Published in final edited form as:
Clin Infect Dis. 2010 August 1; 51(3): 280–285. doi:10.1086/653931.

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

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Abstract

Background—Fluoroquinolones (FQs) are the most commonly prescribed antimicrobials. The epidemiology of fecal colonization with *Escherichia coli* demonstrating reduced susceptibility to FQs remains unclear.

Methods—Over a three year period, all patients hospitalized >3 days were approached for fecal sampling. All *E. coli* with reduced susceptibility to FQs (MIC ≥0.125μg/mL to levofloxacin) were identified. We characterized *gyrA* and *parC* mutations and organic solvent tolerance. Isolates were compared using pulsed field gel electrophoresis.

Results—Of 353 subjects colonized with *E. coli* demonstrating reduced FQ susceptibility, 300 (85%) had ≥ 1 *gyrA* mutation, 161 (45.6%) had ≥ 1 *parC* mutation, and 171 (48.6%) demonstrated organic solvent tolerance. The mean number of total mutations (i.e., *gyrA* + *parC*) for *E. coli* isolates with a levofloxacin MIC ≥8 μg/ml vs. <8.0 μg/ml was 2.70 and 0.82, respectively (p<0.001). Of the 136 *E. coli* isolates with a levofloxacin MIC ≤8 μg/ml, 90 (66.2%) demonstrated a nalidixic acid MIC ≥16 μg/ml. There were significant differences over time in the proportion of *E. coli* isolates demonstrating *gyrA* mutation, *parC* mutation, and organic solvent tolerance. There was little evidence for clonal spread of isolates.

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Presented in part at the 19th Annual Meeting of the Society for Healthcare Epidemiology of America (SHEA) of March 19–22, 2009 San Diego, CA.

All other authors report no potential conflict of interest.

Conclusions—GI tract colonization with *E. coli* demonstrating reduced susceptibility to levofloxacin is common. Although 40% of study isolates exhibited a levofloxacin MIC <8 μg/ml (and would thus be missed by current CLSI 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.

Keywords

E. coli; fluoroquinolone; colonization; genotype; phenotype

INTRODUCTION

The fluoroquinolone (FQ) antibiotics have become the most commonly used class of antibiotics [1,2]. As such, the increasing prevalence of FQ resistance in *Escherichia coli*, the most common gram negative pathogen, is most concerning [3,4]. This is particularly true given the negative impact of FQ 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 step-wise accumulation of FQ resistance determinants in *E. coli* (e.g., 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 FQ-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 one or a few fecal surveys at specific points in time 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 *E. coli* with reduced susceptibility to FQs among the hospitalized patient population using continuous enrollment of hospitalized patients over a three year time period. In addition, we sought to comprehensively characterize the resistance genotypes and phenotypes of fecal *E. coli* isolates with reduced susceptibility to FQs in this patient population.

METHODS

The study was performed at two University of Pennsylvania Health System hospitals: 1) The Hospital of the University of Pennsylvania (HUP), an academic tertiary care medical center with 725 patient beds; and 2) Penn Presbyterian Medical Center (PMC), 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 September 15, 2004 through October 19, 2007. We approached all patients hospitalized at the two study sites. All hospital floors and units were included. To be eligible, a patient had to have been hospitalized for at least three 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, one additional attempt to approach the patient was made the following day. Each subject could only be enrolled once. For subjects who agreed to enroll, a peri-rectal swab was obtained by research staff. Of note, a peri-rectal swab has been shown to be highly sensitive and specific for detection of FQ-resistant *E. coli* when compared to stool culture [17].

Microbiological Methods

To detect *E. coli* isolates with reduced susceptibility to FQs, peri-rectal swabs were obtained from enrolled patients and samples were inoculated to MacConkey agar plates supplemented with levofloxacin (0.125 μg/ml). Levofloxacin was used as a marker for susceptibility to FQ antibiotics. Plates were streaked for isolation of colonies and incubated at 37°C in atmospheric air supplemented with 5% to 10% CO_2 and were checked for growth at 24 and 48 hours. Colonies suspected of being *E. coli* based on morphological appearance were subcultured to blood agar plates (tryticase soy agar with 10% sheep blood) and MacConkey agar without levofloxacin. The subcultured isolates were examined for the appropriate colony morphology on the MacConkey agar (i.e., pink colonies) and tested for oxidase production on the blood agar plate. All oxidase negative colonies with the appropriate colony morphology were definitively identified using the semi-automated Vitek 2 identification and susceptibility system [18].

