Diversity of Individual Dynamic Patterns of Emergence of Resistance to Quinolones in *Escherichia coli* From the Fecal Flora of Healthy Volunteers Exposed to Ciprofloxacin

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(See the editorial commentary by Rice, on pages 1334–5.)

**Background.** Emergence of quinolone-resistant *Escherichia coli* (QREC) is an increasing clinical challenge mostly originating in fecal microbiota. The dynamics of the emergence of QREC in feces from individuals exposed to ciprofloxacin is unknown.

**Methods.** A total of 48 healthy volunteers received oral ciprofloxacin for 14 days. Fecal specimens were collected on days 0, 8, 14, and 42. Subpopulations of QREC were detected on selective agar, genetically characterized, and compared with quinolone-susceptible *E. coli* (QSEC) strains collected on different days.

**Results.** On day 42, 34 subjects carried QSEC, and 14 carried QREC. Of the 14 who carried QREC, 9 carried quinolone-susceptible *E. coli* on day 0, 1 carried *E. coli* with a lower level of quinolone resistance on day 0, and 4 carried *E. coli* with similar levels of resistance and RAPD-genotypes on days 0 and 42. No plasmid acquisition and no selection of resistant mutants from the initial microbiota was evidenced in any case.

**Conclusions.** In QREC emerging under ciprofloxacin pressure in the fecal microbiota, no proof of selection of quinolone-resistant mutants from the initial microbiota was evidenced, suggesting that QREC strains on day 42 were either present at undetectable levels in the initial microbiota or that exogenous acquisition of QREC strains occurred.

**Clinical Trials Registration.** NCT00190151.

*Escherichia coli* resistance to fluoroquinolones is a growing problem worldwide, leading to prolonged hospital stays and increased antibiotic use, morbidity, mortality, and costs [1, 2]. The emergence of *E. coli* resistance to antibiotics, including fluoroquinolones, may be the result of direct selection at the site of infection or in the fecal microbiota. The role of the fecal microbiota as a natural reservoir of bacterial resistance may even be more important than the infectious foci because the greater numbers of bacteria and various species present in this region allow multiple horizontal gene transfers to pathogens and direct infection by commensal bacteria [3–5]. The fecal microbiota is repeatedly affected by antibiotic receipt, whether prescribed as empirical treatment for documented bacterial infections or as prophylaxis [6, 7]. Consequently, high rates of quinolone resistance among bacteria in the feces of hospitalized patients [8–10], community patients [11], and even healthy volunteers [12] have been observed during or after treatment with fluoroquinolones. In France, up to 11% of hospitalized patients were carriers of quinolone-resistant *E. coli* (QREC) at hospital admission [13], and previous fluoroquinolone treatment is a major risk factor for QREC.
carriage [14]. The emergence of resistance in fecal microbiota may result either from the selection of resistant mutants from the initial microbiota, from interspecies or intraspecies transfer of resistant genes from bacterial cells belonging to the same microbiota, or from exogenous acquisition of resistant bacterial cells transiting the gut [4].

The composition of the human distal microbiota, when studied by high-speed sequencing analysis of the metagenome, is significantly altered quantitatively and qualitatively after ciprofloxacin treatment [15]. Fluoroquinolones diffuse particularly well in the gut, and very high concentrations of ciprofloxacin are detectable during treatment [12, 16], which may explain their particular impact on the fecal microbiota. However, the impact of ciprofloxacin treatment on clinically relevant bacteria included in the microbiota, particularly E. coli, and the dynamics of the emergence of QREC strains over time have not been described at the individual level.

In a previous work by our team, we described the results of a clinical study involving 48 healthy volunteers who received ciprofloxacin for 14 days, and we analyzed the relationship between antibiotic exposure and emergence of QREC strains [12]. In the present study, we used fecal samples collected from these volunteers during and after ciprofloxacin treatment to analyze the dynamics of the emergence of resistance in E. coli over time in each volunteer. We determined the molecular mechanisms involved in quinolone resistance and compared the phenotypic and genetic characteristics of E. coli strains colonizing individuals as the dominant or subdominant populations before, during, and after ciprofloxacin exposure.

