M. Oethinger, A. S. Jellen-Ritter, S. Conrad, R. Marre, W. V. Kern

Colonization and Infection with Fluoroquinolone-Resistant *Escherichia coli* among Cancer Patients: Clonal Analysis

Summary: *Escherichia coli* with high-level fluoroquinolone resistance were isolated from feces and/or various body sites of 16 cancer patients who were on oral fluoroquinolone prophylaxis. Population analysis of fecal isolates in 11 patients showed that fluoroquinolone-resistant *E. coli* was the only aerobic gram-negative bacillus present and exhibited a relatively homogenous fluoroquinolone MIC distribution. Molecular typing by pulsed field gel electrophoresis of chromosomal DNA digests or by random amplified polymorphic DNA fingerprinting confirmed the clonal nature of gastrointestinal tract colonization with *E. coli*. Genotyping of ten colonies picked from the same fecal culture demonstrated identical strains in four of four patients examined. Identical genotypes from the same patient were isolated over prolonged periods of time in 12 of 12 cases examined, with one patient (with the longest follow-up of 14 months) who lost his initial genotype and became persistently colonized with a new genotype. In the 11 patients who developed infection due to fluoroquinolone-resistant *E. coli*, molecular typing also indicated that fecal colonization was associated with, and presumably preceded infection due to an indistinguishable genotype of fluoroquinolone-resistant *E. coli*.

Introduction

Fluoroquinolones have been widely used in patients given cytotoxic chemotherapy to prevent infection during episodes of neutropenia. This type of antibacterial chemoprophylaxis has been particularly effective in reducing the incidence of bacteremia due to *Escherichia coli* and other enteric bacteria [1, 2]. Unfortunately, increasing rates of resistance to fluoroquinolones among *E. coli* have now been described for clinical isolates from cancer patients who were given fluoroquinolone prophylaxis [3–5]. This observation has been surprising since the risk for development of fluoroquinolone resistance in *E. coli* was considered extremely low: multiple chromosomal mutations are required and there is no transferable resistance to fluoroquinolones.

Recently, we studied the molecular epidemiology of such isolates that had caused bacteremia and had been obtained at different cancer centers located across Europe and the Middle East [6]. Molecular typing of isolates from different centers demonstrated broad genetic diversity, and, secondly, some risk for sharing identical genotypes between patients admitted to a single, given center. No information, however, has been available about the clonal nature of colonization with fluoroquinolone-resistant *E. coli* at the level of the individual patient, since only bloodstream isolates were analyzed.

Previous studies with *E. coli* have suggested colonization and, in the case of infection, expansion of a single clone causing urinary tract infection [7]. The corresponding scenario in the cancer patient on fluoroquinolone prophylaxis could be: endogenous emergence or exogenous acquisition of one (or more) resistant strains, clonal expansion in the gut, and urinary tract infection (ascending route) or translocation to mesentrial lymph nodes and the bloodstream. In the present paper we show that, in fact, colonization of the cancer patient with fluoroquinolone-resistant *E. coli* in the gut is homogenous, remains clonal over time and is frequently associated with invasion into the bloodstream.

Patients and Methods

A prospective study on colonization with fluoroquinolone-resistant *E. coli* among cancer patients was performed in adult patients with neutropenia (<1000 neutrophils/μl) secondary to cancer or cytotoxic therapy who received oral ofloxacin (daily dose: 600 mg) as antibacterial prophylaxis. Surveillance cultures from oropharyngeal washings and feces were obtained at least once weekly from the patients. Cultures from blood or other sites were obtained as clinically indicated. Isolates were identified as *E. coli* by standard methods [8], including biochemical reactions in the API 20E reaction profile (BioMérieux, Nürtingen, Germany). Adult hospitalized patients with fluoroquinolone-susceptible *E. coli* were used as control patients.

