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Ciprofloxacin-Resistant Gram-Negative Bacilli in the Fecal Microflora of Children

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The extent to which antibiotic-resistant bacteria are excreted by humans who have not been exposed to antibiotics is not known. Children, who rarely receive fluoroquinolones, provide opportunities to assess the frequency of fecal excretion by fluoroquinolone-naïve hosts of fluoroquinolone-resistant gram-negative bacilli. Fresh nondiarrheal stools from children were processed by screening them on agar containing ciprofloxacin to recover ciprofloxacin-resistant gram-negative bacilli. Resistant isolates were identified, and ciprofloxacin MICs were determined. Resistant *Escherichia coli* isolates were also analyzed for urovirulence-associated loci. Thirteen (2.9%) of 455 stools yielded ciprofloxacin-resistant *E. coli* (seven children), *Stenotrophomonas maltophilia* (four children), and *Achromobacter xylosoxidans* and *Enterobacter aerogenes* (one child each). Neither the subjects themselves nor members of their households used fluoroquinolones in the 4 weeks preceding collection. Six of the seven resistant *E. coli* isolates belonged to phylogenetic groups B2 and D, in which extraintestinal pathogenic *E. coli* bacteria are frequently found. All resistant *E. coli* isolates contained at least three putative *E. coli* virulence loci. Most ciprofloxacin-resistant bacteria were resistant to additional antibiotics. Potentially pathogenic bacteria that are resistant to therapeutically important antimicrobial agents are excreted by some humans, despite these persons' lack of exposure to the particular drugs. The sources of these resistant organisms are unknown. This underrecognized reservoir of drug-resistant potential pathogens poses public health challenges.

Acquired antimicrobial resistance in bacteria poses major challenges for medical practice, public health, and the pharmaceutical industry. Organisms with reduced susceptibilities might proliferate and become more likely to disseminate when selective pressure is exerted by antimicrobial agents, as has been observed during prophylaxis of traveler's diarrhea with trimethoprim or trimethoprim-sulfamethoxazole (24). Human populations that have not been exposed to antibiotics provide opportunities to test the hypothesis that antibiotic-resistant microflora can be acquired in the community in the absence of selective pressure in a particular human host. If this hypothesis is correct, efforts to determine the origins of such resistant organisms may need to focus on determining the sources of their nonselective acquisition by human hosts, such as foods or other colonized humans.

Children are an easily defined population in which to determine if resistance to certain classes of antimicrobial agents can occur in unexposed individuals. Fluoroquinolones (e.g., ciprofloxacin), although extensively used in adults, are not approved for routine childhood administration because of concern about cartilage toxicity (10). Consequently, pediatric usage of fluoroquinolones, except for the treatment of selected illnesses, such as cystic fibrosis, is quite unusual in North America. Gram-negative bacilli, which are potential pathogens, are intrinsically susceptible to fluoroquinolones. Because children are rarely exposed to fluoroquinolones, gram-negative bacilli resistant to this class of antimicrobial agents in the fecal microflora of children probably emerged via selective pressure exerted in a different setting or host, before these individuals ingested the organisms. To attempt to determine if such resistant bacteria exist in the feces of children within the general population who have no history of relevant antimicrobial exposure, we conducted a prospective analysis of stools from children who had not been directly exposed to fluoroquinolones.

MATERIALS AND METHODS

Subjects and specimens. Between September 2001 and June 2002, stools were provided anonymously by children without diarrhea who attended a general ambulatory pediatric office in Seattle (Virginia Mason Sand Point Clinic). Subjects were solicited by brochures provided to families during their visits. Families completed questionnaires providing demographic information and details con-

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cerning any use of antimicrobial agents by the child during the 4 weeks preceding collection of the stool specimen. The questionnaires also were used to record antimicrobial usage by others in the household during this 4-week interval to attempt to address the possibility that excretion of resistant flora could be related to intrahousehold utilization of these drugs (11).

The stools were refrigerated at the pediatric office for up to 4 hours before being processed. This study was approved by the Children's Hospital and Regional Medical Center (the sponsoring academic institution) and the Virginia Mason Medical Center Institutional Review Boards.