SUBJECTS, MATERIALS, AND METHODS

Subjects and Study Design
A total of 48 healthy volunteers were selected for the original study trial and gave informed consent [12]. The following 14-day therapeutic regimens of oral ciprofloxacin were randomly assigned to participants: 250 mg every 12 hours, 500 mg every 24 hours, 500 mg every 12 hours, 750 mg every 24 hours, 750 mg every 12 hours, or 1000 mg every 24 hours. Subjects had not received any antimicrobials in the 3 months before the trial and received none during the follow-up period. Fecal samples were collected on days 0, 8, 14, and 42. Because detectable concentrations of ciprofloxacin are present in stool up to 10 days after a single dose of ciprofloxacin [16], the end point of day 42 was chosen to make sure that no ciprofloxacin would be found in the feces. Drug intake was strictly monitored (drugs were taken under the supervision of an investigator), and drug concentrations were measured in plasma and feces. Subject selection, ciprofloxacin regimens, drug concentrations in feces, and plasma and fecal sample collection were described elsewhere [12]. Ciprofloxacin concentrations in feces on days 8 and 14 were 1000–2000 μg per gram of feces, whatever the ciprofloxacin dosing regimen. On day 42, ciprofloxacin concentrations were undetectable in the feces of all subjects.

Analysis of E. coli Strains From Fecal Samples
After collection, fecal samples were mixed in 1 mL of brain heart infusion (BHI) broth. A 50% dilution was performed (500 μL of mix in 500 μL of BHI); 100 μL of the final mix was plated on Drigalski agar that contained no antibiotics (Pasteur Diagnostics, Paris, France). On the basis of the quantity of feces (10–20 mg) in each sample, we estimated the lower limit of detection of E. coli strains to be 10^3 colony-forming units (CFU) per gram of feces. The number of CFU per plate was determined, and up to 10 CFU were randomly picked to represent the dominant flora on that day.

QREC strains were detected by plating 100 μL of the final mix on 2 Drigalski plates, one containing 16 μg/mL nalidixic acid, to detect first-step E. coli mutants, and the other containing 1 μg/mL ciprofloxacin, to detect ciprofloxacin-resistant mutants. The number of CFU per plate was quantified, and 5 CFU were randomly picked for further analysis.

Antibiotic Susceptibility Patterns
For each of the 10 colonies of dominant flora and the 5 colonies of subdominant flora, the susceptibility to quinolones was determined by measuring the minimal inhibitory concentrations (MICs) of ciprofloxacin, ofloxacin, levofloxacin, and nalidixic acid by the agar dilution method in duplicate [17]. Full antimicrobial susceptibility patterns to beta-lactams with and without beta-lactamase inhibitors, aminoglycosides, tetracycline, colimycin, fosfomycin, chloramphenicol, and cotrimoxazole were determined by the disk-diffusion method. Breakpoints were those recommended by the European Committee on Antimicrobial Susceptibility Testing 2011 (available at: http://www.eucast.org). E. coli strains with a high level of resistance to quinolones were defined by a ciprofloxacin MIC of >1 μg/mL. E. coli strains with a low level of resistance to quinolones were defined by a nalidixic acid MIC of >16 μg/mL and a ciprofloxacin MIC of ≤1 μg/mL.

