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Characterization of Clinical Isolates of *Escherichia coli* Showing High Levels of Fluoroquinolone Resistance

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During the years 1992 to 1994, an increase in fluoroquinolone-resistant *Escherichia coli* was observed at the Medical Center of the Technical University in Munich, Germany. Nineteen strains were collected and were thus available for further analysis. Pulsed-field gel electrophoresis showed clonal diversity in all but two strains. The majority of the patients from whom the strains were isolated had been previously treated with fluoroquinolones. Quinolone resistance was associated with mutations of the quinolone resistance-determining region of the *gyrA* gene in all cases. Direct sequencing of *gyrA* PCR amplification products revealed a mutation in codon 83 of the *gyrA* gene. In some instances the Ser-83→Leu mutation was accompanied by an Asp-87→Asn or Asp-87→Gly mutation. Furthermore, the strains exhibited two different genotypes: in almost half of the fluoroquinolone-resistant *E. coli* strains as well as in the fluoroquinolone-susceptible *E. coli* reference strains ATCC 25922 and 35218, silent mutations were detected at bases 255, 273, 300, and 333. Although fluoroquinolones solved major problems in antimicrobial chemotherapy, in certain departments of our hospital the number of resistant *E. coli* isolates has become so high that susceptibility to fluoroquinolones can no longer be taken for granted.

Fluoroquinolones are antimicrobial agents that are very effective against most gram-negative microaerophilic and aerobic bacteria. Fluoroquinolones are essential in the treatment of urinary tract infections in both hospitals and primary health care. MICs of fluoroquinolones for enterobacteria are very low compared with the obtainable serum and tissue levels. Fluoroquinolones interfere with DNA supercoiling by interacting with the bacterial enzyme DNA gyrase (bacterial topoisomerase II), which consists of two subunits (A and B) (12). Two main mechanisms of resistance have been described: (i) point mutations in the so-called quinolone resistance-determining region (QRDR) of the *gyrA* gene (35), and (ii) nonspecific, low-level resistance by modification or altered expression of the OmpF protein, resulting in decreased uptake of antimicrobial substances (11).

Escherichia coli is the most frequent cause of some of the most common bacterial infections, ranging from urinary tract infections to pneumonia and bacteremia. Besides causing a variety of clinical infections, *E. coli* physiologically colonizes the large intestine (28). So far, resistance of members of the family *Enterobacteriaceae* to fluoroquinolones has rarely been observed (14, 18, 30). However, the number of reports of fluoroquinolone-resistant *E. coli* strains seems to be on the increase (1, 10, 17, 22).

In 1994, we observed a significant increase in the number of *E. coli* clinical isolates resistant to fluoroquinolones. Here we describe 19 isolates, all but 2 of which proved to be clonally distinct by pulsed-field gel electrophoresis (PFGE), suggesting independent development of resistance instead of clonal spread within the hospital associated with lack of hygiene. By direct sequencing of PCR amplification products, three types of mutation of the *gyrA* gene were detected.

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MATERIALS AND METHODS

E. coli isolates were identified by routine microbiological diagnostic procedures, including biochemical tests (API 20 E or ID 32 E; BioMérieux, Nürtingen, Germany). Resistant strains were identified by routine agar diffusion test assays, using a 5-μg ofloxacin disk. Ofloxacin resistance was verified by disk diffusion (M2-A5; National Committee for Clinical Laboratory Standards [NCCLS]), E test (Difco, Augsburg, Germany), dilution in microtiter plates (DIN 58640), and agar dilution (M7-A3; NCCLS). *E. coli* ATCC 25922 and ATCC 35218 were employed as controls; each exhibited a MIC of ofloxacin of <0.25 μg/ml. In disk diffusion, diameters of ≤ 12 mm are considered to reveal resistance and those of ≥ 16 mm are considered to reveal susceptibility to ofloxacin (M2-A5; NCCLS). In the dilution assays, all bacteria for which the MIC is ≥ 8 mg/liter are defined as resistant to ofloxacin and those for which the MIC is ≤ 2 mg/liter are defined as susceptible (M7-A3; NCCLS). The grouping by resistance pattern is explained in Table 4.