Susceptibility testing: Antimicrobial susceptibility was determined by disk diffusion and confirmed by standard broth microdilution tests with cation-adjusted Mueller-Hinton broth and a final inoculum of 5×10^5 CFU/ml according to NCCLS performance and interpretive guidelines [9]. Microtiter plates were purchased from Merlin Diagnostics (Bornheim, Germany). The breakpoint for ofloxacin resistance was 8 μg/ml

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Dr. med. *Margret Oethinger*, Institut für Laboratoriumsmedizin und Transfusionsmedizin, D-32545 Bad Oeynhausen; Dr. rer. nat. *Angelika S. Jellen-Ritter*, PD Dr. med. *W. V. Kern*, Sektion Infektiologie und Klinische Immunologie, Medizinische Universitätsklinik und Poliklinik, D-89070 Ulm; Dr. rer. nat. *Siglinde Conrad*, Prof. Dr. med. *R. Marre*, Institut für Mikrobiologie und Immunologie, Universitätsklinikum, D-89070 Ulm, Germany.
Correspondence to: Dr. *W. V. Kern*

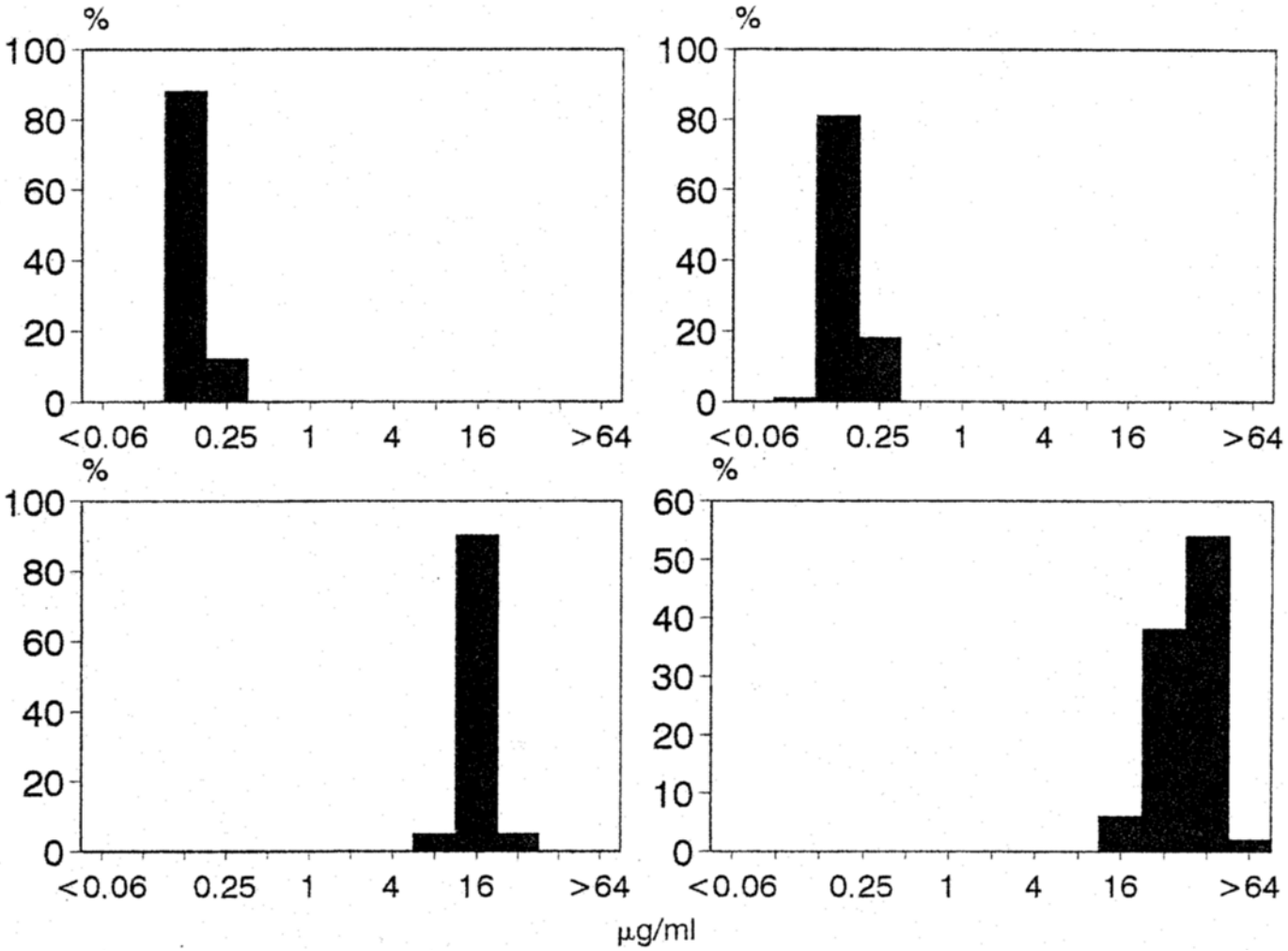

Figure 1: Distribution of fluoroquinolone MIC values of 100 colonies of *E. coli* isolates from a fecal sample. Colonies were replica-plated on ofloxacin-containing agar plates and incubated for 18 h at 36°C (modified agar dilution test). Upper row: results of samples taken from control patients with fluoroquinolone-susceptible *E. coli*. Lower row: results from two cancer patients given fluoroquinolone prophylaxis and colonized by fluoroquinolone-resistant *E. coli*.

Population analyses on fecal samples: When the routine surveillance culture demonstrated fluoroquinolone-resistant *E. coli* in the feces, the specimen was stored at 4°C, if processed within 24 h of sampling, or otherwise frozen at −20°C. At the time of analysis, 0.1 g feces was solubilized in 1 ml PBS and further diluted serially up to 10^{-6}. For colony counts and assessment of colony morphology, 100 µl of the dilution was spread onto MacConkey agar plates (Biotest, Dreieich, Germany) and incubated at 36°C for 18 h. The concentration