The Prevalence of Fluoroquinolone Resistance Mechanisms in Colonizing Escherichia coli Isolates Recovered from Hospitalized Patients

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(See the article by Johnson et al, on pages 286–294.)

Background. Fluoroquinolones are the most commonly prescribed antimicrobials. The epidemiology of fecal colonization with Escherichia coli demonstrating reduced susceptibility to fluoroquinolones remains unclear.

Methods. During a 3-year period (15 September 2004 through 19 October 2007), all patients hospitalized for >3 days were approached for fecal sampling. All E. coli isolates with reduced susceptibility to fluoroquinolones (minimum inhibitory concentration [MIC] of levofloxacin, ≥0.125 μg/mL) were identified. We characterized gyrA and parC mutations and organic solvent tolerance. Isolates were compared using pulsed-field gel electrophoresis.

Results. Of 353 patients colonized with E. coli demonstrating reduced fluoroquinolone susceptibility, 300 (85.0%) had ≥1 gyrA mutation, 161 (45.6%) had ≥1 parC mutation, and 171 (48.6%) demonstrated organic solvent tolerance. The mean numbers of total mutations (ie, gyrA and parC) for E. coli isolates with a levofloxacin MIC of ≥8 μg/mL versus <8.0 μg/mL were 2.70 and 0.82 (P<.001). Of the 136 E. coli isolates with a levofloxacin MIC of ≤8 μg/mL, 90 (65.2%) demonstrated a nalidixic acid MIC of ≥16 μg/mL. Significant differences were found over time in the proportion of E. coli isolates demonstrating gyrA mutation, parC mutation, and organic solvent tolerance. There was little evidence of clonal spread of isolates.

Conclusions. Gastrointestinal tract colonization with E. coli demonstrating reduced susceptibility to levofloxacin is common. Although 40% of study isolates exhibited a levofloxacin MIC of <8 μg/mL (and would thus be missed by current Clinical and Laboratory Standards Institute breakpoints), nalidixic acid resistance may be a useful marker for detection of such isolates. Significant temporal changes occurred in the proportion of isolates exhibiting various resistance mechanisms.

The fluoroquinolone antibiotics have become the most commonly used class of antibiotics [1, 2]. As such, the increasing prevalence of fluoroquinolone resistance in Escherichia coli, the most common gram-negative pathogen, is concerning [3, 4]. This is particularly true given the negative impact of fluoroquinolone resistance on clinical outcomes [5].

The human gastrointestinal (GI) tract serves as a natural reservoir for E. coli [6], and E. coli isolates causing clinical infection are almost always derived from organisms colonizing the GI tract [7, 8]. In addition, the stepwise accumulation of fluoroquinolone resistance determinants in E. coli (eg, in response to selection pressure from antimicrobial use) in the clinical setting likely occurs at the level of the GI tract [9]. Despite this, most studies seeking to characterize fluoroquinolone-resistant E. coli isolates have focused on isolates derived from clinical infections rather than on fecal colonizing isolates [10–14]. Studies that have focused on E. coli colonization in the clinical setting have typically assessed E. coli colonization with only 1 or a few fecal surveys at specific points or used sampling approaches that changed over time [12, 14–16]. This
approach limits the ability to assess secular changes and person-to-person transmission over time.

The goal of this study was to characterize GI tract colonization due to \textit{E. coli} with reduced susceptibility to fluoroquinolones among the hospitalized patient population using continuous enrollment of hospitalized patients during a 3-year period. In addition, we sought to comprehensively characterize the resistance genotypes and phenotypes of fecal \textit{E. coli} isolates with reduced susceptibility to fluoroquinolones in this patient population.

**METHODS**

The study was performed at 2 University of Pennsylvania Health System hospitals: (1) The Hospital of the University of Pennsylvania (hospital 1), an academic tertiary care medical center with 725 patient beds; and (2) Penn Presbyterian Medical Center (hospital 2), a 344-bed urban community hospital. This study was reviewed and approved by the institutional review board of the University of Pennsylvania.

Patients were enrolled in this study from 15 September 2004 through 19 October 2007. We approached all patients hospitalized at the 2 study sites. All hospital floors and units were included. To be eligible, a patient had to have been hospitalized for at least 3 days and be deemed capable by research staff of providing consent. Research staff approached all patients on the third day of hospitalization to obtain informed consent. All eligible patients were enrolled if informed consent was provided. If a patient was unavailable, 1 additional attempt to approach the patient was made the next day. Each patient could only be enrolled once. For patients who agreed to enroll, a perirectal swab was obtained by research staff. Of note, a perirectal swab has been shown to be highly sensitive and specific for detection of fluoroquinolone-resistant \textit{E. coli} when compared with stool culture [17].