For preparation of bacterial DNA for PFGE, bacteria were cultured in 50 ml of LB medium (Difco) at 37°C on a gyratory shaker (1-in. [ca. 3-cm] pivot, 200 rpm; Braun, Melsungen, Germany) to late log phase (optical density at 600 nm = 1). Chloramphenicol (Sigma, St. Louis, Mo.) was added to give a final concentration of 0.16 mg/liter, and the bacterial suspension was incubated for 30 min at 37°C. Bacteria were harvested by centrifugation, washed in phosphate-buffered saline, and adjusted to yield 5×10^7 bacteria per ml in cell suspension buffer (10 mM Tris [pH 7.2], 20 mM NaCl, 50 mM EDTA). Agarose plugs were cast by mixing 500 μl of the cell suspension with 2% Incert agarose (FMC, Risingerej, Denmark). The plugs were incubated in 2.5 ml of proteinase K reaction buffer (100 mM EDTA [pH 8.0], 0.2% sodium deoxycholate, 1% sodium lauryl sarcosine, 1 mg of proteinase K per ml; Boehringer, Mannheim, Germany) overnight at 50°C. DNA plugs were washed four times in buffer A (20 mM Tris [pH 8.0], 50 mM EDTA) for 1 hour on each occasion at room temperature. The first wash was accomplished in the presence of 1 mM phenylmethylsulfonyl fluoride to inactivate residual proteinase K. After equilibration of the DNA plugs in 1 ml of restriction enzyme reaction buffer at room temperature, DNA was digested in 0.3 ml of fresh reaction buffer containing 50 U of *Xba*I (Gibco BRL, Berlin, Germany) per 100-μl plug overnight at 37°C. The digestion was stopped with 1 ml of buffer A, and the plugs were equilibrated with 0.5× Tris-borate-EDTA. PFGE was performed in a CHEF-DRIII electrophoresis chamber (Bio-Rad, Munich, Germany) for 18 h at 6 V/cm and 14°C in a 1% (wt/vol) fast-lane agarose (FMC) gel, using a linear pulse ramp of 5 to 25 s in 0.5× Tris-borate-EDTA electrophoresis buffer.

For amplification of the *gyrA* gene, DNA was isolated from a single bacterial colony grown on a Columbia agar plate containing 5% sheep blood (Becton Dickinson, Heidelberg, Germany). The cells were lysed in 100 μl of lysis buffer (10 mM Tris [pH 7.6], 1 mM EDTA, 50 μg of proteinase K per ml) for 15 min at 55°C. Proteinase K was then heat inactivated at 80°C for 15 min. Five microliters was added to the reaction mixture to amplify the *gyrA* gene of *E. coli* by

TABLE 1. Oligonucleotides used in the amplification of *gyrA*

Oligonucleotide	Sequence[a]	Position[b]
*gyrA-Eco*RI	5′-CCGT**GAATTC**GCATTCATTGGCACTTCTACTCCG-3′[c]	−154–−131
*gyrA-Xba*I	5′-AGCG**TCTAGA**CTACAAGACCATAGAACCGCCAGC-3′[c]	2752–2776
*gyrA-Sst*I	5′-GAGGAAGAGCTGAA**GAGCTC**CT-3′	40–61
*gyrA-Hin*dIII	5′-CCGGTACGGT**AAGCTT**CTTCAA-3′	686–707
*gyrA-Seq*1	5′-CGTGCGCTGCCAGATGTCCG-3′	94–113

[a] Boldface letters indicate recognition sequences of the specified restriction enzymes.
[b] Corresponding to the position of the published sequence of *gyrA* from *E. coli* K-12 (accession number X57174 in the EMBL database).
[c] Numbering of position starts immediately downstream of the boldface letters.

PCR, using the oligonucleotides listed in Table 1: *gyrA-Sst*I and *gyrA-Hin*dIII were used for nested PCR of the product amplified by *gyrA-Eco*RI and *gyrA-Xba*I. The reaction mixture contained 20 mM Tris (pH 8.4), 5 mM $MgCl_2$, 50 mM KCl, 200 μM (each) deoxynucleoside triphosphate, 50 pmol of the primers *gyrA-Eco*RI and *gyrA-Xba*I, and 2.5 U of *Taq* DNA polymerase (Gibco BRL) in a total volume of 100 μl. The cycling program consisted of an initial denaturation step for 5 min at 95°C and then 30 cycles of annealing for 1 min at 63°C, extension for 2 min at 72°C, and denaturation for 1 min at 95°C and a final extension-termination step of 10 min at 72°C (Biometra Triblock, Göttingen, Germany). For nested PCR, 1 μl of the initial PCR product was used as a template with the primers *gyrA-Sst*I and *gyrA-Hin*dIII to amplify the QRDR. PCR was performed as mentioned above except that the annealing temperature was 55°C. Samples, 20 μl, of the reaction mixture were visualized by 1% agarose gel electrophoresis.