of *E. coli* in CFU/g feces was calculated from plates containing between 30 and 300 colonies of typical morphology. One hundred colonies were replica-plated onto MacConkey agar plates supplemented with ofloxacin in twofold dilutions from 0.03 µg/ml to 64 µg/ml (modified agar dilution test). Control strains *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27858 were included on each plate. A preliminary screen of fluoroquinolone-susceptible *E. coli*, reproducibly, established ofloxacin MICs between 0.06 and 0.25 µg/ml with this method. Comparison of the modified agar dilution test with the standardized broth microdilution test on 20 fluoroquinolone-resistant isolates showed one dilutional step lower MICs with the latter method.

Molecular typing: Pulsed field gel electrophoresis (PFGE) of whole chromosomal DNA was used for molecular typing as previously described [6]. Briefly, *E. coli* cells were embedded in agarose plugs, lysed and cleaved with the restriction enzyme XbaI (Pharmacia Biotech, Freiburg, Germany). PFGE was performed in 1% agarose gels in a Rotaphore Type IV apparatus (Biometra, Göttingen, Germany). Pulse time was 5 sec for 4 h and then ramped from 5 to 35 sec over 18 h at 240 V. Restriction fragment patterns were analyzed visually. Dice coefficient of similarity was calculated as described [6]. Isolates with Dice coefficients of similarity $F \geq 0.90$ were considered genetically related.

Since a significant proportion of isolates was not typeable by PFGE, isolates were also subjected to random amplified polymorphic DNA (RAPD) fingerprinting in order to increase the discriminatory power of molecular typing. This method was used as previously described [6, 10], with modifications. Briefly, for isolation and purification of genomic DNA the RapidPrep kit of Pharmacia Biotech was used. For PCR, dried beads containing all the necessary reagents (Ready-to-go RAPD analysis beads; Pharmacia Biotech) were used with 5–10 ng of template DNA.