Determination of Molecular Mechanisms of Quinolone Resistance
Mechanisms of resistance were investigated for all QREC strains, whatever the level of resistance and MICs. The quinolone-resistance-determining region (QRDR) was amplified by polymerase chain reaction (PCR) and sequenced in the gyrA (521–base pair fragment), gyrB (287–base pair fragment), parC (230–base pair fragment) and parE (201–base pair fragment) genes, as described elsewhere [16]. qnr and qepA genes were detected by real-time PCR [18]. aac(6′)-Ib-cr was detected by pyrosequencing: one amplification product targeted the 77–base pair portion with T304C/A, and another targeted the 102–base pair portion with G535T. T304C/A and G535C were detected on pyrograms, as described elsewhere [19].
**Genotypic and Phenotypic Comparison of E. coli Strains**

In subjects with at least 1 QREC strain isolated during the study, the phenotypes and genotypes of these QREC strains were compared with those of the quinolone-susceptible *E. coli* (QSEC) strains isolated from the dominant flora. If QREC strains were detected on >1 sampling day, the strains were also compared to those isolated at different sampling days. We also compared genetically the different QREC strains isolated on day 42 from different subjects to determine if they belonged to the same clone. In addition, we determined whether these strains belonged to 2 QREC clones circulating in Europe, O25b-ST131 and O15:K52: H1 [20], using methods described elsewhere [21, 22].

Patterns of susceptibility to other antibiotics were compared. If the QREC strain had a marker of resistance to ≥1 additional antibiotic, the fecal sample was directly plated on Drigalski agar containing the drug (or drugs) to which the strain was coresistant (eg, 100 µg/mL for amoxicillin or 200 µg/mL for cotrimoxazole), to detect subdominant populations of coresistant *E. coli* strains. To increase the sensitivity of the method and the chances of detecting coresistant *E. coli* on these plates, we plated a final quantity of 10 mg of feces on Drigalski plates for each volunteer.

For genotyping, random amplification of polymorphic DNA (RAPD) PCR was performed using the primer 1254 (5′-CCGCAGCCAA-3′), as described previously [23]. When RAPD PCR patterns differed, strains were considered to be different. When RAPD PCR patterns were similar, genomic DNA was digested with *Xba*I (New England Biolabs, Boston, MA) and subjected to pulsed-field gel electrophoresis (PFGE), using the Gene Navigator System (Amersham Biosciences) [24]. Fragments were separated at 200 V with a pulse time of 15–35 seconds for 16 hours. The DNA patterns obtained on PFGE were analyzed according to criteria of Tenover [25].

**RESULTS**

**Presence of *E. coli* Strains in Feces With Regard to Ciprofloxacin Exposure**

All 48 volunteers carried fecal *E. coli* on day 0, before ciprofloxacin administration. On days 8 and 14, no subject carried QSEC strains, and only 3 (6.25%) harbored *E. coli* strains that were highly resistant to quinolones. At day 42, *E. coli* was isolated again from feces of all subjects at the same quantity than before exposure (>10⁷ CFU per gram of feces). Strains from 14 subjects (29.2%) were resistant to quinolones, with low-level resistance in strains from 10 and high-level resistance in strains from 4. For the remaining 34 subjects, strains isolated on day 42 were susceptible to quinolones (Figure 1).

**Characterization of QREC Strains**

The MICs of quinolones and the associated gene(s) conferring quinolone resistance are presented in Table 1. None of the described plasmid-mediated quinolone resistance genes (ie, *qnr*, *qepA*, or *aac[6]-Ib-cr*) were found in QREC strains, and none carried *gyrB* or *parE* mutations. Strains with low-level resistance to quinolones harbored 1 *gyrA* mutation, either alone (in strains from 9 subjects) or in association with a *parC* mutation (in strains from 5). Strains with high-level resistance to quinolones harbored 2 mutations in *gyrA* and 1 (in strains from 8 subjects) or 2 (in strains from 4) additional mutations in *parC*.

For subjects who experienced QREC strains at least once, the 5 different colonies randomly picked on the same day of
collection had similar RAPD PCR patterns. We also compared RAPD PCR patterns between QREC from day 42 and QSEC strains from day 0 isolated from the same subject. For strains from the 14 subjects with QREC, the patterns were different, which conflicts with the hypothesis that the QREC strains were mutants of the QSEC strains from the initial dominant flora.

