginosa</i>			<i>S. aureus</i>			<i>E. faecalis</i>		
		<i>n</i>	I ^a	II ^b	<i>n</i>	I	II	<i>n</i>	I	II	<i>n</i>	I	II
Ciprofloxacin	1983	1,495	— ^c	0.3	288	3.1	0.7	297	0	0	272	2.2	2.2
	1986	1,334	— ^c	0.1	186	10.5 ^d	1.0	393	1.0	0.5	288	0.7	0.7
Enoxacin	1983	1,495	3.1	1.4	288	2.4	2.4	297	0.7	0.7	272	0.7	73.2
	1986	1,334	2.2	1.0	286	8.7 ^d	8.7	393	2.8	2.8	288	2.1	89.2
Norfloxacin ^e	1986	1,334	2.4	0.3	286	8.0	4.2	383	2.0	2.0	288	1.7	23.2
Ofloxacin	1983	1,485	3.5	0.6	288	3.8	3.8	297	1.3	0	272	0.4	4.8
	1986	1,334	2.3	0.8	286	10.8 ^d	20.6	393	1.3	0.5	288	2.4	12.8

^a Percentage of strains for which MICs were greater than fourfold higher than the mode MIC.

^b Percentage of strains for which MICs were $>2 \mu\text{g/ml}$.

^c —, Evaluation was not possible as the mode MIC was equal to or less than the lowest concentration tested.

^d Difference between 1983 and 1986 is statistically significant ($P < 0.001$).

^e Norfloxacin was not tested in 1983.

used data from these two years since they were the most complete.

In Table 4, we list the data for members of the *Enterobacteriaceae*, *P. aeruginosa*, *S. aureus*, and *E. faecalis* on the basis of the percentage of strains for which the MIC was greater than fourfold higher than the mode MIC, which in all cases was identical to the MIC₅₀. We used a conventional method of looking at the percentage of strains for which MICs were greater than $2 \mu\text{g/ml}$. For members of the *Enterobacteriaceae*, *S. aureus*, and *E. faecalis*, there seemed to be no change, either by the use of the mode MIC or by the use of the $2\text{-}\mu\text{g/ml}$ breakpoint. However, for *P. aeruginosa*, there was an increase of resistant strains for all drugs. The difference between 1983 and 1986 was about 7% for strains for which MICs were fourfold above the mode MIC. This difference was statistically significant ($P < 0.001$, Fisher's exact test, two sided).

In 1984, when the first fluoroquinolone, norfloxacin, was introduced in the Federal Republic of Germany, there was a marked increase in the use of this drug. Despite the marked increase, the incidence of isolates of the *Enterobacteriaceae* resistant to nalidixic acid remained at a low level. However, differences between individual hospitals were evident. In one hospital, we found about 30% of *K. pneumoniae* strains to be resistant to nalidixic acid and chemically unrelated drugs. This finding suggests that multiply resistant isolates with simultaneous nalidixic acid resistance can spread by cross infection.

At present, resistance to fluoroquinolones in members of the *Enterobacteriaceae* is rare (lower than 1%). The majority of nalidixic acid-resistant strains were classified as susceptible to fluoroquinolones. *P. aeruginosa*, *S. aureus*, and *E. faecalis* isolates, intrinsically resistant to nalidixic acid, were less susceptible to fluoroquinolones than isolates of *Enterobacteriaceae*. Resistance to fluoroquinolones in these species was generally at a low level, although differences with various drugs were evident. From 1983 to 1986, a 7% increase was observed in the number of *P. aeruginosa* strains for which the MIC was four times higher than the mode MIC.

The mechanism of action of 4-quinolones is complex and involves inhibition of bacterial DNA gyrase (4). Resistance to nalidixic acid and the fluoroquinolones results from various chromosomal genes but has not been found to be carried on plasmids or transposons (2), except for one report, which has not yet been confirmed (12). Since ciprofloxacin, enoxa-

cin, and ofloxacin are intended for systemic therapy it is important to know the frequency of variants resistant to low drug concentrations. The frequency of resistant variants ranged from 10^{-7} to $<10^{-9}$ with concentrations of fluoroquinolones which were four to sixteen times the MIC (3, 6, 8, 16). Resistant *P. aeruginosa* strains were usually obtained at a frequency 100 times higher than that of resistant *E. coli* strains (5, 6, 8, 13, 20). Several investigators have also demonstrated a stepwise increase in MICs after exposure of bacteria to subinhibitory concentrations (1, 8, 10, 14, 20). The level of resistance buildup is similar to the levels of resistance obtained by the frequency of resistance experiments.

From our data, it is evident that resistance to quinolones did not increase dramatically after a dramatic increase in their use. However, the *P. aeruginosa* resistance must be followed as a further increase seems likely. Even with those species in which a single step mutation does not lead to clinically relevant resistance, as in *Klebsiella* spp., sequential mutations and following cross infection can lead to an increase in resistance.

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