The PCR reaction was carried out in a thermocycler (Personal Cycler, Biometra) programmed for 1 cycle at 95°C for 5 min followed by 45 cycles at 95°C for 1 min, 36°C for 1 min and 72°C for 2 min. The 10-nucleotide primer no. 5 (AAC GCG CAA C) and, when needed, primer 1283 (GCG ATC CCC A) were used. Primer no. 5, purchased from Pharmacia Biotech, was recently evaluated in this laboratory as useful for discriminating *E. coli*. Primer 1283 had earlier been evaluated for RAPD fingerprinting of *E. coli* by *Wang* et al. [11] and used by us [6], and was synthesized on a 381A DNA synthesizer (Applied Biosystems, Weiterstadt, Germany). The amplified fragments were separated on a 2% agarose gel using TBE buffer. Gels were photographed under UV light and visually evaluated for banding patterns. A difference of > 2 bands distinguished different RAPD genotypes.

Results

Patients and Clinical Characteristics

In an 18-month study period, fluoroquinolone-resistant *E. coli* were cultured from at least one body site in 16 cancer patients (seven female, nine male). All patients had been pretreated with ofloxacin before the first isolation of the resistant *E. coli*. The median cumulative ofloxacin dose before first isolation was 13.8 g (range: 3.6–28.8 g), corresponding to 23 daily doses (range: 6–48). The median time between the most recent susceptible and first resistant *E. coli* isolate was 34 days (range: 12–102 days). Five out of 16 patients (31%) were colonized with fluoroquinolone-resistant *E. coli* in the intestinal tract without signs of infection, while

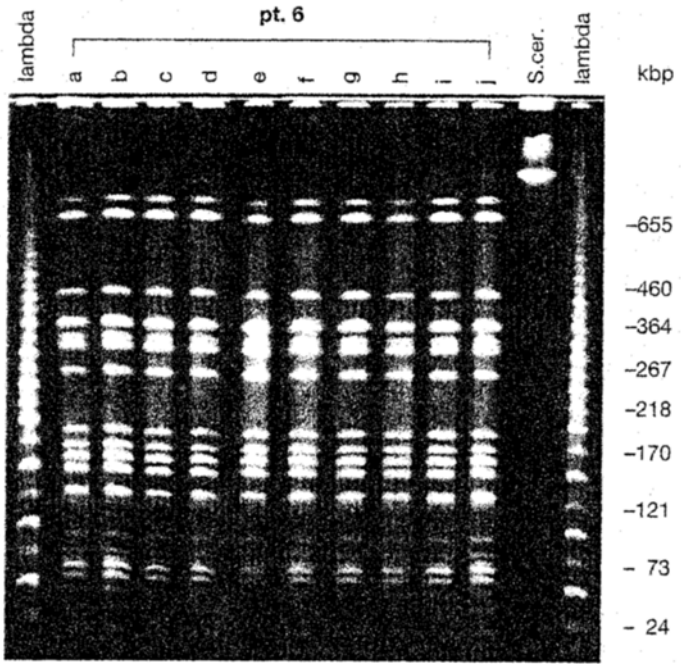

Figure 2: Pulsed-field gel electrophoresis (PFGE) of *Xba*I digested chromosomal DNA from fluoroquinolone-resistant *E. coli* isolates recovered from a single fecal sample of a cancer patient (patient no. 6) given fluoroquinolone prophylaxis. Single colonies (lanes a through h) were picked at random from an agar plate to assess clonality of the intestinal tract colonization. Lane lambda: Lambda concatemers MidRange II. Lane S.cer.: yeast chromosomes. Molecular sizes are given in the margin as kilobase pairs.

Table 1. Details of samples and isolates analyzed and results of molecular typing by PFGE and RAPD fingerprinting.