All QREC strains collected from different subjects had different RAPD PCR profiles. None belonged to either the 2 clonal groups tested (O25b-ST131 and O15:K52:H1).

Dynamic Patterns of the Emergence of Quinolone Resistance
We distinguished 3 different dynamic patterns of emergence of quinolone resistance in E. coli strains among the 48 volunteers. These patterns are shown in Figure 1.

Group 1 included 34 subjects with no QREC detected on day 42. Thirty-three harbored only QSEC strains on day 0. One subject (V43) had a subdominant population of E. coli with low-level resistance to quinolones detected on day 0 but not on days 8–42. This strain harbored a single gyrA mutation and was genetically distinct from the QSEC retrieved on day 42.

Group 2 included 10 subjects carrying E. coli strains with low-level resistance on day 42. Among these subjects, 2 (V44 and V45) already carried strains with low-level resistance on day 0. These strains were genetically similar to those found on day 42 (Figure 2 shows the results of RAPD PCR and PFGE comparisons for V45). The strains with low-level resistance to quinolones harbored a single gyrA mutation (V44) and 1 gyrA and 1 parC mutation (V45). In both cases, no E. coli was evidenced on days 8 or 14. For the remaining 8 subjects (V34,
V35, V36, V37, V38, V39, V40, and V42), no QREC strains were detected on day 0. These subjects thus experienced the emergence of QREC strains after ciprofloxacin exposure. Interestingly, when the strains with low-level resistance to quinolones isolated on day 42 were compared with the QSEC strains collected on day 0, strains were genetically distinct by RAPD PCR. For the strains with low-level resistance to quinolones that were coresistant to a nonquinolone antibiotic, such as ampicillin (V36 and V40) or ampicillin and cotrimoxazole (V34, V38, V39, and V42), fecal samples collected on day 0 were cultivated on agar containing the relevant antibiotics. No colony grew on these plates; therefore, specimens obtained on day 0 did not contain susceptible strains that were parents of the strains with low-level resistance to quinolones that were isolated on day 42. Subject V42 intermittently carried a strain with high-level resistance, on days 8 and 14, but these strains differed genetically from the strain with low-level resistance to quinolones isolated on day 42. Finally, we found 3 subjects (V35, V40, and V42) with a dominant population of QREC on day 42: the same number of colonies was found on agar without and with nalidixic acid. A comparison of 10 colonies randomly picked from each agar revealed that they were genetically identical and carried the same mutations in gyrA and parC.

Group 3 included 4 subjects harboring E. coli with high-level resistance to quinolones on day 42. Two subjects (V47 and V48) had been found to be carrying such strains on days 0, 8, and 14. For both, the strains isolated before and after ciprofloxacin exposure were genetically similar by RAPD PCR and PFGE, and they harbored the same QRDR mutations. For subject V48, the strain with high-level resistance was subdominant on day 0 (i.e., it was only found on plates containing ciprofloxacin) but dominant on day 42 (i.e., it constituted the largest population present in fecal microbiota). The same number of colonies was found on agar with and without ciprofloxacin, and comparison of 10 colonies randomly picked from each agar showed that they were genetically identical and carried the same mutations in gyrA and parC. Of the remaining 2 subjects, one (V46) carried E. coli with low-level resistance to quinolones on day 0, while the other (V41) only harbored QSEC. They thus also experienced the emergence of QREC or an increase in the level of quinolone resistance following ciprofloxacin exposure. For both subjects, the E. coli strains with high-level resistance to quinolones isolated on day 42 were genetically distinct from the E. coli strains collected on day 0. Because strains with a high level of quinolone resistance isolated on day 42 were also resistant to ampicillin and cotrimoxazole, fecal samples obtained on day 0 were cultivated on selective agar containing both antibiotics, but no colony grew on either of them.