Antibiotic resistance screening. To isolate ciprofloxacin-resistant gram-negative bacteria, stools were plated on MacConkey agar containing ciprofloxacin (1 mg per liter), prepared by adding antibiotic to autoclaved, cooling MacConkey agar base (Becton-Dickinson, Sparks, Maryland). If there was growth on these plates after overnight incubation at 35°C, a single resulting colony ("index ciprofloxacin-resistant bacteria") was selected at random and its identity was determined by conventional tube biochemical assays, Vitek GNI+ (bioMérieux, Inc., Hazelwood, MO), API 20E (bioMérieux), and the Rapid NF Plus System (Remel, Inc., Lenexa, KS). The identities of presumptive *Stenotrophomonas maltophilia* and *Achromobacter xylosoxidans* isolates were confirmed by partial 16S rRNA gene sequencing (22). MICs to ciprofloxacin and moxifloxacin were determined using the Etest (AB Biodisk, Solna, Sweden) (27) according to the manufacturer's recommendations. An isolate was considered resistant to ciprofloxacin or moxifloxacin if its MIC for these agents was ≥4 mg per liter. Index ciprofloxacin-resistant bacteria were stored in Luria-Bertani broth (26) with 15% glycerol and ciprofloxacin (1 mg per liter) at −80°C.

To determine the proportion of total gram-negative bacteria excreted by the subjects that were resistant to ciprofloxacin, all stool specimens were plated on MacConkey agar containing no antibiotics. After overnight incubation at 35°C, five lactose-fermenting colonies (as available) were selected at random ("coisolated lactose-fermenting bacteria") and frozen in Luria-Bertani broth with 15% glycerol at −80°C until they were analyzed further. These coisolated lactose-fermenting bacteria were subsequently tested for ciprofloxacin resistance if they had been recovered from a patient whose stool yielded ciprofloxacin-resistant *Escherichia coli*.

Index bacteria determined to be ciprofloxacin-resistant *E. coli* were O:H serotyped at the Centers for Disease Control and Prevention by standard agglutination methods. The primer pairs 5′ACGTACTAGGCAATGACTGG3′-5′AGAACTCGCCGTCGATAGAAC3′ and 5′TGTATGCGATGTCTGAACTG3′-5′CTCAATAGCAGCTCGGAATA3′ were used to amplify *gyrA* and *parC*, respectively, from boiled lysates of ciprofloxacin-resistant *E. coli*. These amplicons, which include regions that can contain mutations associated with quinolone resistance (7), were then sequenced bidirectionally.

Index resistant *E. coli* were tested for susceptibilities to ampicillin, ampicillin-clavulinic acid, aztreonam, cefazolin, cefepime, ceftazidime, ceftriaxone, cefuroxime, ciprofloxacin, gentamicin, meropenem, piperacillin-tazobactam, and trimethoprim-sulfamethoxazole, using first the Vitek GNS-122 system (bioMérieux) for screening and then a standard antimicrobial disk diffusion test for confirmation. Susceptibilities of *A. xylosoxidans* to other antibiotic agents were tested by the disk diffusion method only. The MICs of ciprofloxacin and moxifloxacin for all quinolone-resistant organisms were determined by the Etest method (AB Biodisk, Solna, Sweden). The antibiotic susceptibilities of *S. maltophilia* to a battery of five other antibiotic agents (ceftazidime, chloramphenicol, doxycycline, ticarcillin-clavulinic acid, and trimethoprim-sulfamethoxazole) were also determined by the Etest method. The results generated from both the disk diffusion tests and the MIC Etests were interpreted using the criteria suggested by the Clinical and Laboratory Standards Institute (3, 4).

Index ciprofloxacin-resistant *E. coli* isolates were assigned to one of four main phylogenetic groups (A, B1, B2, or D) (12) by multiplex PCR (2). They were also tested for 47 virulence genes, including 34 virulence-associated genes of extraintestinal pathogenic *E. coli* and 13 *papA* (structural-subunit) alleles, using established PCR-based assays (15, 16). Appropriate positive and negative controls were included. An aggregate virulence score was calculated for each isolate as the number of virulence genes detected. *E. coli* isolates were additionally classified as extraintestinal and pathogenic if they contained at least two of the following five genes: *papA* or *papC*, *sfa*/*foc*, *afa*/*dra*, *iutA*, and *kpsM*II (14). Index and coisolated ciprofloxacin-resistant *E. coli* isolates were tested for clonal relatedness by pulsed-field gel electrophoresis, following XbaI digestion (1).