The QRDR of the *gyrA* gene was sequenced with the Cycle Sequencing System (Gibco BRL), using the ^{33}P-labelled primer *gryA-Seq*1 (Table 1).

RESULTS

During 1992 and 1993, the frequency of *E. coli* isolates resistant to ofloxacin in our hospital was ≤1% (1992, 6 in 1,190 isolates; 1993, 10 in 1,524 primary isolates); in 1994, this rate rose to 2.4% (38 of 1,578 isolates) (Table 2). Less than 10% of all specimens sent to our laboratory and only two fluoroquinolone-resistant *E. coli* isolates were from intensive care units.

Nineteen fluoroquinolone-resistant *E. coli* isolates were collected and were thus available for further analysis. Most of the strains (8 of 19) were isolated from specimens supplied by the urology department. The remaining samples originated from the departments of internal medicine (5 samples), surgery (4 samples), and gynecology (2 samples) (Table 3). Isolates were recovered from urine (12 samples), wounds (3 samples), bile (1 sample), cervical swabs (2 samples), and other sources (1 sample). Underlying diseases included cystic disorders (mainly carcinomas), diseases of the prostate, kidneys, pancreas, and the biliary system, and AIDS.

Six of nine patients for whom histories of antibiotic treatment were available had previously received fluoroquinolones at least while in hospital, which might account for the observed antibiotic-mediated selection of resistant mutants (Table 3).

The ofloxacin MICs for the fluoroquinolone-resistant *E. coli* strains first isolated from each patient (primary isolates; n = 19) are shown in Table 3: the MIC at which 50% of the isolates are inhibited (MIC_{50}) was 32 mg/liter, and the MIC_{90} was 128 mg/liter. All strains exhibited cross-resistance to nalidixic acid, norfloxacin, and ciprofloxacin, with MIC_{90} values of >256, >256, and >32 mg/liter, respectively. A considerable fraction of these 19 primary isolates proved resistant to aminoglycosides, co-trimoxazole, ampicillin, ampicillin plus sulbactam, and piperacillin. One strain was resistant to the expanded-spectrum cephalosporin cefotiam, but none of the strains were resistant to cefotaxime or imipenem. The resistance data were placed in four different resistance groups. Most of the strains, however, were found in only two resistance groups (Table 4). Thus, horizontal distribution appeared likely.

Seventeen of 19 primary isolates were typeable by PFGE, and all were clonally distinct except for strains 13 and 18b (Fig.

TABLE 2. Numbers of all *E. coli* strains isolated and of susceptible and resistant isolates and rate of fluoroquinolone-resistant clinical isolates of *E. coli* in our hospital broken down into 3-month periods (I to IV)[a]

Yr and period	Total no. of isolates	No. of susceptible isolates	No. of resistant isolates	Resistance rate (%)
1992				
II	415	413	2	0.5
III	395	393	2	0.5
IV	380	378	2	0.5
1993				
I	388	387	1	0.3
II	390	386	4	1.0
III	394	392	2	0.5
IV	352	349	3	0.9
1994				
I	376	366	10	2.7
II	358	351	7	2.0
III	450	443	7	1.6
IV	394	380	14	3.6

[a] For 1994, an increase in resistance rates was observed.

TABLE 3. Fluoroquinolone-resistant primary clinical isolates of *E. coli*

Strain no.	Source	Department	Previous fluoroquinolone therapy[a]	MIC of ofloxacin (mg/liter)
5	Urine	Urology	?	32
6	Cervix	Gynecology	?	16
7	Urine	Urology	+	32
8	Urine	Internal medicine	−	32
10	Wound	Internal medicine	+	64
11	Wound	Surgery	+	16
13	Urine	Urology	−	32
14	Wound	Internal medicine	+	32
15	Urine	Urology	+	32
17	Urine	Urology	?	16
18	Urine	Urology	?	64
21	Urine	Urology	?	32
23	Urine	Surgery	?	32
24	Urine	Surgery	+	128
25	Urine	Surgery	−	64
26	Urine	Urology	?	16
27	Cervix	Gynecology	?	16
28	Bile	Internal medicine	?	16
30	Other	Internal medicine	?	128

[a] ?, complete history of antibiotic treatment not available; +, yes; −, no.

