Gut Colonization of Healthy Children and Their Mothers With Pathogenic Ciprofloxacin-Resistant *Escherichia coli*

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(See the editorial commentary by Spellberg and Doi on pages 1853–5.)

Background. The reservoir of pathogenic ciprofloxacin-resistant *Escherichia coli* remains unknown.

Methods. We conducted a prospective cohort study of 80 healthy twins and their mothers to determine the frequency of excretion of ciprofloxacin-resistant, potentially pathogenic *E. coli*. Stool specimens were cultured selectively for ciprofloxacin-resistant gram-negative bacteria. Isolates were categorized on the basis of additional resistance and virulence profiles. We also prospectively collected clinical metadata.

Results. Fifteen children (19%) and 8 mothers (20%) excreted ciprofloxacin-resistant *E. coli* at least once. Overall, 33% of 40 families had at least 1 member whose stool specimen yielded ciprofloxacin-resistant *E. coli* on culture. Fifty-seven submitted stool specimens (2.8%) contained such organisms; clones ST131-*H*30 and ST405 accounted for 52 and 5 of the positive specimens, respectively. Length of hospital stay after birth ($P = .002$) and maternal colonization ($P = .0001$) were associated with subsequent childhood carriage of ciprofloxacin-resistant *E. coli*; antibiotic use, acid suppression, sex, mode of delivery, and maternal perinatal antibiotic use were not. Ciprofloxacin-resistant *E. coli* were usually resistant to additional antibiotic classes, and all had virulence genotypes typical of extraintestinal pathogenic *E. coli*.

Conclusions. Healthy children and their mothers commonly harbor ciprofloxacin-resistant *E. coli* with pathogenic potential.

Keywords. ciprofloxacin-resistant *E. coli*; extra-intestinal pathogenic *E. coli*; *E. coli* ST131-H30; *E. coli* ST405; urinary tract infections.

Ciprofloxacin-resistant *Escherichia coli* that are capable of causing extraintestinal infections, and especially infections of the urinary tract, are increasingly recovered from diverse populations throughout the world [1–3].

Most such infections have been identified in adults. Though it is postulated that the habitat of ciprofloxacin-resistant *E. coli* is the gastrointestinal tract, their human reservoir remains unknown. A decade-old survey determined that 7 of 455 healthy Seattle children (1.5%) excreted ciprofloxacin-resistant *E. coli* in their stools [4], even though these children had never been prescribed fluoroquinolones, because such antibiotics were not then (and are not now) approved for use in individuals <18 years of age in the United States. However, few subsequent studies have measured the colonization of community-dwelling children by ciprofloxacin-resistant *E. coli*, and during this interval the global dissemination of these pathogens, which are often resistant to multiple additional antibiotics, has continued

unabated. Here, we defined the extent to which healthy children in a St. Louis twin cohort who are not exposed to prescription fluoroquinolones, and their mothers, excrete ciprofloxacin-resistant *E. coli* in their stools. We also characterized the resistant isolates for susceptibility to antibiotics other than fluoroquinolones, determined their sequence types, profiled their complement of virulence loci, and then compared these isolates to those from the study performed in Seattle children in the early 2000s.

MATERIALS AND METHODS

Cohorts and Sample Collection

After receiving Washington University and Missouri-Baptist Medical Center institutional review board approval, we obtained consent from women with twin pregnancies to collect stool specimens from them (semiannually) and their twins (monthly to age 2 years and bimonthly thereafter). Stool specimens obtained from January 2010 through then end of May 2013 were couriered to the laboratory in insulated envelopes containing frozen packs and stored at −80°C until analyzed. Specimens of stool produced on weekends and in the evenings were stored temporarily at −20°C until the next opportunity to transport them on the morning of the next working day. Data were collected regarding pregnancy, labor, delivery, medications, and feeding by interviewing parents and/or reviewing medical records.