To determine the minimum inhibitory concentration (MIC) of levofloxacin between the concentrations of 0.002 μg/ml and 32 μg/ml, *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 other antimicrobials using the semi-automated Vitek 2 identification and susceptibility system (bioMerieux, Inc.) [18].

For all *E. coli* with decreased susceptibility to FQs, the quinolone resistance determining region of *gyrA* and *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 V 3.1. (Applied Biosystems, Foster City, CA). Sequence data were analyzed and compared to reference sequences using the LASERGENE software package (DNASTAR, Inc., Madison, WI).

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:cylohexane (3:1) mixture was interpreted as positive for AcrAB overexpression.

The genetic relatedness of *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 *Xba*I enzyme and separated by PFGE using the CHEF Mapper XA System (Bio-Rad, Hercules, CA). All results were analyzed using the Fingerprinting II Informatix Software v 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 ≥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 (≥80% similarity), and (2) they were defined as epidemiologically related on the basis of any overlap in the dates of hospitalization [27].

Statistical Methods

The proportion of subjects with fecal colonization due to *E. coli* with reduced susceptibility to levofloxacin was calculated. We also calculated the proportion of *E. coli* isolates with a levofloxacin MIC ≥8 μg/ml. We summarized the frequency of genetic mechanisms of resistance for all *E. coli* isolates exhibiting reduced susceptibility to FQs focusing specifically on mutations in *gyrA* and *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 ≥8 μg/ml vs. MIC <8 μg/ml on the basis of: 1) median number of *gyrA* mutations; 2) median number of *parC* mutations; 3) median number of total mutations (i.e., *gyrA* + *parC* mutations); and 4) presence of organic solvent tolerance. We also investigated the association between specific FQ resistance mechanisms (i.e., *gyrA* muatation, *parC* mutation, organic solvent tolerance) and susceptibility to the following antibiotics: chloramphenicol, trimethoprim-sulfamethoxazole (TMP-SMZ), amikacin, gentamicin, imipenem, tetracycline, and tobramycin.

Categorical variables were compared using the Fisher's exact test while continuous variables were compared using the Student's t test or the Wilcoxon rank sum test, depending on the validity of the normality assumption [28]. For all calculations, a two-tailed P value of <0.05 was considered significant. All statistical calculations were performed using standard programs in STATA v 10.0, (Stata Corp, College Station TX).

RESULTS

During the study period, a total of 353 subjects were identified as colonized with *E. coli* demonstrating reduced FQ susceptibility. These 353 represented 15.1% of all subjects who agreed to have a sample obtained. Among the 353 subjects, the median age was 56 (interquartile range = 48–65) and 187 (53.0%) were male. With regard to race and 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 unknown. Of the 353 subjects, 271 (76.7%) were hospitalized at Hospital 1 while 82 (23.2%) were hospitalized at Hospital 2.

Of the 353 study isolates, 217 (61.5%) demonstrated a levofloxacin MIC ≥8 μg/ml. Among these 353 isolates, the mean number of *gyrA* mutations per isolate was 1.45 (range 0–4), while the mean number of *parC* mutations per isolate was 0.51 (range 0–2). The mean number of total mutations (*gyrA* + *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 FQs, the total number of mutations (*gyrA* + *parC*) was as follows: zero (n=48); one (n=77); two (n=85); three (n=121); and four (n=22). Of note, no isolate exhibited a *parC* mutation without also having a *gyrA* mutation.