**Microbiological methods.** To detect \textit{E. coli} isolates with reduced susceptibility to fluoroquinolones, perirectal swabs were obtained from enrolled patients and samples were inoculated to MacConkey agar plates supplemented with levofloxacin (0.125 \(\mu\)g/mL). Levofloxacin was used as a marker for susceptibility to fluoroquinolone antibiotics. Plates were streaked for isolation of colonies and incubated at 37°C in atmospheric air supplemented with 5% to 10% carbon dioxide and were checked for growth at 24 and 48 h. Colonies suspected of being \textit{E. coli} based on morphologic appearance were subcultured to blood agar plates (trypticase soy agar with 10% sheep blood) and MacConkey agar without levofloxacin. The subcultured isolates were examined for the appropriate colony morphologic characteristics on MacConkey agar (ie, pink colonies) and tested for oxidase production on the blood agar plate. All oxidase-negative colonies with the appropriate colony morphologic characteristics were definitively identified using the semiautomated Vitek 2 identification and susceptibility system [18].

To determine the minimum inhibitory concentration (MIC) of levofloxacin between the concentrations of 0.002 and 32 \(\mu\)g/mL, \textit{E. coli} isolates were subsequently tested for susceptibility to levofloxacin using the E-test method [19]. Isolates were also tested for susceptibility to a variety of other antimicrobials using the semiautomated Vitek 2 identification and susceptibility system (bioMérieux) [18].

For all \textit{E. coli} isolates with decreased susceptibility to fluoroquinolones, the quinolone resistance determining region of \textit{gyrA} and \textit{parC} were amplified and sequenced using previously described primers [20]. Sequencing was performed by the University of Pennsylvania DNA Sequencing Facility using an ABI 3730 DNA analyzer with BigDye Taq FS Terminator, version 3.1 (Applied Biosystems). Sequence data were analyzed and compared with reference sequences using the Lasergene software package (DNASTAR).

Increased drug efflux via the AcrAB efflux pump was measured indirectly by the organic solvent tolerance assay [21, 22]. Overexpression of AcrAB was measured indirectly by the organic solvent tolerance assay [20, 23]. The appearance of confluent growth in the presence of a hexane:cyclohexane (3:1) mixture was interpreted as positive for AcrAB overexpression.

The genetic relatedness of \textit{E. coli} isolates was determined by molecular typing using pulsed-field gel electrophoresis (PFGE). Chromosomal DNA was extracted and digested from isolates using the procedure described by Gautom [24]. Chromosomal DNA was digested with the XbaI enzyme and separated by PFGE using the CHEF Mapper XA System (Bio-Rad). All results were analyzed using the Fingerprinting II Informatix Software, version 3.0 (Bio-Rad). The band patterns were compared by means of the Dice coefficient using the unweighted pair-group method to determine band similarity and interpreted according to established criteria [25]. Genetic relatedness was determined by isolates that had \(\geq 80\%\) similarity. Although there are no standard criteria to determine whether isolates are due to person-to-person transmission [26], we used the following criteria to classify isolates as related via person-to-person transmission: (1) the isolates were defined as similar on the basis of the PFGE type (\(\geq 80\%\) similarity), and (2) they were defined as epidemiologically related on the basis of any overlap in the dates of hospitalization [27].

**Statistical analyses.** The proportion of patients with fecal colonization due to \textit{E. coli} with reduced susceptibility to levofloxacin was calculated. We also calculated the proportion of \textit{E. coli} isolates with a levofloxacin MIC of \(\geq 8\ \mu\)g/mL. We summarized the frequency of genetic mechanisms of resistance for all \textit{E. coli} isolates exhibiting reduced susceptibility to fluoroquinolones focusing specifically on mutations in \textit{gyrA} and \textit{parC}, as well as the presence of organic solvent tolerance. Finally, we
analyzed the frequency of different resistance mechanisms by study hospital and calendar year, using Fisher’s exact test.