TABLE 4. Composition of resistance groups: ampicillin, co-trimoxazole, expanded-spectrum cephalosporin (cefotiam), and gentamicin[a]

Resistance group	Ampicillin	Co-trimoxazole	Cephalosporin II	Gentamicin	*n*
1	R	S	S	S	2
2	R	R	S	R	6
3	R	R	S	S	10
4	R	S	R	S	1

[a] Intermediate and moderate resistance was considered resistant. R, resistant; S, susceptible.

1). This indicates individual development of resistance in most patients rather than the horizontal transmission associated with lack of hygiene. Surprisingly, these two strains belong to different resistance groups (Table 4).

From all fluoroquinolone-resistant strains of *E. coli*, the QRDR of *gyrA* was amplified by PCR. The amplification product was cut with the restriction enzyme *Hin*fI. The restriction pattern in all fluoroquinolone-susceptible control strains showed three fragments, in accord with the wild-type sequence of the *gyrA* gene. In contrast, *Hin*fI restriction of DNA from any of the fluoroquinolone-resistant *E. coli* strains produced only two fragments, which is consistent with the occurrence of the known point mutations at bp 247 (T) or 248 (C) resulting in a loss of the *Hin*fI site in codon 83 (Fig. 2).

Nucleotide sequencing revealed three different types of mutation inside the QRDR: in all of the 19 fluoroquinolone-resistant *E. coli* strains, a change of C→T (Ser→Leu) at position 248 was detected. Sixteen strains exhibited an additional mutation at position 259 G→A (Asp→Asn). In two strains, position 260 was mutated A→G (Asp→Gly) (Fig. 3). Moreover, in 8 of 19 fluoroquinolone-resistant *E. coli* isolates and in both of the ATCC strains, the nucleotide sequences of the *gyrA* gene differed at positions 255, 273, 300, and 333. However,

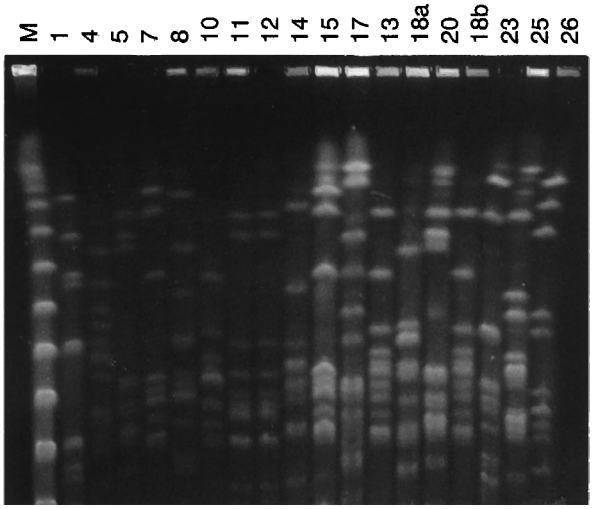

FIG. 1. PFGE of *Xba*I-restricted chromosomal DNA from primary isolates of fluoroquinolone-resistant *E. coli* (each strain from a different patient). The lanes are numbered with the strain numbers. Strains 1 and 4 are fluoroquinolone-susceptible control strains (ATCC 35218 and ATCC 25922). Only isolates 13 and 18b from different patients are of clonal origin; patient 18 was infected by two different strains at the same time. Strain 12 was included for control and isolated from the same patient as strain 11. M, lambda DNA molecular weight standard (monomer size, 48.5 kbp) (Boehringer).