Overall, QREC emerged in 9 subjects (V34–42). We did not evidence the selection of resistant mutants from the initial E. coli population in any of these cases. In strains from 1 subject, the level of quinolone resistance increased, but the resistant strains collected before and after treatment were genetically different. The increase in resistance was thus not due to additional mutations occurring in the resistant mutants from the initial population. This indicates that QREC strains were probably acquired exogenously between day 14 (when ciprofloxacin exposure ended) and day 42 (when the last sample was collected). To determine whether the strains with low-level resistance and the strains with high-level resistance collected from different subjects belonged to endemic clones, side-by-side comparisons of all QREC strains by RAPD PCR was performed, and they were all found to be different. In one group of 4 subjects (V44, V45, V47, and V48), the QREC strains remained from days 0 to 42. In another group of 4 subjects (V35, V40, V42, and V48), the QREC strain replaced the dominant population on day 42. Finally, in 1 subject (V43), E. coli strains with a low level of quinolone resistance before treatment cleared after ciprofloxacin therapy.

**DISCUSSION**

In this study, we investigated at the individual level the emergence of quinolone resistance in E. coli from the human microbiota. Despite the major global impact of ciprofloxacin treatment on the human microbiota [15], only a minority of volunteers experienced emergence of resistance among E. coli in their feces. Indeed, in strains of 33 of 48 volunteers (69%), no resistance was detected either during or after exposure, suggesting an efficient protective role played by the anaerobes forming the microbiota [5]. This phenomenon, known as colonization resistance, prevents colonization of the intestinal ecosystem by exogenous bacteria and helps maintain the stability of the intestinal microbiota [26].

During antibiotic exposure (on days 8 and 14), no E. coli could be detected in most volunteers. This was explained by the high fecal concentrations of ciprofloxacin, which was several thousand times greater than both the MIC and the mutant-prevention concentration against the dominant flora [12]. Therefore, selection of resistance was unlikely during treatment [12]. The appearance of QREC strains 4 weeks after the end of ciprofloxacin therapy was also explained by the pharmacokinetics of ciprofloxacin in stool, because ciprofloxacin concentrations slowly decreased from day 14 to day 42, when they were undetectable, with ciprofloxacin concentrations passing through the mutant selection window between days 14 and 42, when emergence of resistance was eventually detected in the fecal microbiota [7].

Several hypotheses may explain the emergence of QREC strains: (1) de novo selection of resistant mutants from parental QSEC strains, (2) enrichment with the QREC subpopulation already present in the initial microbiota, (3) transmission...
of plasmid-mediated quinolone resistance genes from Enterobacteriaceae that were or were not resident [27], and environmental acquisition of exogenous quinolone-resistant bacteria during or after treatment. After ciprofloxacin exposure, on day 42, 9 subjects (V34–V42) experienced emergence of resistance to quinolones in E. coli, and 1 (V46) harbored E. coli strains with a higher level of quinolone resistance than before exposure on day 0. We precisely studied the dynamic patterns of emergence of resistance in these subjects by comparing strains isolated before and those isolated after exposure and by determining the mechanisms of resistance to quinolones.