The statistical significances of differences between proportions were determined using the two-tailed Fisher's exact test.

RESULTS

Stools from 455 children without diarrhea were studied. The median age was 3.4 years, and the range was 18 days to 19 years, 8 months. Only two subjects were older than 18 years, the age below which the U.S. Food and Drug Administration does not recommend the use of fluoroquinolones. Of these, 14 (3.1%) yielded gram-negative bacteria on ciprofloxacin-supplemented MacConkey agar. Of these 14 bacteria, 13 organisms, consisting of *E. coli* (seven children) representing five different serogroups, *S. maltophilia* (four children), and *E. aerogenes* and *A. xylosoxidans* (one child each), had MICs of ciprofloxacin and moxifloxacin ranging from 4 to ≥32 mg per liter, with the *E. coli* isolates demonstrating the highest degrees of resistance (Table 1). All seven resistant *E. coli* isolates contained Ser83Leu and Asn87Asp mutations in *gyrA* and a Ser83Ile mutation in *parC*; two also contained a Glu84Val mutation in *parC*. The resistant *E. aerogenes* isolate had a Ser83Ile mutation in *gyrA*.

Six of the seven ciprofloxacin-resistant *E. coli* isolates were derived from phylogenetic groups B2 and D (three isolates each), which are epidemiologically associated with extraintestinal virulence (14). The virulence-associated traits of extraintestinal pathogenic *E. coli* found in the seven isolates, in descending order of prevalence, consisted of *malX*/pathogenicity island marker and *fyuA*/yersiniabactin system (six isolates each); *fimH*/type 1 fimbriae and *iutA*/aerobactin system (five isolates each); *traT*/serum resistance associated (four isolates), *iha*/putative adhesin/siderophore, and *kpsM*II/group II capsule (three isolates each); *ompT*/outer membrane protease (two isolates); and *papEF*/P fimbria minor subunits, K1/*kpsM* variant, and *ibeA*/invasion of brain endothelium A (one isolate each). The median aggregate virulence score was 7 (range, 3 to 8). Three ciprofloxacin-resistant isolates contained both *iutA* and *kpsM*II and one contained both *papEF* and *kpsM*II and were classified as extraintestinal pathogenic *E. coli* (14).

On plain MacConkey agar plates, stools from six of the seven children with ciprofloxacin-resistant *E. coli* yielded at least one additional lactose-fermenting colony that also proved to be a ciprofloxacin-resistant *E. coli* isolate. All such coisolated ciprofloxacin-resistant *E. coli* isolates were indistinguishable from the corresponding index resistant isolates, according to XbaI pulsed-field gel electrophoresis patterns.

The spectrum of antimicrobial resistance in the 13 resistant organisms was not limited to fluoroquinolones. Five of the seven index *E. coli* isolates were fully resistant to trimethoprim-sulfamethoxazole; three were also resistant to narrow- and broad-spectrum cephalosporins. None of the 13 children whose stool specimens yielded ciprofloxacin-resistant gram-negative bacilli either used a fluoroquinolone or resided in a household where a fluoroquinolone was used during the 4 weeks before specimen collection, based on recall. We detected no influence of other antibiotics on the detection of a quinolone-resistant organism. Fifty-three (11.6%) of the 455 children used antibiotics during the 4 weeks before stool collection, including 2 (15.4%) of the 13 children with ciprofloxacin-resistant gram-negative bacilli in their stools ($P = 0.65$). Eighty-nine (19.6%) of the children lived in households where at least one other household member used antibiotics during

TABLE 1. Summary of resistant microflora[a]