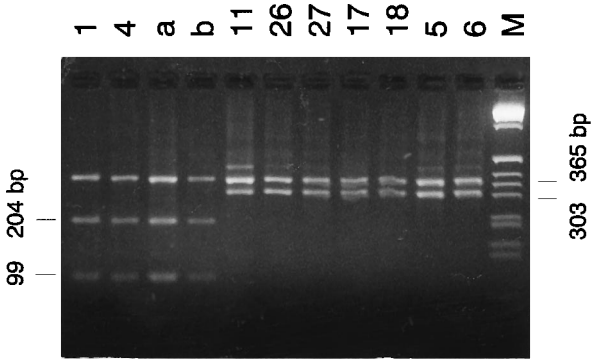

FIG. 2. Amplification of a 668-kb *gyrA*-specific fragment, using PCR and restriction by *Hin*fI. Strains 1 and 4 are fluoroquinolone-susceptible control strains (ATCC 35218 and ATCC 25922); a and b are two susceptible clinical isolates. Strains 11, 26, 27, 17, 18, 5, and 6 are representative of all fluoroquinolone-resistant *E. coli* strains displayed in Table 5. The amplification products were cut in the fluoroquinolone-susceptible control strains but not in the fluoroquinolone-resistant strains, indicating a change of bases in codon 82 or 83. M, DNA molecular weight standard, 1-kb ladder (Gibco BRL).

these mutations do not change the amino acid sequences (Table 5).

DISCUSSION

In the majority of resistance surveillances, fluoroquinolones are still considered to be very active against members of the *Enterobacteriaceae*. The reported rate of quinolone resistance of *E. coli* is only about 1% (14, 18, 30). In contrast, we observed a value of 2.4%. This high value is due mainly to isolates from the department of urology, which had resistance rates exceeding 10%.

Resistance to fluoroquinolones is not restricted to *E. coli*; it has also been reported for such bacteria as *Staphylococcus aureus* and *Pseudomonas aeruginosa* (2, 4). In the case of *Campylobacter* sp., resistance rates were reported to exceed 50% (24).

There have been several case reports on fluoroquinolone-resistant *E. coli* (6, 15, 17, 19, 22). In a report by Kern et al. on bacteremia in leukemia patients in a cancer unit, rates of fluoroquinolone-resistant *E. coli* rose from <0.5 to 4.5% (16). PFGE of nine primary isolates showed only four different genotypes, indicating both the independent development and the horizontal spread of resistant clones of *E. coli.*

When we used resistance as an epidemiological marker, 16 of 19 primary isolates clustered into only two groups, suggesting horizontal spread. However, the results of genotyping by PFGE revealed clonal diversity in all but two isolates, implying independent development of resistance in our strains. These data underline the discriminatory power of genotyping with PFGE; resistance grouping obviously lacks epidemiological value.

The observed clonal diversity of fluoroquinolone-resistant *E. coli* is even more remarkable when one considers that the majority of the isolates originated from a single department of the hospital (urology). Recently, Bauernfeind et al. described a similar observation with nine clinical isolates of *E. coli* from one hospital (1).

Some of the known mutations in the QRDR of the *gyrA* gene result in the loss of the *Hin*fI restriction enzyme recognition sequence. This allows fast screening for such mutations by digestion of the PCR products with *Hin*fI to detect a mutation in codon 82 or 83 (8). This loss was detected in all fluoroquin-