The most noteworthy feature was that the emerging QREC strains did not have a quinolone-susceptible counterpart in the initial microbiota. These findings conflict with the hypothesis that de novo mutations occurred in the initial microbiota. Because no plasmid-related mechanism of resistance was found in any of the resistant strains, 2 hypotheses remain: (1) resistant subpopulations of E. coli were present at undetectable concentrations in the initial microbiota, and (ii) resistant strains were exogenously acquired. Several arguments suggest that exogenous acquisition of resistant E. coli after treatment may be the explanation. First, in 7 of 10 subjects in whom the emergence of or increase in resistance was evidenced, the QREC strains also harbored resistance to other antibiotics. However, no parental E. coli strain harboring these coresistance mechanisms were evidenced. Although bacteria may be able to acquire several resistance-conferring mechanisms at once, particularly if the mechanisms are plasmid mediated (which was not found here with regard to quinolone resistance), this result conflicts with the hypotheses that preexisting subdominant clones with coresistance to several antibiotics were selected and that de novo selection of QREC mutants carrying resistance to other antibiotics occurred. Second, within-household sharing of commensal E. coli, including QREC strains, among human and animal members has been widely documented [28–33]. One investigation reported that 2.9% of 455 healthy children who had never been exposed to quinolones (because these antibiotics are not approved for use in children) excreted QREC in their feces, suggesting that they had acquired QREC exogenously, either directly through food or indirectly via a household member [30]. Moreover, the close community environment of antibiotic use, as occurs in urban industrialized centers, contributes to an increased prevalence of resistant bacteria in an individual’s fecal microbiota, probably because of the circulation of resistant commensal enterobacteria among individuals [34]. These different studies suggest that horizontal transmission of E. coli (either through close contact or through the food supply [35, 36]) is an important phenomenon. The current study was done in Paris, where the prevalence of resistant commensal enterobacteria is high [13, 37]. Volunteers were therefore at risk of absorbing resistant bacteria, either through food or through contacts with

Figure 2. Comparison of quinolone-resistant Escherichia coli strains detected on days 0 and 42 in subject V45 by random amplification of polymorphic DNA (RAPD) PCR (A) and pulsed-field gel electrophoresis (PFGE); (B). A, RAPD PCR profiles of quinolone-resistant E. coli from subject V45 on day 0 (first 3 bands after molecular weight marker) and day 42 (last 4 bands). B, PFGE patterns of quinolone-resistant E. coli from V45 on day 0 (lanes 1 and 2) and day 42 (lane 3).
other individuals. Because they were also taking ciprofloxacin daily for 14 days, conditions were combined to allow the settlement of resistant bacteria in their feces. We found no evidence of clonal dissemination of resistant *E. coli* between the different subjects.

Our study also raises the issue of the complex relationship between fluoroquinolone resistance and bacterial fitness. Obviously, ciprofloxacin-resistant strains detected in volunteers V47 and V48 before ciprofloxacin exposure and through day 42 were perfectly adapted to the fecal microbiota and unaffected by massive antibiotic exposure. In 4 subjects (V35, V40, V42, and V48), the resistant strains had replaced the dominant flora by day 42. This suggests that even after ciprofloxacin withdrawal, these resistant strains had a selective advantage during days 14–42. It has been shown that some resistance mutations can be selected because they decrease susceptibility and reduce fitness costs associated with resistance mutations [38]. Whether the persistence of these strains is only explained by their level of resistance to quinolones remains to be determined. This question is also accurate in the cases of volunteers V44 and V45 in whom the same strain present at day 0 was detected again at day 42 while it only exhibited a low-level of resistance, suggesting that factors other than resistance probably drove the survival of these strains.

Potential limitations of this study need to be addressed. First, we estimated the lower limit of detection of *E. coli* strains to be 10³ CFU per gram of feces. Considering that healthy subjects carry 10⁸–10⁹ *E. coli*, we analyzed the vast majority of these *E. coli*, but resistant strains present in very low proportions may have been missed. Second, only 10 random colonies were picked to represent the dominant susceptible *E. coli* population on each collection day. However, the probability that we missed the dominant *E. coli* population was small: others have shown that the probability of including at least 1 isolate from the dominant clone was 99.3% for 5 randomly selected colonies [39].

The emergence of quinolone resistance in *E. coli* from the fecal microbiota is a complex phenomenon that does not depend only on the concentrations of ciprofloxacin in the feces and on the level of resistance of the strains. Among the several mechanisms by which the fecal microbiota becomes enriched with resistant *E. coli* strains, our results suggest that exogenous acquisition of resistant strains when ciprofloxacin concentrations fall within the selective window plays an important role.

### Notes

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