Isolation and Characterization of Ciprofloxacin-Resistant Gram-Negative Bacteria

Approximately 10 mg of frozen stool specimen was placed into 180 μL of tryptic soy broth, mixed, and incubated at 37°C for 65 minutes in a brief nonselective amplification step [5]. A total of 20 μL of this outgrowth was inoculated into 180 μL of MacConkey broth containing vancomycin (20 mg/L), ciprofloxacin (2 mg/L), and amphotericin (2 mg/L). These antimicrobial-containing broths were incubated overnight at 37°C, and turbid outgrowths were plated to MacConkey agar containing the same antimicrobials. A single ciprofloxacin-resistant isolate on each plate with such growth was identified and frozen (at −80°C in MacConkey broth with 15% glycerol) until further studied. In spiking experiments, this method was sufficiently sensitive to detect as few as 141 ciprofloxacin-resistant *E. coli* per gram of stool. Ciprofloxacin-resistant *E. coli*, defined as *E. coli* that grow on MacConkey agar containing ciprofloxacin, then underwent multilocus sequence typing [6]. These isolates were also subjected to disk diffusion testing to confirm their resistance to ciprofloxacin and, additionally, to determine their susceptibility to ampicillin, cefazolin, cefotetan, ceftazidime, ceftriaxone, cefepime, piperacillin-tazobactam, trimethoprim-sulfamethoxazole, gentamicin, doxycycline, and meropenem [7].

DNA extracted from ciprofloxacin-resistant *E. coli* was screened by polymerase chain reaction for genes encoding diverse virulence-associated traits: adhesins *fimH, iha, papAH, papC, papEF, papG, papG* alleles I, II, and III, *sfa/focDE, sfaS, focG, afa/draBC, afaE8, bmaE, gafD, F17, clpG, yfcV,* and *hra*; toxins *sat, hlyD, hlyF, cnf1, cdtB; pic, vat, tsh,* and *astA;* siderophores/iron-acquisition proteins *iroN, ireA, fyuA,* and *iutA;* protectins kpsMIII and the *K1, K15, K2/K100, kpsII, K5* capsule variants, *rfc, iss,* and *traT;* and miscellaneous virulence factors microcin *cvaC,* flagellin variant *H7,* the colibactin polyketide synthesis genes *clbB* and *clbN;* bacteriocin *usp;* outer membrane protein *ompT;* and pathogenicity island marker *malX* [8, 9]. Finally, isolates underwent clonal typing for ST131, ST131-*H*30, and ST405 SNPs by PCR [8, 9]. The 7 archival ciprofloxacin-resistant *E. coli* from Seattle [4] were also characterized using these techniques, to determine their antibiotic resistances, virulence profiles, and clonal types.

Positive stool specimens were defined as those from which ciprofloxacin-resistant *E. coli* were isolated. Stool specimens containing ciprofloxacin-resistant gram-negative bacteria other than *E. coli* were not considered positive. Similarly, stool specimens that yielded isolates that initially grew on the ciprofloxacin-containing MacConkey agar but that could not be propagated on agar or in broth were also considered negative. The day of first colonization was defined as the midpoint between the first positive sample and the immediately preceding sample. If the first sample obtained from the subject was positive, the midpoint between birth and the date of collection of that first positive stool was considered to be the day of first colonization for that subject.

To determine the proportion of the lactose-fermenting coliform bacteria that were resistant to ciprofloxacin in the stool specimens that were known to contain ciprofloxacin-resistant *E. coli* (ie, positive stool specimens), frozen samples that were previously determined to contain such resistant bacteria were processed as described above, except ciprofloxacin was omitted from the broth and agar in the first plating. Five random lactose-fermenting colonies on these nonselective agar plates were then tested for growth on ciprofloxacin-supplemented agar.

Statistic Analyses

Statistically significant differences (defined as those with a *P* value of <.05) in characteristics between groups were determined by the Mann–Whitney or Fisher exact tests. *P* values were 2 tailed.

RESULTS

Frequency of Positive Stool Specimens

The stool specimens obtained from the twins were sampled longitudinally for a median of 890 days (interquartile range [IQR], 678–1118 days). Ciprofloxacin-resistant *E. coli* were detected in at least 1 stool specimen among 15 of 80 twins (19%) and 8 of 40

Table 1. Demographic, Clinical, and Maternal of Children Whose Stool Specimens Did or Did Not Contain Ciprofloxacin-Resistant *Escherichia coli*

Characteristic	Ciprofloxacin-Resistant *E. coli* in Stools		P Value
	Present in ≥1 Tested Sample (n = 15)	Absent From All Tested Samples (n = 65)	
Gestational age at birth, wk	36 (34–36)	37 (36–37)	.06
Birth weight, g	2455 (2325–2583)	2693 (2393–3008)	.13
Day of life discharged home after birth	10 (4–13)	4 (2, 5)	.002
Male sex	11 (73)	30 (45)	.09
African American/White	4 (27)	6 (9)	.09
Hispanic/not Hispanic	1 (7)	4 (7)	1.0
Monozygotic:dizygotic	8 (53)	34 (53)	1.0
Vaginal birth	2 (13)	27 (42)	.07
Maternal perinatal antibiotics[a]	14 (93)	52 (80)	.45
Antibiotics early in life[b]	6 (40)	14 (22)	.18
Day of life of first positive specimen	341 (49–641)	NA	
Specimens studied per subject, no.	22 (15–26)	24 (21–28)	.1
Specimens positive/tested, no.	43/299 (26.9)	0/1514 (0)	
Mothers with positive specimens	8 (53)	4 (6)	.0001
Day of life when final specimen submitted	681 (624–1060)	912 (694–1129)	.21
Acid suppression early in life[c]	2 (13)	12 (18)	1.0
Exclusively breastfed[d]	0 (0)	10 (15.4)	.19
Exclusively formula fed[d]	6 (40)	16 (24.6)	.34
Combined breastfed and formula fed[d]	9 (60)	39 (60)	1.0