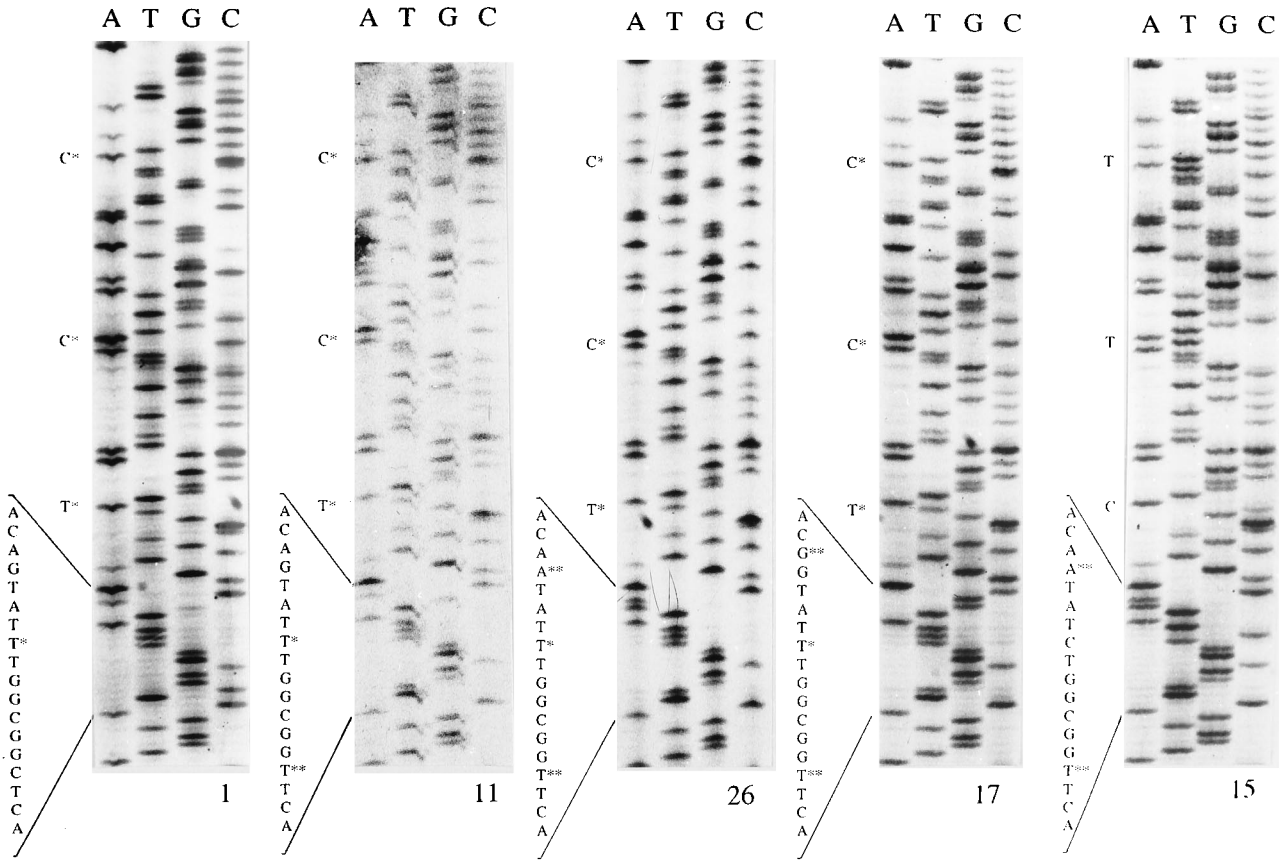

FIG. 3. Autoradiography of sequence gels of a 668-kb *gyrA*-specific fragment. Strain 1 is a fluoroquinolone-susceptible control strain (ATCC 35218); strains 11, 26, 17, and 15 are fluoroquinolone-resistant *E. coli* strains with different mutations and genotypes.

olone-resistant *E. coli* isolates. Because several different point mutations may contribute to this change of the *Hin*fI recognition sequence, and additional mutations cannot be identified in this way, the complete QRDR was sequenced. Nucleotide sequencing disclosed that an exchange of C for T in bp 248 (Ser-83→Leu) was uniformly responsible for the change in the restriction pattern. In addition, two different mutations affecting codon 87 (bp 259 G to A [Asp→Asn] and bp 260 A to G [Asp→Gly]) were detected. Moreover, half of the strains (including the two fluoroquinolone-susceptible ATCC control strains) exhibited a different genotype with four silent mutations (bp 255 C to T, bp 273 C to T, bp 300 T to C, and bp 333 T to C). This genotype was also described by Heisig et al. in a highly fluoroquinolone-resistant clinical isolate of *E. coli* (10).

That 16 of 19 of our isolates have identical double mutations at positions 248 (T) and 259 (A) confirms the importance of this region for the molecular basis of fluoroquinolone resistance in *E. coli* (10, 33, 34). A change of Ser-83 seems to be sufficient to generate a high-level resistance to nalidixic acid, whereas a second mutation at Asp-87 may play a complementary role in developing a high level of fluoroquinolone resistance; mutations in the *gyrB* gene contributing to resistance seem to be rare in clinical isolates (31). In the fluoroquinolone-resistant *E. coli* strains described here, 3 of 13 published types of *gyrA* mutation were detected. Similar mutations in *gyrA* are responsible for fluoroquinolone resistance in bacteria other than *E. coli* (3, 9, 13, 29, 32). For ofloxacin, a MIC as high as 64 or 128 mg/liter might not be explainable merely by the mutations detected in the QRDR of *gyrA*.

Altered expression of OmpF porin is an additional known mechanism of resistance to fluoroquinolones. However, the MICs of fluoroquinolones did not correlate with resistance against aminoglycosides and co-trimoxazole, suggesting that changes in the outer membrane are of minor importance. At least in vitro, modification of outer membrane proteins does not lead to a high level of resistance. Reduced uptake of fluoroquinolones in vivo may favor the survival of DNA gyrase mutants instead (5). In addition, Chamberland et al. demonstrated that protein F deficiency in subsequent clinical isolates of *P. aeruginosa* was not associated with resistance to quinolones (4).