We then investigated the relationship between the mechanism(s) of resistance and the level of reduced susceptibility. We compared *E. coli* isolates with a levofloxacin MIC of $\geq 8 \mu g/mL$ versus $<8 \mu g/mL$ on the basis of (1) median number of *gyrA* mutations, (2) median number of *parC* mutations, (3) median number of total mutations (ie, *gyrA* and *parC* mutations), and (4) presence of organic solvent tolerance. We also investigated the association between specific fluoroquinolone resistance mechanisms (ie, *gyrA* mutation, *parC* mutation, organic solvent tolerance) and susceptibility to the following antibiotics: chloramphenicol, trimethoprim-sulfamethoxazole, amikacin, gentamicin, imipenem, tetracycline, and tobramycin.

Categorical variables were compared using the Fisher exact test, whereas continuous variables were compared using the Student *t* test or the Wilcoxon rank-sum test, depending on the validity of the normality assumption [28]. For all calculations, a 2-tailed *P* $< .05$ was considered significant. All statistical calculations were performed using standard programs in Stata statistical software, version 10.0 (StataCorp).

**RESULTS**

During the study period, 353 patients were identified as colonized with *E. coli* demonstrating reduced fluoroquinolone susceptibility. These 353 represented 15.1% of all patients who agreed to have a sample obtained. Among the 353 patients, the median age was 56 years (interquartile range, 48–65 years) and 187 (53.0%) were male. With regard to race/ethnicity, 140 (39.7%) were white, 100 (28.3%) were African American, 3 (0.9%) were Native American, 6 (1.7%) were Asian, 3 (0.9%) were Hispanic, 5 (1.4%) were classified as other, and 99 (28.1%) were of unknown race/ethnicity. Of the 353 patients, 271 (76.7%) were hospitalized at hospital 1, whereas 82 (23.2%) were hospitalized at hospital 2.

Of the 353 study isolates, 217 (61.5%) demonstrated a levofloxacin MIC of $\geq 8 \mu g/mL$ (Figure 1). Among these 353 isolates, the mean number of *gyrA* mutations per isolate was 1.45 (range, 0–4), whereas the mean number of *parC* mutations per isolate was 0.51 (range, 0–2). The mean number of total mutations (*gyrA* and *parC*) per isolate was 1.98 (range, 0–4).

The number of *gyrA* and *parC* mutations among study isolates is noted in Table 1. Among all *E. coli* isolates with reduced susceptibility to fluoroquinolones, the total number of mutations (*gyrA* and *parC*) was as follows: 0 (*n* = 48), 1 (*n* = 77), 2 (*n* = 85), 3 (*n* = 121), and 4 (*n* = 22). Of note, no isolate exhibited a *parC* mutation without also having a *gyrA* mutation.

For *E. coli* isolates with a levofloxacin MIC of $\geq 8 \mu g/mL$, the mean number of *gyrA* mutations per isolate was 1.93 compared with 0.70 mutations for isolates with a levofloxacin MIC of $<8 \mu g/mL$.
of <8.0 μg/mL (P < .001). Similarly, the mean number of parC mutations for E. coli isolates with a levofloxacin MIC of ≥8.0 μg/mL versus <8.0 μg/mL was 0.77 and 0.12 (P < .001). Finally, the mean number of total mutations (ie, gyrA and parC) for E. coli isolates with a levofloxacin MIC of ≥8.0 μg/mL versus <8.0 μg/mL was 2.70 and 0.82 (P < .001).

For E. coli isolates with a levofloxacin MIC of ≥2.0 μg/mL, the mean number of gyrA mutations per isolate was 1.93, compared with 0.69 mutations for isolates with a levofloxacin MIC of <2.0 μg/mL (P < .001). Similarly, the mean number of parC mutations for E. coli isolates with a levofloxacin MIC of ≥2.0 μg/mL versus <2.0 μg/mL was 0.75 and 0.11 (P < .001). Finally, the mean number of total mutations (ie, gyrA and parC) for E. coli isolates with a levofloxacin MIC of ≥2.0 μg/mL versus <2.0 μg/mL was 2.72 and 0.81 (P < .001). Presence of gyrA or parC mutations was not significantly associated with resistance to other antibiotics.