Patient no.	Number of fecal samples analyzed by population analysis	Period for which isolates were available	Site(s) of infection	Number of isolates obtained at different times analyzed by molecular typing and typing results		
				n	PFGE	RAPD
1	3	9/94– 3/95		3	nt	VI
2	1	1/94– 2/94	Wound, urine	3	A	I
3	1	11/93– 3/94	Blood, urine	5	nt	VI
4	–	11/93– 5/94	Blood	3	E	IV
5	1	11/94		1	nt	VI
6	1	12/94–12/94	Blood	3	G	XI
7	3	12/93– 2/95	Blood	7	A/nt	I/VI
8	–	1/95		1	H	VIII
9	–	11/93– 2/94	Blood, lung	2	A	I
10	–	10/94–10/94		1	nt	VI
11	2	3/95– 6/95	Blood	3	H	VIII
12	1	5/94– 6/94	Urine	3	nt	IX
13	2	10/94– 1/95	Blood	3	nt	VI
14	–	6/95	Blood	1	H	VIII
15	1	10/94–11/94	Blood	3	nt	VI
16	2	9/94–11/94		4	F	X

All patients except no. 9 and no. 14 had one or more *E.coli* stool isolates included in the molecular typing studies. If no site of infection is indicated, the patient was colonized in the intestinal tract only. PFGE = pulsed field gel electrophoresis; RAPD = random amplified polymorphic DNA, nt = nontypeable. Note that patient no. 7 was colonized by two *E.coli* genotypes, initially type A/I, and subsequently type nt/VI.

11 patients (69%) had infections caused by fluoroquinolone-resistant *E. coli* at the following sites: bloodstream (seven patients), bloodstream and urinary tract (one patient), bloodstream and bronchoalveolar lavage fluid (one patient), urinary tract and wound (one patient) and urinary tract alone (one patient). As indicated in Table 1, the period for which isolates from the patients were available ranged from a single time point to more than a year (patient no. 7).

Antimicrobial Susceptibility

All isolates were resistant to both ofloxacin and ciprofloxacin. Many were resistant to trimethoprim-sulfamethoxazole (at least one isolate from 13/16 patients), while none was resistant to cefuroxime, third generation cephalosporins, piperacillin-tazobactam and imipenem. Thus, none of the patients was colonized by strains that were resistant to the β-lactam agents used for empirical therapy in case of fever.

Population Analyses of Fecal Samples

In 14 patients at least one fecal culture was positive for fluoroquinolone-resistant *E. coli*. In the remaining two patients (one with bacteremia, the other with bacteremia and subsequent lung infection), fecal cultures had not been done. Population analyses on one or more fecal samples obtained at different times could be performed in 11 cancer patients (see Table 1) and on a single fecal sample cultured in seven control patients. In the fecal samples colony counts of fluroquinolone-resistant *E. coli* ranged between 10^4 and 10^{11} CFU/g. In the cancer patients, fluoroquinolone-resistant *E. coli* was the only species of aerobic gram-negative bacilli present. Colony morphology and the level of fluoroquinolone resistance, as judged by the modified agar dilution test, were extraordinarily homogeneous. The 100 colonies analyzed in each case clustered virtually around one ofloxacin MIC value (± one dilutional step): these values were 8 μg/ml (one patient), 16 μg/ml (five patients), 32 μg/ml (one patient), 64 μg/ml (two patients), and >64 μg/ml (two patients). Figure 1 shows representative examples for the MIC distribution of patient isolates and controls. When we repeated the population analysis on specimens taken up to 3 months after the first sample during periods of fluoroquinolone treatment, both morphology and level of fluoroquinolone resistance of *E. coli* were comparable to the first analysis.
Fecal isolates of four patients who had population analyses done were typeable by PFGE. Genotyping by this method of ten colonies picked from the same specimen demonstrated identical PFGE patterns in these cases (Figure 2). The results were slightly different for control patients: three of the seven controls had more than one PFGE genotype (up to six) of fluoroquinolone-susceptible *E. coli* in the same fecal sample.