Data are median (interquartile range) or no. (%) of children, unless otherwise indicated.

Abbreviation: NA, not applicable.

[a] Defined as any perinatal antibiotics administered to the mother within 7 days of birth.

[b] Defined as antibiotics administered to children during the first 2 weeks of life.

[c] Defined as proton-pump inhibitors, H2-blockers, or cytoprotective agents administered to children during the first 2 months of life.

[d] Defined as postdischarge feeds during the first 2 months of life.

mothers (20%). At least 1 member of 13 of 40 households (33%) had at least 1 positive stool specimen, defined as a stool specimen in which ciprofloxacin-resistant *E. coli* were identified. Overall, 57 of 1977 stool specimens (2.9%) from all subjects were positive; 44 of 1813 specimens (2.4%) were from twins, and 13 of 164 (7.9%) were from mothers (Table 1 and Supplementary Table 1).

Ten stool specimens obtained from 10 different children yielded ciprofloxacin-resistant gram-negative bacilli that were not *E. coli*. These included *Stenotrophomonas maltophilia* (8 children), *Citrobacter freundii* complex (1 child), or *Citrobacter werkmanii* (1 child). Five specimens yielded ciprofloxacin-resistant small colonies that could not be propagated and were considered negative.

Epidemiological Correlates of Positive Stool Specimens

The 15 children who had 1 or more positive stool specimen had a median of 1 such specimen (IQR, 1–3 specimen). The median day of life on which the first positive stool specimen was obtained was 341 (IQR, 49–641). Infants who subsequently had

a positive stool specimen stayed in the hospital a median of 6 days longer than were children who had no positive stool specimens (*P* = .002) and were more likely to have mother who also had a positive stool specimen (*P* = .0001; Table 1). No other variable, including gestational age at birth, sex, race, zygosity, mode of delivery, feeding practices, antibiotic use in the first 2 months of life, maternal receipt of perinatal antibiotics, receipt of medications by children to counteract symptoms potentially related to gastric acidity, or sampling duration, differed between the children who had 1 or more positive stool specimen and controls who did not. Only 2 of 15 children who had a positive stool specimen had received any antibiotics in the 9 months preceding their first positive stool, and 6 received no antibiotics at all before their first positive stool (Table 1, Supplementary Table 1). Eleven of the 23 colonized subjects had multiple, and usually consecutive, positive stool samples (Supplementary Table 1). In 6 families, both twins had positive stool specimens collected in close time proximity to each other, usually in multiple samples, whereas in 3 families, only 1 twin tested positive, and these 3 infants had only 1 positive stool specimen each.

Table 2. Antibiotic Resistance Patterns of the 57 Ciprofloxacin-Resistant *Escherichia coli* Isolates From Children and Mothers

Pattern	Isolates With Pattern, No.	Ampicillin and/or Cefazolin (n = 35[a])	Trimethoprim-Sulfamethoxazole (n = 37[a])	Piperacillin-Tazobactam (n = 1[a])	Gentamicin (n = 15[a])	Doxycycline (n = 35[a])
1	1	R	R	R	R	R
2	5	R	R	S	R	R
3	2	R	S	S	R	R
4	3	R	R	S	R	S
5	4	R	S	S	R	S
6	1	R	R	S	S	S
7	11	R	R	S	S	R
8	16	S	R	S	S	R
9	8	R	S	S	S	S
10	6	S	S	S	S	S

All isolates were susceptible to cefotetan, ceftazidime, ceftriaxone, cefepime, and meropenem.

Abbreviations: R, resistant; S, susceptible.

[a] Values represent the number of isolates resistant to antibiotics described in top row.