Of the 353 study isolates, 171 (48.6%) demonstrated organic solvent tolerance. Of the 171 isolates, 101 (59.1%) had a levofloxacin MIC of ≥8 μg/mL, whereas 116 (64.1%) of 181 isolates without organic solvent tolerance had a levofloxacin MIC of ≥8 μg/mL (P = .38). E. coli isolates exhibiting organic solvent tolerance were significantly more likely to be resistant to chloramphenicol (17.5% vs 6.6%; P = .002). However, the presence of organic solvent tolerance was not associated with increased resistance to other antibiotics tested.

As noted previously, 48 isolates exhibited no mutations in gyrA or parC. Among these isolates, 45 (94%) had a levofloxacin MIC of <0.25 μg/mL. Also, 37 (77%) of these 48 isolates demonstrated organic solvent tolerance.

Among all 353 E. coli isolates, 306 (86.7%) demonstrated a nalidixic acid MIC in the nonsusceptible range (ie, ≥16 μg/mL). Of the 217 E. coli isolates with a levofloxacin MIC of ≥8 μg/mL, 216 (99.6%) exhibited a nalidixic acid MIC of ≥16 μg/mL. Of the 136 E. coli isolates with a levofloxacin MIC of ≤8 μg/mL, 90 (66.2%) demonstrated a nalidixic acid MIC of ≥16 μg/mL.

No significant differences were found when comparing isolates from the 2 study sites. For hospitals 1 and 2, respectively, the proportion of isolates demonstrating organic solvent tolerance was 48.0% and 50.0% (P = .42), and the proportion of isolates exhibiting a levofloxacin MIC of ≥8 μg/mL was 60.9% and 63.4% (P = .39). Similarly, the proportion of isolates at hospitals 1 and 2, respectively, demonstrating at least 1 gyrA mutation was 84.5% and 86.6% (P = .40), whereas the proportion of isolates exhibiting at least 1 parC mutation was 45.0% and 47.6% (P = .39).

Among E. coli with reduced susceptibility to levofloxacin, the annual proportion of isolates with a levofloxacin MIC of ≥8 μg/mL did not change significantly over time: 75% (15 of 20) in 2004, 64.8% (46 of 71) in 2005, 58.9% (86 of 146) in 2006, and 60.3% (70 of 116) in 2007 (P = .50). However, significant differences were seen across study years in the proportion of E. coli isolates demonstrating various mechanisms of resistance. For example, the proportion of isolates with at least 1 gyrA mutation was 90% (18 of 20) in 2004, 94.4% (67 of 71) in 2005, 77.4% (113 of 146) in 2006, and 87.9% (102 of 116) in 2007 (P = .005). Similarly, the proportion of isolates with at least 1 parC mutation was 55.0% (11 of 20) in 2004, 52.1% (37 of 71) in 2005, 50.7% (74 of 146) in 2006, and 33.6% (39 of 116) in 2007 (P = .02). Finally, the proportion of isolates exhibiting organic solvent tolerance was 25% (5 of 20) in 2004, 36.6% (26 of 71) in 2005, 54.5% (79 of 145) in 2006, and 52.6% (61 of 116) in 2007 (P = .02).

Among all E. coli isolates with reduced susceptibility to fluoroquinolones, there were 49 PFGE types. Within these, there was 1 large cluster of related isolates (ie, PFGE types 12a–12f) comprising 48 isolates. However, within this cluster, only 2 of 48 patients also met epidemiologic criteria for relatedness (ie, overlapping period of hospitalization with another patient from the cluster). There was also a smaller related large cluster of PFGE type 16 (16a–16c) comprising 17 isolates. Within this cluster, only 3 met epidemiologic criteria for relatedness. Thus, there were only 5 patients (1.5%) whose isolates met criteria for person-to-person transmission.

**DISCUSSION**

Of 353 patients colonized with E. coli with reduced susceptibility to fluoroquinolones, 217 (61.5%) were colonized with an E. coli meeting the Clinical and Laboratory Standards Institute (CLSI) breakpoint for fluoroquinolone resistance (ie, a levofloxacin MIC of ≥8 μg/mL) and demonstrating organic solvent tolerance.
floxacin MIC of \( \geq 8 \mu g/mL \). Thus, 136 isolates (or nearly 40%) would not have been identified as having reduced susceptibility to fluoroquinolones by current CLSI standards. Of these 136 E. coli isolates, 90 (66.2%) were nonsusceptible to nalidixic acid. This finding suggests that nalidixic acid may be a useful marker for reduced fluoroquinolone susceptibility among fecal E. coli isolates. These data support recent suggestions that routinely reporting nalidixic acid susceptibilities might effectively identify many isolates already harboring an early gyrA mutation or without a parC mutation (ie, early mutations that result in an increased fluoroquinolone MIC but an MIC that nevertheless does not meet the established threshold for resistance) [14, 29]. Indeed, these are precisely the isolates most likely to become fully resistant in the presence of antimicrobial selective pressure [30]. Efforts to study the potential for optimizing fluoroquinolone resistance surveillance efforts and/or fluoroquinolone prescribing based on nalidixic acid susceptibilities should be pursued.