For *E. coli* isolates with a levofloxacin MIC ≥8 μg/ml, the mean number of *gyrA* mutations per isolate was 1.93 compared to 0.70 mutations for isolates with a levofloxacin MIC <8.0 μg/ml (p<0.001). Similarly, the mean number of *parC* mutations for *E. coli* isolates with a levofloxacin MIC ≥8.0 μg/ml vs. <8.0 μg/ml was 0.77 and 0.12, respectively (p<0.001). Finally, the mean number of total mutations (i.e., *gyrA* + *parC*) for *E. coli* isolates with a levofloxacin MIC ≥8.0 μg/ml vs. <8.0 μg/ml was 2.70 and 0.82, respectively (p<0.001).

For *E. coli* isolates with a levofloxacin MIC ≥2.0 μg/ml, the mean number of *gyrA* mutations per isolate was 1.93 compared to 0.69 mutations for isolates with a levofloxacin MIC <2.0 μg/ml (p<0.001). Similarly, the mean number of *parC* mutations for *E. coli* isolates with a levofloxacin MIC ≥2.0 μg/ml vs. <2.0 μg/ml was 0.75 and 0.11, respectively (p<0.001). Finally, the mean number of total mutations (i.e., *gyrA* + *parC*) for *E. coli* isolates with a levofloxacin MIC ≥2.0 μg/ml vs. <2.0 μg/ml was 2.72 and 0.81, respectively (p<0.001).

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 ≥8 μg/ml while, 116 of 181 (64.1%) isolates without organic solvent tolerance had a levofloxacin MIC ≥8 μg/ml (p=0.38). *E. coli* isolates exhibiting organic solvent tolerance were significantly more likely to be resistant to chloramphenicol (17.5% vs 6.6%, respectively; p=0.002). However, 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 <0.25ug/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 non-susceptible range (i.e., ≥16 μg/ml). Of the 217 *E. coli* isolates with a levofloxacin MIC ≥8 μg/ml, 216 (99.6%) exhibited a nalidixic acid MIC ≥16 μg/ml. Of the 136 *E. coli* isolates with a levofloxacin MIC ≤8 μg/ml, 90 (66.2%) demonstrated a nalidixic acid MIC ≥16 μg/ml.

There were no significant differences when comparing isolates from the two study sites. For Hospitals 1 and 2, respectively, the proportion of isolates demonstrating organic solvent tolerance was 48.0% and 50.0% (p=0.42) and the proportion of isolates exhibiting a levofloxacin MIC of >8ug/ml was 60.9% and 63.4% (p=0.39). Similarly the proportion of isolates at Hospitals 1 and 2 demonstrating at least one *gyrA* mutation was 84.5% and 86.6% (p=0.40) while the proportion of isolates exhibiting at least one *parC* mutation was 45.0% and 47.6% (p=0.39).

Among *E. coli* with reduced susceptibility to levofloxacin, the annual proportion of isolates with a levofloxacin MIC ≥8 μg/ml did not change significantly over time: 15/20 (75%) in 2004; 46/71 (64.8%) in 2005; 86/146 (58.9%) in 2006; and 70/116 (60.3%) in 2007 (p=0.50). However, there were significant differences across study years in the proportion of *E. coli* isolates demonstrating various mechanisms of resistance. For example, the proportion of isolates with at least one *gyrA* mutation was 18/20 (90%) in 2004; 67/71 (94.4%) in 2005; 113/146 (77.4%) in 2006; and 102/116 (87.9%) in 2007 (p=0.005). Similarly, the proportion of isolates with at least one *parC* mutation was 11/20 (55.0%) in 2004; 37/71 (52.1%) in 2005; 74/146 (50.7%) in 2006; and 39/116 (33.6%) in 2007 (p=0.02). Finally, the proportion of isolates exhibiting organic solvent tolerance was 5/20 (25%) in 2004; 26/71 (36.6%) in 2005; 79/145 (54.5%) in 2006; and 61/116 (52.6%) in 2007 (p=0.02).

