ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Dec. 2004, p. 4556–4561 Vol. 48, No. 12
0066-4804/04/$08.00+0 DOI: 10.1128/AAC.48.12.4556–4561.2004

Detection of CTX-M-Type β-Lactamase Genes in Fecal *Escherichia coli* Isolates from Healthy Children in Bolivia and Peru

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Received 18 March 2004/Returned for modification 31 May 2004/Accepted 30 July 2004

A survey was carried out from August to November 2002 to evaluate the antimicrobial susceptibilities of fecal *Escherichia coli* isolates from 3,208 healthy children from four different urban areas of Latin America, two in Bolivia (Camiri and Villa Montes) and two in Peru (Yurimaguas and Moyobamba). Ceftriaxone-resistant *E. coli* isolates were detected in four children, one from each of the areas sampled. The isolates exhibited a multidrug-resistant phenotype, including resistance to oxyimino-cephalosporins and aztreonam, and the MICs of ceftazidime for the isolates were lower than those of cefotaxime. By PCR and sequencing, the $bla_{\text{CTX-M-2}}$ determinant was detected in three isolates and the $bla_{\text{CTX-M-15}}$ determinant was detected in one isolate (from Peru). The CTX-M-2-producing isolates belonged to three different phylogenetic groups (groups A, B2, and D), while the CTX-M-15-producing isolate belonged to phylogenetic group D. The $bla_{\text{CTX-M-2}}$ determinants were transferable to *E. coli* by conjugation, while conjugative transfer of the $bla_{\text{CTX-M-15}}$ determinant was not detectable. Plasmids harboring the $bla_{\text{CTX-M-2}}$ determinant exhibited similar restriction profiles, and in all of them the gene was located on a 2.2-kb PstI fragment, suggesting a genetic environment similar to that present in In35 and InS21. The findings of the present study confirm the widespread distribution of CTX-M-type β-lactamases and underscore the role that commensal *E. coli* isolates could play as a potential reservoir of these clinically relevant resistance determinants. This is the first report of CTX-M-type enzymes in Bolivia and Peru and also the first report of the detection of CTX-M-15 in Latin America.

CTX-M-type enzymes are a lineage of molecular class A extended-spectrum β-lactamases (ESBLs) active against oxyimino-cephalosporins and monobactams, with an overall preference for cefotaxime and ceftriaxone (10). Some 40 different variants of these enzymes have been identified, and these are distributed in five major sublineages indicated with the names of the prototype enzymes (CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25) (10; http://www.lahey.org/studies/webt.stm).

CTX-M-type β-lactamases were first detected in the late 1980s as secondary plasmid-mediated enzymes in clinical isolates of the family *Enterobacteriaceae* in Europe (4, 7, 9) and Argentina (8, 29). Since the mid-1990s the emergence of CTX-M enzymes has been observed in most parts of the world, including Europe, North and South America, Asia, and Africa (10). Along with the TEM- and SHV-type variants, CTX-M-type β-lactamases are acknowledged to be among the most important acquired ESBLs spreading in *Enterobacteriaceae* worldwide (10, 28).

CTX-M producers have been detected among clinical isolates of several species of the family *Enterobacteriaceae*, as well as in *Vibrio cholerae* and *Aeromonas hydrophila* (3, 15–17, 21–23, 28, 35–39; see also reference 10 and the references therein).

Most strains producing CTX-M-type enzymes have been implicated in nosocomial infections, but unlike what happens with SHV and TEM ESBLs, these enzymes have also been reported in clinical isolates from community-acquired infections (1, 10, 31) and even from healthy animals (11), suggesting that the latter could act as reservoirs for these resistance genes.

In this paper we describe the detection of $bla_{\text{CTX-M}}$ genes of two different lineages ($bla_{\text{CTX-M-2}}$ and $bla_{\text{CTX-M-15}}$) in the commensal *Escherichia coli* microbiota from healthy children living in Bolivia and Peru. This is the first report of CTX-M-type enzymes in those countries and is also the first report of the detection of CTX-M-15 in Latin America. The present findings confirm the widespread distribution of similar resistance genes and underscore the role that commensal *E. coli* isolates could play as a potential reservoir of these clinically relevant resistance determinants.

MATERIALS AND METHODS

Bacterial isolates. The four *E. coli* strains investigated in this study were isolated from fecal swab specimens taken from healthy children living in four different urban areas of Latin America, two in Bolivia (Camiri, Santa Cruz Department, and Villa Montes, Tarija Department) and two in Peru (Moyobamba, San Martin Department, and Yurimaguas, Loreto Department), following a large screening carried out from August to November 2002 to investigate antimicrobial resistance rates in commensal *E. coli* isolates from healthy children (ages, 6 to 72 months) from those study areas of the ANTRES research project

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(ANTRES: Towards controlling antibiotic use and resistance in low-income countries. An intervention study in Latin America; ICA4-CT-2001-10014). The screening was carried out by a rapid method essentially as described previously (5, 20). *E. coli* Y23-8 and *E. coli* M35-6 were isolated from a 26-month-old girl living in Yurimaguas, Peru, and from a 42-month-old boy living in Moyobamba, Peru, respectively; *E. coli* C52-5 and *E. coli* V55-6 were isolated from a 7-month-old girl living in Camiri, Bolivia, and from a 19-month-old boy living in Villamontes, Bolivia, respectively.