We noted that most isolates had at least 1 gyrA mutation, with many demonstrating additional gyrA and/or parC mutations. These findings suggest that, in colonization in the clinical setting, the first step in the evolution of fluoroquinolone-resistant E. coli is a gyrA mutation with subsequent steps, likely including additional gyrA or parC mutations and/or enhanced efflux [31, 32]. Prospective studies with serial fecal sampling are needed to confirm the nature of longitudinal changes in E. coli GI colonization over time.

We also found that nearly 50% of isolates demonstrated efflux pump overexpression as indicated by organic solvent tolerance. This percentage is somewhat higher than that of prior reports, including our own [12, 14–16], and suggests that this mechanism of resistance may be becoming more widespread over time. The clinical implications of widespread organic solvent tolerance are clear in the fact that efflux overexpression typically confers resistance to multiple other antimicrobial agents [33]. Despite the many recognized substrates of efflux pumps, we found that the presence of organic solvent tolerance was associated with a greater likelihood of resistance to chloramphenicol but not other antibiotics.

Finally, we found several temporal changes in isolate characteristics. In particular, we found significant differences across study years for the presence of gyrA and parC mutations and organic solvent tolerance. Because this study enrolled patients continuously over time, these results extend considerably findings from our earlier work that only assessed colonization through several point prevalence surveys [12, 14–16]. Our results suggest substantial changes over time in the prevalence of organic solvent tolerance among E. coli with reduced susceptibility to fluoroquinolones [16]. Although an outbreak of a specific E. coli strain might be 1 explanation, results of the PFGE analysis argue against substantial person-to-person spread. Likewise, there were no major changes in the antimicrobial formulary in the 2 hospitals that might explain these results. Given the consistent findings across studies, future investigations of temporal changes in resistance mechanisms may provide valuable insights into the evolution of these resistant pathogens.

Our study had a few potential limitations. Although selection bias is of potential concern, we sought to enroll all eligible patients. Although only 51% of eligible patients were enrolled, participants and nonparticipants were similar with regard to available data (ie, age, sex, hospital location, duration of hospitalization before invitation to enroll), suggesting no substantial bias was introduced by nonparticipation.

In sampling patients, only 1 colony was selected for evaluation. However, recent work has noted that patients may on occasion be colonized with multiple distinct strains of fluoroquinolone-resistant E. coli [34, 35]. Despite these recent findings, our goal in the current study was to identify patients colonized with E. coli with reduced susceptibility to fluoroquinolones, regardless of the number of strains with which a given patient was colonized. Because our goal was not to examine strain diversity, we believe obtaining only 1 strain per person was reasonable. However, this approach clearly limits the ability to comment on the phenotypic and genotypic characterization of multiple isolates per patient.

In addition, our study focused only on identifying the most common, and clinically important, mechanisms of fluoroquinolone resistance. As such, we did not identifying less common mechanisms (e.g., qnr, aac\([6]’)-lb-cr). For those E. coli isolates with reduced susceptibility to fluoroquinolones that did not manifest either gyrA/parC mutations or efflux overexpression, it is possible that 1 of these less common resistance mechanisms may have contributed to reduced susceptibility. In addition, our study was conducted in a large tertiary care medical center and a smaller urban community hospital; the results may not be generalizable to other dissimilar institutions.

In summary, GI tract colonization with E. coli demonstrating reduced susceptibility to fluoroquinolones is common in hospitalized patients. Although \( \sim 40\% \) of study isolates exhibited a levofloxacin MIC of \(< 8 \mu g/mL \) (and would thus be missed by current CLSI breakpoints), nalidixic acid resistance may be a useful marker for detection of such isolates. Significant differences occurred across study years in the proportion of isolates exhibiting various resistance mechanisms, suggesting that future research should more clearly elucidate potential evolution of fluoroquinolone resistance mechanisms in the clinical setting over time.

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