Organism (and serotype, if *E. coli*)	No. of ciprofloxacin-resistant coisolated lactose-fermenting colonies, if *E. coli* (of 5 tested)	Age (yr)	Gender[b]	Antibiotic used in previous 4 wk by subject or member of subject's household	MIC for ciprofloxacin (mg/liter)	Phylogenetic group	Virulence loci, if *E. coli*	Antibiotic resistances other than ciprofloxacin[c]
E. coli O26:NM	5	0.9	M		>32	B2	*iha*, *fimA*, *iutA*, *kpsMII*, *ompT*, *malX*	SXT
E. coli O26:H4	3	5.3	M		>32	B2	*iha*, *fimA*, *fyuA*, *iutA*, *kpsMII*, *ompT*, *malX*	
E. coli O26:H4	5	7.8	M		>32	B2	*iha*, *fimA*, *fyuA*, *iutA*, *kpsMII*, *ompT*, *malX*	AM, SXT
E. coli O rough:H6	3	6.9	F		>32	D	*papEF*, *iha*, *fimA*, *fyuA*, *kpsMII*, *K1*, *malX*	AM, CZ
E. coli related to O2, O50:H34	4	1.6	M	Azithromycin[d], minocycline[e]	4	D	*fimA*, *iutA*, *ibeA*	AM, AMC, CZ, GM, SXT
E. coli O untypeable:NM	0	9.3	F	Clindamycin[e]	>32	A	*fimA*, *fyuA*, *iutA*	AM, SXT
E. coli O102:H6	2	4.8	M		>32	D	*fimA*, *iutA*, *malX*	AM, CZ, GM, SXT
E. aerogenes		0.5	F		4[f]			AM, AMC, CAZ, CRO, CXM, CZ
A. xylosoxidans		0.2	F		6			ATM, CRO
S. maltophilia		0.6	M	Cephalexin[e]	4			CL
S. maltophilia		0.8	F		4			CAZ
S. maltophilia		1.3	M		6			CAZ, CL
S. maltophilia		1	F	Amoxicillin[d]	8			CL

[a] Characteristics of isolates that were resistant to fluoroquinolones, and of the subjects from whom they were recovered. For fluoroquinolone-resistant *E. coli*, additional data are reported, including O:H serotypes, frequency of coisolated resistant *E. coli*, and virulence genotypes. The virulence loci not detected in any of the ciprofloxacin-resistant *E. coli* were *papA*, *papC*, *papG*, *sfa*/*focDE*, *sfaS*, *focG*, *afa*/*draBC*, *bmaE*, *gafD*, *hlyD*, *cnf1*, *cdtB*, K2 *kpsM* variant, *kpsMT*III, *rfc*, *cvaC*, *iss*, and H7 *fliC*.

[b] M, male; F, female.

[c] AM, ampicillin; AMC, ampicillin-sulbactam; ATM, aztreonam; CAZ, ceftazidime; CRO, ceftriaxone; CXM, cefuroxime; CZ, cefazolin; CL, choramphenicol; GM, gentamicin; SXT, trimethoprim-sulfamethoxazole.

[d] Taken by subject.

[e] Taken by another person in subject's household.

[f] Rounded up from an intermediate value of 3 in the Etest.

this interval, including 3 (23.1%) of the 13 children excreting ciprofloxacin-resistant gram-negative bacilli ($P = 0.72$).

DISCUSSION

In this prospective study, we found ciprofloxacin-resistant gram-negative bacteria in the stools of children, even though the children and the other members of their households had not used these antimicrobial agents in the 4 weeks before culture. Thus, our data demonstrate that resistant bacteria belonging to species that are normally intrinsically susceptible to fluoroquinolones can be found in, and probably have colonized, the intestinal tracts of individuals who had not been exposed to fluoroquinolones. This finding suggests that humans can acquire bacteria resistant to clinically useful antimicrobial agents without having experienced selective antimicrobial pressure for those agents either directly or indirectly via a household member.

Even though gram-negative bacilli are members of the commensal gastrointestinal microflora, these organisms can cause serious extraintestinal infections in both healthy and immunocompromised hosts (20). Fluoroquinolones are often used to treat extraintestinal infections with gram-negative bacteria. Though our study focused on children, the findings of ciprofloxacin-resistant gram-negative bacilli in antibiotic-naïve children seems likely to also be relevant to adults who have not been exposed to these antimicrobial agents.

The pathogenic potentials of the resistant organisms identified in this study are uncertain, but it is of concern that most of the ciprofloxacin-resistant *E. coli* isolates had characteristics that implicate them as uropathogens. Thus, these resistant organisms should not be dismissed as merely harmless members of the commensal microflora. It is also noteworthy that the resistant *E. coli* isolates, when present, were usually quantitatively predominant within the facultative coliform microflora, rather than a minority clone, as demonstrated by their presence among five arbitrarily picked colonies of gram-negative fecal bacteria that grew on nonselective media. The apparent urovirulence potentials of these *E. coli* isolates contrast with previous surveys, in which ciprofloxacin-resistant *E. coli* isolates from humans contained few such pathogenicity-associated loci (14, 16, 17).