In vitro susceptibility testing. In vitro susceptibility was determined by a broth macrodilution procedure with cation-supplemented Mueller-Hinton (MH) broth (Difco Laboratories, Detroit, Mich.), as recommended by the National Committee for Clinical Laboratory Standards (NCCLS) (24). The results of the susceptibility tests were interpreted according to the criteria of the NCCLS (25). Antimicrobial agents were from Sigma Chemical Co. (St. Louis, Mo.) unless otherwise specified. Cefepime and aztreonam were from Bristol-Myers Squibb (Wallingford, Conn.), imipenem was from Merck Sharp & Dohme (Rome, Italy), piperacillin and piperacillin-tazobactam were from Wyeth (Catania, Italy), and ciprofloxacin was from Bayer (Milan, Italy). *E. coli* ATCC 25922 was used for quality control purposes in susceptibility testing.

β-Lactamase assays. The double-disk synergy test for the detection of ESBL activity was carried out as described previously (18) by screening for synergistic activities between clavulanate (represented by a disk of amoxicillin-clavulanate) or tazobactam (represented by a disk of piperacillin-tazobactam) and cefotaxime, ceftazidime, cefepime, or aztreonam (Oxoid, Milan, Italy) against the isolates. A potentiation of the inhibitory zones of any of the expanded-spectrum β-lactams by any inhibitor was considered suggestive of ESBL production. Analytical isoelectric focusing (IEF) of crude bacterial lysates for the detection of β-lactamases was carried out on polyacrylamide gels containing ampholines (pH range, 3.5 to 10), as described previously (19). Crude extracts were prepared by sonication from early-stationary-phase cultures grown aerobically at 37°C in antibiotic-free MH broth. β-Lactamase bands were visualized with the chromogenic substrate nitrocefin (Oxoid), as described previously (19).

Molecular analysis techniques. Basic procedures for DNA extraction, analysis, and manipulation were performed as described by Sambrook and Russel (33).

PCR amplification of $bla_{\text{CTX-M}}$ alleles was carried out with primers CTX-MU1 (5′-ATGTGCAGYACCAGTAARGT) and CTX-MU2 (5′-TGGGTRAARTA RGTSACCAGA), designed on the basis of conserved regions of $bla_{\text{CTX-M}}$ genes, as described previously (27). PCR amplification of the allele belonging to the $bla_{\text{CTX-M-1}}$ group was carried out with primers CTX-M3G-F (5′-GTTACAAT GTGTGAGAAGCAG) and CTX-M3G-R (5′-CCGTTTCCGCTATTACAA AC), as described previously (27). PCR amplification of the alleles belonging to the $bla_{\text{CTX-M-2}}$ group was carried out with primers Orf513-fw (5′-GATCCATC ACAGAGTCGTCTCT) and Orf3-rev (5′-GGCAGCTCATACAGGTAAC TCT) and the following reaction parameters: initial denaturation at 94°C for 5 min; denaturation at 94°C for 45 s, annealing at 56°C for 45 s, and elongation at 72°C for 5 min, repeated for 35 cycles; and a final extension at 72°C for 10 min. PCR amplification of bla_{TEM} determinants was carried out with primers TEMp-for (5′-ATAAAATTCTTGAAGACGAA) and TEM-rev (5′-ATATGAGTAAG CTTGGTCTGACAG), designed for amplification of bla_{TEM} genes and some of the upstream region, and the following reaction parameters: initial denaturation at 94°C for 5 min; denaturation at 94°C for 45 s, annealing at 46°C for 45 s, and elongation at 72°C for 1 min, repeated for 35 cycles; and a final extension at 72°C for 10 min. PCR was always carried out in a 50-μl volume with 30 pmol of each primer, 200 μM deoxynucleoside triphosphates, 1.5 mM $MgCl_2$, and 0.5 U of the enzyme provided with the Expand PCR system (Roche Biochemicals, Mannheim, Germany) in the reaction buffer provided by the enzyme manufacturer. Direct sequencing of the PCR products was carried out as described previously (30) with custom sequencing primers. Both strands were sequenced. Plasmid DNA was extracted by the alkaline lysis method (33). Electroporation of *E. coli* DH5α was performed with a Gene-Pulser apparatus (Bio-Rad Laboratories, Richmond, Calif.), according to the instructions of the manufacturer. Southern hybridization was carried out directly on the dried gels, as described previously (34), with DNA probes labeled with ^{32}P by the random-priming technique. The $bla_{\text{CTX-M}}$ probe was made by use of a 1:1 mixture of amplicons generated with primers CTX-MU1 and CTX-MU2 from $bla_{\text{CTX-M-2}}$ and $bla_{\text{CTX-M-15}}$, respectively. Colony blot hybridization was carried out with the same probe, as described previously (32).