Among all *E. coli* isolates with reduced susceptibility to FQs, there were 49 PFGE types. Within these, there was one large cluster of related isolates (i.e., PFGE types 12a through 12f) comprising 48 isolates. However, within this cluster, only 2 of 48 subjects also met epidemiologic criteria for relatedness (i.e., overlapping period of hospitalization with another subject from the cluster). There was also a smaller related large cluster of PFGE type 16 (16a through 16c) comprising 17 isolates. Within this cluster, only three met epidemiologic criteria for relatedness. Thus, there were only five subjects (1.5%) whose isolates met criteria for person-to-person transmission.

DISCUSSION

Of 353 subjects colonized with *E. coli* with reduced susceptibility to FQs, 217 (61.5%) were colonized with an *E. coli* meeting the Clinical and Laboratory Standards Institute (CLSI) breakpoint for FQ resistance (i.e., a levofloxacin MIC ≥8 μg/ml). Thus, 136 isolates (or nearly 40%) would not have been identified as having reduced susceptibility to FQs by current CLSI

standards. Of these 136 *E. coli* isolates, 90 (66.2%) were non-susceptible to nalidixic acid. This suggests nalidixic acid may be a useful marker for reduced FQ 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* with or without a *parC* mutation (i.e., early mutations that result in an increased fluoroquinolone MIC, but an MIC which nevertheless does not meet the established threshold for resistance) [14,29]. Indeed, these are precisely those isolates most likely to become fully resistant in the presence of antimicrobial selective pressure [30]. Efforts to study the potential for optimizing FQ resistance surveillance efforts and/or FQ prescribing based on nalidixic acid susceptibilities should be pursued.

We noted that the large majority of isolates had at least one *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 FQ-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 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 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 as well as organic solvent tolerance. As this study enrolled patients continuously over time, these results extend considerably findings from our earlier work which 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 FQs [16]. While an outbreak of a specific *E. coli* strain might be one 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 two hospitals which 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. While selection bias is of potential concern, we sought to enroll all eligible subjects. Although only 51% of eligible subjects were enrolled, participants and non-participants were similar with regard to available data (i.e., age, sex, hospital location, duration of hospitalization prior to invitation to enroll) suggesting no substantial bias was introduced by non-participation.

In sampling subjects, only one colony was selected for evaluation. However, recent work has noted that subjects may on occasion be colonized with multiple distinct strains of FQ-resistant *E. coli* [34,35]. Despite these recent findings, our goal in the current study was to identify subjects colonized with *E. coli* with reduced susceptibility to fluoroquinolones, regardless of the number of strains with which a given subject was colonized. As our goal was not to examine strain diversity, we believe obtaining only one strain person was reasonable. However, this

approach clearly limits the ability to comment on the phenotypic and genotypic characterization of multiple isolates per subject.

In addition, our study focused only on identifying the most common, and clinically important, mechanisms of FQ resistance. As such, we did not identifying less common mechanisms (e.g., qnr, aac(6')-lb-cr). For those *E. coli* with reduced susceptibility to fluoroquinolones that did not manifest either *gyrA/parC* mutations or efflux overexpression, it is possible that one 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 FQs is common in hospitalized patients. Although approximately 40% of study isolates exhibited a levofloxacin MIC <8 μg/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 future research should more clearly elucidate potential evolution of FQ resistance mechanisms in the clinical setting over time.

Acknowledgments

This study was primarily supported by National Institutes of Health (NIH) grant R01-AI055008 (EL). Support was also provided by NIH Grant K24-AI080942 (EL), K24-AI073957 (JPM), as well as an Agency for Healthcare Research and Quality (AHRQ) Centers for Education and Research on Therapeutics cooperative agreement (U18-HS10399).

Dr. Lautenbach has received research support from Merck, Ortho-McNeil, Cubist, and AstraZeneca.