Our finding of ciprofloxacin-resistant gram-negative lactose-fermenting bacteria other than *E. coli* also has potential clinical implications. For example, because fluoroquinolones are used to treat *S. maltophilia* infections (5, 9), fluoroquinolone resistance in this species could lead to therapeutic failures if the resistant organisms were to cause infection.

It is not possible from our data to determine the source of acquisition of these resistant enteric bacteria. However, it is plausible that the source was food (28, 29, 31). In the United States, fluoroquinolones (e.g., enrofloxacin) have been used in cattle since 1998 and in chickens and turkeys from 1995 to

2005. Their use provides a selective advantage to bacteria that are resistant to these agents and potentially contributes to dissemination of these resistant bacteria to humans through the food supply. Bacteria resistant to enrofloxacin are typically resistant to ciprofloxacin. It is therefore conceivable that the resistance in these human source strains was derived from the use of these antimicrobial agents in food animals. Provocatively, in Australia, where fluoroquinolones are banned in food animals, domestically acquired fluoroquinolone-resistant *Campylobacter* infections are quite rare (30). It is also possible that sources for the resistant bacteria isolated in this study could have been family members who received a fluoroquinolone more than 4 weeks before the children's stool samples were submitted (because it is not known how long such organisms persist in human fecal microflora) or people in the community outside the subjects' households. Indeed, daycare studies have demonstrated nonfamilial transmission of *E. coli* isolates resistant to trimethoprim (8, 25). Some of the children in our study who were <1 year of age and whose stools contained resistant organisms might have consumed exclusively breast milk or infant formula and not been exposed directly to food-borne bacteria.

The possibility that the use of antimicrobial agents in food animals has contributed to the selection of antibiotic-resistant bacteria and their dissemination to humans through the food supply is a concern raised by several previous studies. For example, human infections with fluoroquinolone-resistant *Campylobacter jejuni* have been attributed to the use of fluoroquinolones in poultry production (6, 14). Additionally, the recent widespread emergence of multidrug-resistant uropathogenic *E. coli* and the rising incidence of fluoroquinolone-resistant urinary *E. coli* (19, 23) demonstrate the risk fluoroquinolone-resistant gram-negative bacilli pose to the general population (13, 14, 16, 18).

We note that our study has several limitations. First, recall biases could have influenced, either positively or negatively, reports of recent antibiotic use in the households. Second, we were selective in the quantity of data we were able to accrue, and we propose that future studies address potentially relevant factors, such as dietary questions, household size, daycare attendance, and breast milk consumption. Third, the 1-month period for exposure assessment, while seemingly reasonable for this exploratory study, might have been too limited. A recent study demonstrated long-term carriage of tetracycline-resistant *E. coli* in stools of infants (21), which raises the possibility of selective events occurring at times well before sampling. Fourth, we were unable to confirm compliance with our request that stools be submitted once from each subject. Fifth, we were unable to monitor how many stools were submitted from children within the same household, though our large sample size mitigates biases introduced by intrafamily repeat submissions. Sixth, in this open U.S. population, in a study in which specimens were submitted anonymously, we were unable to use pharmacy databases to confirm medication use or nonuse. Despite these limitations, we have demonstrated that children who were highly unlikely to have been exposed to fluoroquinolones shed in their stools bacteria that had acquired resistance to these antibiotics. Acquisition of an antimicrobial-resistant gastrointestinal microflora by persons in the community who had not been exposed to antimicrobial agents poses considerable challenges. Further research should determine the prevalence, and spectrum, of antibiotic resistance in human reservoirs in the community. Such investigations ideally should also address the age-specific duration and intensity of shedding of the resistant organisms, mechanisms of development of resistance in these organisms, and molecular comparisons of antimicrobial-resistant human, food, and animal isolates to clarify the sources of the resistant isolates encountered in humans. These data will be helpful in confirming or refuting the potential association between the use of antimicrobial agents in food animals and intestinal carriage of antibiotic-resistant fecal organisms by non-antibiotic-exposed humans.

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