Increased use of fluoroquinolones might facilitate selection of spontaneous mutations. Between 1992 and 1994, the use of fluoroquinolones at our hospital increased steadily from 19,000- to 26,000-unit doses. However, more than twice the amount of fluoroquinolones is prescribed in general practice compared with that prescribed in hospitals, and in Germany, the use of fluoroquinolones is still increasing (18). That the majority of the strains were isolated from specimens of urine might be attributed to the frequent use of fluoroquinolones for the treatment of urinary tract infections. In a clinical case control study by Richard et al., the selective pressure by fluoroquinolone use was one of the main exogenous risk factors involved in the emergence of resistance (27). In a study by Muder et al., previous administration of a fluoroquinolone was the single most significant risk factor for isolation of a ciprofloxacin-resistant gram-negative organism (23). In a report on emergence of fluoroquinolone-resistant *E. coli* at a cancer cen-

TABLE 5. Ofloxacin MICs and *gyrA* sequences of fluoroquinolone-resistant *E. coli* strains and two susceptible control strains

Strain[a]	MIC (mg/liter)[b]	Nucleotide in *gyrA* at bp[c]:						
		248 (83)	255 (85)	259 (87)	260 (87)	273 (91)	300 (100)	333 (111)
K-12		C (Ser)	C (Val)	G (Asp)	A (Asp)	C (Arg)	T (Tyr)	T (Ser)
1	0.12		**T**			**T**	**C**	**C**
4	0.12		**T**			**T**	**C**	**C**
11	16	*T* (*Leu*)	**T**			**T**	**C**	**C**
26	16	*T*	**T**	*A* (*Asn*)		**T**	**C**	**C**
27	16	*T*	**T**	*A*		**T**	**C**	**C**
8	32	*T*	**T**	*A*		**T**	**C**	**C**
24	128	*T*	**T**	*A*		**T**	**C**	**C**
30	128	*T*	**T**	*A*		**T**	**C**	**C**
17	16	*T*	**T**		*G* (*Gly*)	**T**	**C**	**C**
18	64	*T*	**T**		*G*	**T**	**C**	**C**
6	16	*T*		*A*				
28	16	*T*		*A*				
5	32	*T*		*A*				
7	32	*T*		*A*				
13	32	*T*		*A*				
14	32	*T*		*A*				
15	32	*T*		*A*				
21	32	*T*		*A*				
23	32	*T*		*A*				
10	64	*T*		*A*				
25	64	*T*		*A*				

[a] Control strains 1 and 4 are strains ATCC 35218 and ATCC 25922, respectively.
[b] Determined by agar dilution.
[c] Numbers in parentheses are codon numbers. Mutations in italics result in amino acid mutations. Mutations in boldface do not result in amino acid changes.

ter, most of the patients had been pretreated with ofloxacin at a median cumulative dose of 14.4 g (16). During prophylactic administration of norfloxacin in cirrhotics, half of the patients developed fecal organisms, including members of the *Enterobacteriaceae* resistant to fluoroquinolones (7). In addition to the frequent use of fluoroquinolones, a reduction in dosage might facilitate selection of resistant organisms. This has been shown both in vitro (26) and in an animal model that used *P. aeruginosa* (21).

Weber et al. (32a) failed to observe any increase in resistance to quinolones in isolates of *E. coli* from general practice. However, resistance to quinolones was more frequent in patients who had received quinolones up to 1 month before specimen collection. Moreover, a short report from Spain shows a dramatic increase in the resistance of *E. coli* to ciprofloxacin in outpatients with urinary tract infections: the resistance rate increased from 0.8% in 1989 to 7.1% in 1992 (25). However, this study did not analyze clonality, and the marked increase may thus be due to less than optimal hygiene.

Fluoroquinolones have helped to overcome major problems in antimicrobial chemotherapy. However, in our hospital the number of resistant *E. coli* isolates has become distressingly high, and susceptibility to fluoroquinolones can no longer be taken for granted. In chromosomally mediated resistance, the spread of resistance to different bacterial species is definitely less than in the plasmid-mediated type. Nevertheless, our study demonstrates the need to prevent further spread of resistant strains, which appear to be able to persist even in the absence of selective pressures (20).

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