Characteristics of Ciprofloxacin-Resistant *E. coli*

Fifty-two 57 ciprofloxacin-resistant *E. coli* isolates (91%) were *E. coli* ST131-*H*30. The remaining 5 (9%) were isolated from members of 1 family and were *E. coli* ST405. In the positive specimens, a median of 2 (IQR, 0–4.5) of 5 randomly selected lactose-fermenting colonies were ciprofloxacin resistant (Supplementary Table 1). Fifty-one of 57 ciprofloxacin-resistant *E. coli* (89%) were resistant to at least 1 other antibiotic (Table 2). All of the ciprofloxacin-resistant *E. coli* contained genes encoding FimH (type 1 fimbriae), which is typical for this species.

Over 90% of the ciprofloxacin-resistant *E. coli* recovered contained genes encoding Iha (an adhesin-siderophore receptor), Sat (a toxin), various iron-acquisition proteins, Usp (a bacteriocin), OmpT (an outer membrane protease), MalX (a pathogenicity island marker), and YfcV (an adhesin; Table 3). Each isolate contained 7–12 virulence loci (median, 10).

Molecular analysis of the 7 archival ciprofloxacin-resistant *E. coli* from the previous Seattle study [4] showed that 3 belonged to the ST131-*H*30 subclone and that 1 each belonged to ST354, ST405, ST964, or ST1291. Each of these 7 isolates possessed

Table 3. Virulence Loci Variably Present in 57 Ciprofloxacin-Resistant *Escherichia coli* Isolates From Children and Mothers

Profile[a]	Virulence Loci, No.[b]	Isolates With Profile, No.	iha (n = 55[c])	sat (n = 55[c])	fyuA (n = 57[c])	iutA (n = 55[c])	kpsII (n = 19[c])	K5 (n = 4[c])	usp (n = 53[c])	traT (n = 32[c])	ompT (n = 53[c])	yfcV (n = 52[c])
A	12	4	+	+	+	+	+	+	+	+	+	+
B	11	1	+	+	+	+	+	−	+	+	+	+
C	10	9	+	+	+	+	+	−	+	−	+	+
D	10	21	+	+	+	+	−	−	+	+	+	+
E	9	1	+	+	+	+	+	−	+	+	−	−
F	9	1	+	+	+	+	+	−	−	+	+	−
G	9	15	+	+	+	+	−	−	+	−	+	+
H	8	3	+	+	+	+	+	−	−	+	−	−
I	7	2	−	−	+	−	−	−	+	+	+	+

Loci shown are those that were variably present among the 57 ciprofloxacin-resistant *E. coli* isolates. Loci and assays are described elsewhere [8, 9]. Numbers below each locus represents number of isolates for that trait as determined by polymerase chain reaction. Not shown are the loci that were found in either all isolates (*fimH* and *malX*) or none of the isolates. The latter included adhesins *papAH*, *papC*, *papEF*, *papG*, *papG* I, II, and III; *sfa/focDE*, *sfaS*, *focG*, *afa/draBC*, *afaE8*, *bmaE*, *gafD*, F17, *clpG*, and *hra*; toxins *hlyD*, *hlyF*, *cnf1*, *cdtB*; *pic*, *vat*, *tsh*, and *astA*; siderophores *iroN* and *ireA*; protectins *kpsMTlll* and the K1, K15, K2/K100 capsule variants, *rfc*, and *iss*; and miscellaneous traits *cvaC* (microcin), H7 (flagellin variant), and *clbB* and *clbN* (colibactin polyketide synthesis genes).

Abbreviations: +, present; −, absent.

[a] Each letter represents a unique set of virulence loci.

[b] Data represent sums of loci present in each pattern, including *fimH* and *malX*, which are present in all isolates.

[c] Values represent the number of isolates possessing each listed locus.

virulence gene profiles and had antibiotic susceptibility patterns that resembled those of the 57 ciprofloxacin-resistant fecal isolates from the St. Louis families in the current study (Supplementary Table 2).

DISCUSSION

Our study has 2 main findings. First, ciprofloxacin-resistant *E. coli* are common in stools of children and their mothers in St. Louis, even in the absence of pressure from prescribed antibiotics that would select for the presence of these organisms in the gut. Second, these bacteria have many traits in common with those of human clinical extraintestinal pathogenic *E. coli*. Such organisms not only are the leading cause of urinary tract infections worldwide, they are also capable of invading the bloodstream and causing bone and soft-tissue infections [10, 11].