Persistent Colonization and Clonal Expansion

Thirteen of the cancer patients harbored resistant organisms at ≥ 2 body sites and/or were colonized with resistant *E. coli* for ≥ 3 weeks. A comparison by PFGE or RAPD of such multiple isolates from the same individual patient demonstrated a single genotype of *E. coli* in each patient, with one exception (see below). Isolates that were identical or closely related upon molecular typing were found up to 7 months (patients no. 1 and no. 4) and even 1 year apart (patient no. 7; genotype nt/VI; see Table 1). Ofloxacin MICs of the multiple *E. coli* isolates from the same patient were almost identical (± one dilutional step) to each other. The exceptional patient with a change in the *E. coli* genotype was a patient who had an initial isolate (on a single occasion) that was different by PFGE and RAPD from subsequent isolates that were cultured during later admissions and that were, in turn, identical to each other covering a period of 1 year.
We studied several fecal specimens from patients after fluoroquinolone discontinuation. Up to 16 days after fluoroquinolone discontinuation, the MIC distribution of fecal *E. coli* isolates remained unchanged compared to fecal samples obtained during fluoroquinolone treatment. Thereafter, a small subpopulation of fluoroquinolone-susceptible *E. coli* (2% and 6% of the 100 colonies examined, respectively) emerged in two of four fecal samples and increased in one case to >90% in a subsequent sample obtained 10 days later. In both cases, the susceptible isolates were different from the resistant isolates according to PFGE/RAPD fingerprints (data not shown).
In all cases in which resistant isolates colonizing the intestinal tract and infecting isolates cultured from other body sites were analyzed, genotyping demonstrated identity or close relatedness showing expansion of a single clone colonizing mucosal surfaces to become an invasive true pathogen.

Sharing of Genotypes between Patients

Overall, molecular typing of the isolates from the 16 patients revealed seven clonal clusters. Sharing of identical or related genotypes of *E. coli*, thus, was not uncommon. Interestingly, the isolates that were untypeable by PFGE belonged to two different RAPD types of which one was detected only in a single patient (patient no. 12), while the second was shared by seven patients (Table 1). Compared with other genotypes, this particular *E. coli* strain, so far, has formed a relatively large cluster and may become a sort of endemic fluoroquinolone-resistant strain within the cancer patient population of our hospital.

Discussion

The findings of the present study indicate that colonization with fluoroquinolone-resistant *E. coli* in the intestinal tract is usually monoclonal, and that infection due to such strains results from expansion of a single clone. The latter

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finding is not unexpected given earlier reports [7, 12]. However, this report is the first specifically investigating the clonality of fluoroquinolone-resistant *E. coli* within individual patients. Previous studies did not include typing of multiple isolates cultured from the same single specimen to exclude heterogenous colonization at a site. Earlier work on the clonality of the fecal *E. coli* population, using serotyping and biochemical profiles, has suggested that at least three colonies plus all morphologically distinct colonies should be examined to detect the predominant *E. coli* strain in fecal samples and to exclude heterogenous colonization [13]. In view of the higher discriminatory power of molecular typing tools, one would expect that even more isolates should be examined to support this suggestion. Consequently, it has not been well known how clonal or diverse the population of *E. coli* colonizing mucosal surfaces would be, in particular under the condition of recent hospital admission. Among the control patients included in our study, the fecal population of susceptible *E. coli* was polyclonal in several cases, and we assume that this finding indicates the normal state.

The steps leading from such polyclonal colonization with fluoroquinolone-susceptible strains to monoclonal colonization by a resistant *E. coli* strain are as yet poorly understood. It is not known, for instance, whether the development of high-level resistance to fluoroquinolones occurs in the same host, or within a single period of uninterrupted drug treatment. In any case, once a patient has become colonized by fluoroquinolone-resistant *E. coli*, this colonization tends to persist as long as the fluoroquinolone is being given. Repopulation of the intestinal tract with susceptible *E. coli* after fluoroquinolone therapy usually can be seen within 1 week after drug discontinuation [14–16]. The finding in this study of persistence of the resistant, colonizing strain in the intestinal tract for several weeks after drug discontinuation and the apparently slow repopulation with susceptible strains is therefore noteworthy. It may explain a rapid reappearance of the resistant strain after repeated drug intake. Persistent colonization of individuals over similar, extended periods of time has also been observed with vancomycin-resistant *Enterococcus faecium* in a pediatric oncology ward [17], and with fluoroquinolone-resistant *Neisseria gonorrhoeae* in a patient population of a defined geographical area [18]. In contrast, long-term shedding of enterohemorrhagic *E. coli*, for example, seems to be rare, with a median time of shedding of 13 days [19]. Also, earlier studies using multilocus enzyme electrophoresis indicate rapid turnover of intestinal tract colonization with *E. coli* strains [20].