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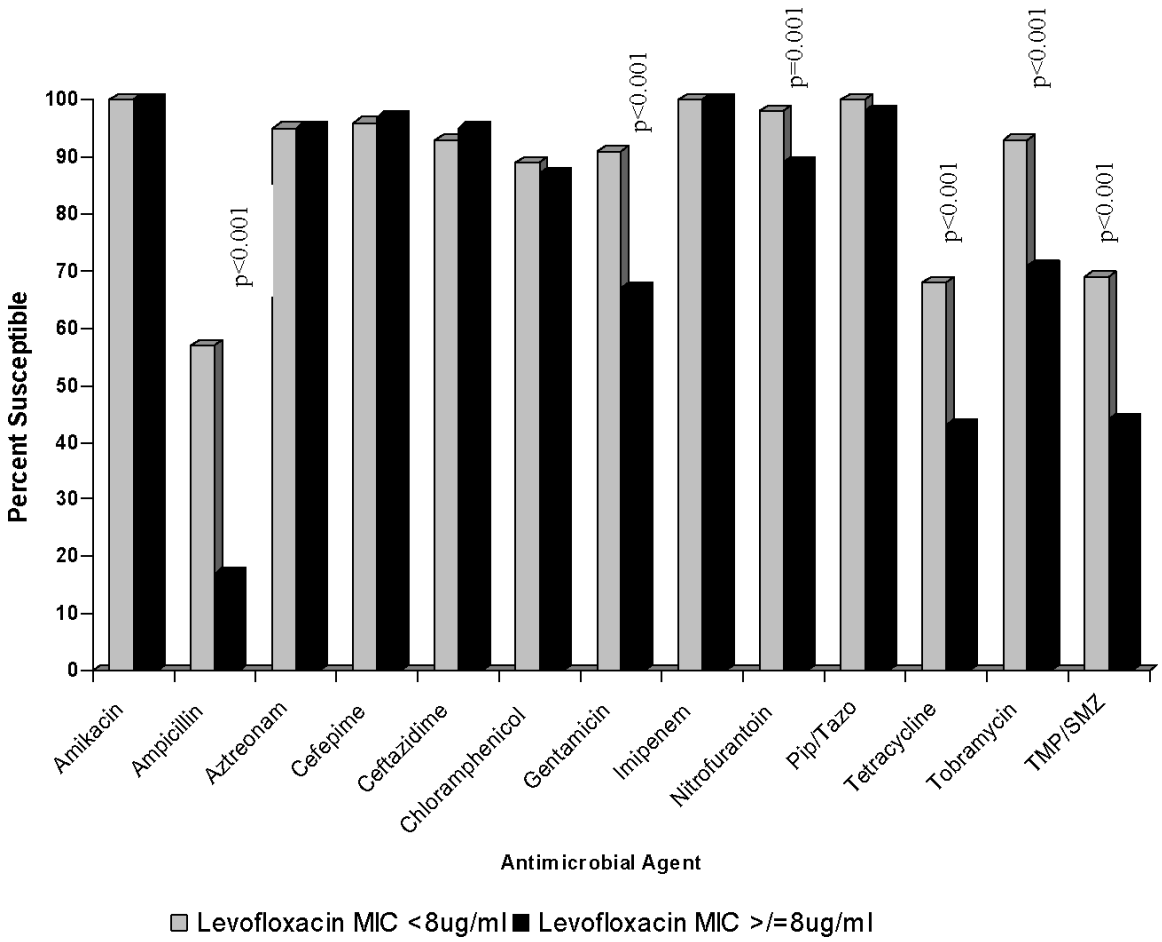

Figure 1.

Levofloxacin MIC and Susceptibility to Other Antimicrobial Agents All statistically significant associations shown

TMP/SMZ: Trimethoprim-sulfamethoxazole

Pip-Tazo: Piperacillin-Tazobactam

Table 1

Mutations in *gyrA* and *parC*

Mutations	N (%) (n=353 total isolates)
gyrA mutations	
None	53 (15.0%)
1 mutation	85 (24.1%)
2 mutations	215 (60.9%)
Specific *gyrA* mutations	
Ser-83-Leu	n=290
Asp-87-Asn	n=208
Asp-87-Tyr	n=11
Asp-87-Gly	n=6
parC mutations	
None	192 (54.4%)
1 mutation	139 (39.4%)
2 mutations	22 (6.2%)
Specific *parC* mutations	
Ser-80-Leu	n=155
Glu-84-Val	n=14
Glu-84-Gly	n=10
Glu-84-Lys	n=4