The ciprofloxacin-resistant *E. coli* identified in this cohort belong to 2 clonal groups: ST131 and ST405. ST131, a globally disseminated clone that was first reported as an important cause of human disease in 2008 [12, 13], attained prominence over the past decade [3, 14–17]. Its pandemic spread can be attributed to its subclone *H*30, which contains an allelic variant, *fimH*30, of FimH [18]. Notably, all ST131 isolates in this study belonged to this subclone. Although generally considered to be a pathogen mainly of adults, *E. coli* ST131 and its *H*30 subclone can cause infections in children [19–22]. A recent paper reports the uropathogenicity of ciprofloxaxin-resistant *E. coli* ST131 in infants [23]. Epidemiologic studies suggest that people can acquire ST131 in the hospital [24], during ambulatory visits [25, 26], or from household members who have been recently discharged from hospital [27].

Our analysis of the 7 archival pediatric fecal isolates from the Seattle study conducted in the early 2000s [4] demonstrates that ST131-*H*30 and ST405 (including its single locus variant ST964) have colonized children in the United States for at least a decade. In contrast to a study of 25 day-care centers in France conducted in 2012, in which 1 facility accounted for most of the children excreting *E. coli* ST131-*H*30 [28], the present household-based cohort suggests community-wide dissemination of these organisms, at least in the recent past, in the St. Louis region. The association between postnatal length of hospital stay and subsequent excretion of ciprofloxacin-resistant *E. coli* suggests that children may acquire such organisms during prolonged stays in hospital after birth. However, the median date of the first positive culture was nearly 1 year after discharge, which points away from in-hospital acquisition. The critical reservoirs and transmission routes for ST131-*H*30 remain to be defined.

The substantial frequency of colonization with ciprofloxacin-resistant *E. coli* and the relative abundance of these organisms among lactose-fermenting coliforms in the positive stool samples suggest that such multidrug-resistant *E. coli* are highly fit members of gut microbial communities. Our study design also enabled us to estimate the durability of colonization: children with a stool specimen containing ciprofloxacin-resistant *E. coli* frequently yielded such organisms in multiple samples despite not having received antibiotics that would favor the persistence of these bacteria. Furthermore, we probably underestimated colonization rates because we inoculated only a small volume of stool from each frozen stock. Indeed, the fact that about half of the children had only a single positive stool specimen raises the possibility that colonization below the level of detection might have been common but not identified.

We cannot state with certainty that the ciprofloxacin-resistant *E. coli* we identified are capable of causing disease in humans. However, the virulence factor profiles that these isolates possess are typical of clinical isolates of ST131-*H*30, ST405 [29–31], and other extraintestinal pathogenic *E. coli* [32–36]. This complement of virulence loci lends credence to their pathogenic potential. Furthermore, the fecal microbiota is a known reservoir for strains that cause urinary tract infection and other extraintestinal infections [37, 38]. Thus, is it highly likely that the identified organisms could cause diseases in humans. Also, even though we did not document prescription use of oral or parenteral fluoroquinolones in the subjects, we would not be able to take into account the risk from fluoroquinolone-resistant *E. coli* in food, possibly related to use of these agents in food animals. Additionally, although the number of samples tested was large, the cohort size overall (only 80 children) might not have been of sufficient magnitude to identify associations that would emerge as significant in larger studies. For example, children whose stool specimens were positive were disproportionally born via cesarean section, but the difference was not quite statistically significant. However, the median time of first demonstrated colonization with fluoroquinolone-resistant *E. coli* was about 1 year after birth, in which case a more extensive interrogation of other exposures in the community, such as day-care attendance and animal exposures, might be informative.

These data might illuminate measures that potentially could prevent colonization of humans with fluoroquinolone-resistant *E. coli*, and various control measures for individuals who are colonized. For example, by understanding the biology underlying acquisition, persistence, and clearance of fluoroquinolone-resistant *E. coli*, new strategies could be formulated to diminish the size of human gut reservoirs. Consumption of foods that might contain, or exposure to animals that might be colonized with, these organisms might areas worthy of study as risk factors for acquisition.

In summary, healthy individuals harbor virulent *E. coli* that are resistant to ciprofloxacin and to multiple additional antibiotics. Antibiotic use appears not to select significantly for gut colonization by these bacteria, because most of the colonized children had not been prescribed recent antibiotics before these fluoroquinolone-resistant *E. coli* appeared in their stools.

Gut microbial colonization of asymptomatic carriers could be a critical control point in persistence of these pathogens in human populations, especially since such fluoroquinolone-resistant *E. coli* are potentially progenitors to *E. coli* that produce extended spectrum β-lactamases. Attempts to interdict dissemination of ciprofloxacin-resistant *E. coli* within communities should address this habitat. Delineation of the biology of asymptomatic carriage could lead to novel strategies to block gut colonization by pathogenic ciprofloxacin-resistant *E. coli* or to hasten the clearance of these organisms from human reservoirs.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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