In vitro data indicate that a number of mutational steps, notably in the *gyrA* and *parC* and perhaps other genes, are required for the emergence of clinically significant, high-level fluoroquinolone resistance in *E. coli* [21]. First step and probably second step mutants are still low-level resistant, at least *in vitro* [22, 23]. In the present study, we did not observe *E. coli* which exhibited low or intermediate levels of fluoroquinolone resistance during drug administration, but we found an apparently homogeneous population with roughly identical MICs. Low-level resistant mutants, thus, may be transient and rapidly mutate to high-level resistance.

The sharing of identical or closely related genotypes between patients has been documented earlier by us and others [5, 6, 24] and was again observed in this study. This finding on the one hand and a certain genetic diversity of susceptible *E. coli* in hospitalized patients on the other hand have important implications when one is attempting to culture fluoroquinolone-susceptible precursor strains in a patient who is at risk of becoming colonized by resistant mutants. Molecular typing of a sufficient number of potential precursor isolates would be required before conclusions concerning superinfection versus endogenous resistance development could be drawn. It can be roughly estimated that only every third of our patients colonized by a fluoroquinolone-resistant strain will have had a true development of resistance of his/her colonizing *E. coli* strain. Given an estimated incidence of 10–20% of colonization with a resistant *E. coli* in our cancer patients given fluoroquinolones [25], the chance to find the wild-type parental strain will be in the order of only 1 per 30 patients at risk to become colonized by a resistant one. Nevertheless, we are currently looking for such precursor strains.

The MIC values of our cancer patient isolates were in the range of 8 to >64 μg/ml for ofloxacin. These values appear to be substantially lower than the drug concentrations achievable in the intestinal tract [16, 26]. While this might indicate that the *E. coli* cells are not killed but only inhibited, the relatively high colony counts in the fecal specimens suggest, on the other hand, proliferation *in vivo*. The reason for this discrepancy is unknown. Inactivation of fluoroquinolones by feces has been suggested as an explanation [27].

We did not evaluate the emergence of fluoroquinolone resistance in organisms other than *E. coli*. Reports of fluoroquinolone resistance among other enteric bacteria have been published, but in cancer patients, the situation has not been alarming [4], and fluoroquinolone prophylaxis may still afford some degree of protection against infection due to *Klebsiella*, *Enterobacter* and other organisms. On the other hand, fluoroquinolone resistance emerged rapidly after clinical use among other potential pathogens such as *P. aeruginosa*, streptococci and staphylococci. There are now controversial discussions about the usefulness of fluoroquinolone prophylaxis in cancer patients [1, 2, 28–30]. The alternative option of using trimethoprim-sulfamethoxazole with or without a polymyxin for prophylaxis is unlikely to provide better efficacy and is less well tolerated. The addition of a polymyxin to a fluoroquinolone might be worth investigating. The decision to use no antibacterial prophylaxis is another option, and certainly needs to be considered if epidemiologic monitoring indicates further loss of efficacy.

In summary, individual cancer patients on prophylactic fluoroquinolone treatment, if colonized in the intestinal

tract by fluoroquinolone-resistant *E. coli*, appear to be colonized by a single clone over prolonged periods of time. Colonization persists in many cases for weeks after drug discontinuation. In many such patients, fecal colonization is associated with and presumably precedes infection due to the same clone.

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