Phylogenetic classification. The phylogenetic grouping of the *E. coli* isolates was determined by the multiplex PCR-based method recently developed by Clermont et al. (13), which allows identification of the four major phylogenetic groups (groups A, B1, B2, and D).

Conjugation assays. Conjugal transfer of the resistance determinants was assayed on MH agar plates with *E. coli* MKD-135 (*argH rpoB18 rpoB19 recA rpsL*) as the recipient and an initial donor/recipient ratio of 0.1. Mating plates were incubated at 30°C for 14 h. Transconjugants were selected on MH agar containing rifampin (400 μg/ml) plus cefotaxime (2 μg/ml) or ampicillin (200 μg/ml). Under these conditions, the detection sensitivity of the mating assay was $\geq 5 \times 10^{-8}$ transconjugants/recipient.

RESULTS

Detection of CTX-M β-lactamase genes in fecal *E. coli* isolates from healthy children from Bolivia and Peru. Following a survey to evaluate the antimicrobial susceptibilities of fecal *E. coli* isolates, carried out by a rapid screening method with fecal swab specimens from 3,208 healthy children from four different urban areas of Latin America, two in Bolivia (Camiri and Villa Montes) and two in Peru (Yurimaguas and Moyobamba), ceftriaxone-resistant *E. coli* isolates were detected in four children, one from each of the areas sampled (*E. coli* C52-5 from Camiri, *E. coli* V55-6 from Villa Montes, *E. coli* Y23-8 from Yurimaguas and *E. coli* M35-6 from Moyobamba).

Analysis of the antimicrobial susceptibilities of the four isolates revealed a multidrug-resistant phenotype that included resistance to most β-lactams (aminopenicillins, ureidopenicillins, narrow-spectrum cephalosporins, oxyimino-cephalosporins, and aztreonam), tetracycline (except for *E. coli* M35-6), chloramphenicol, trimethoprim-sulfamethoxazole, and most aminoglycosides. All isolates except Y23-8 were susceptible to amikacin, nalidixic acid, and ciprofloxacin. The ceftazidime MICs for the isolates varied notably, but they were always at least eightfold lower than those of cefotaxime (Table 1). A double-disk diffusion test revealed synergy between clavulanate or tazobactam and cefotaxime, ceftazidime, cefepime, and aztreonam against the four isolates (data not shown), suggesting the production of ESBL activity. Overall, these results suggested the production of ESBLs with preferential cefotaximase activities.

Analytical IEF analysis of crude extracts prepared from the four isolates, grown in antibiotic-free medium, showed the presence of two β-lactamase bands in all cases. Extracts of isolates C52-5, V55-6, and M35-6 yielded β-lactamases that focused at pI 5.4 and 8.0, while extracts of isolate Y23-8 yielded β-lactamases that focused at pI 5.4 and 8.9 (Table 1).

PCR analysis with primers CTX-MU1 and CTX-MU2, designed for universal amplification of CTX-M-encoding genes (27), yielded an amplicon of the expected size (0.6 kb) from each of the four isolates. Analysis of the restriction profiles of the amplification products with PstI and PvuII yielded a profile compatible with genes of the $bla_{\text{CTX-M-2}}$ group (including $bla_{\text{CTX-M-2/4/5/6/7/20/31}}$ and $bla_{\text{TOHO-1}}$) for three isolates (isolates C52-5, V55-6, and M35-6) and a profile compatible with most genes of the $bla_{\text{CTX-M-1}}$ group (including $bla_{\text{CTX-M-1/3/10/11/12/15/22/23/28/29/30/32/33}}$ and $bla_{\text{FEC-1}}$) in one isolate (isolate Y23-8). The nature of the $bla_{\text{CTX-M}}$ determinants was further investigated by PCR amplification of the entire genes with primers Orf513-fw and Orf3-rev, designed on the basis of the genetic environment of the $bla_{\text{CTX-M-2}}$ gene in In35 and InS21 (2, 14), for genes of the $bla_{\text{CTX-M-2}}$ group and with primers CTX-M3G-F and CTX-M3G-R for the gene of the $bla_{\text{CTX-M-1}}$ group (27). Sequencing of the amplification products identified the genes carried by isolates C52-5, V55-6, and M35-6 as $bla_{\text{CTX-M-2}}$ and the gene carried by isolate Y23-8 as $bla_{\text{CTX-M-15}}$ (Table 1). These results were consistent with the

TABLE 1. Antimicrobial susceptibilities and β-lactamase determinants of the four ceftriaxone-resistant *E. coli* isolates and transconjugants[a]

Isolate or transconjugant	MIC (μg/ml)[b]																			IEF result (band pI)	Gene(s) detected
	AMP	AMC	PIP	TZP	CEF	FOX	CTX	CRO	CAZ	ATM	TET	CHL	SXT	GEN	TOB	KAN	AMK	NAL	CIP		
Isolates																					
Y23-8	>128	32	>128	64	>128	8	>512	>512	64	64	>8	>16	≥4/76	>64	>16	>64	>64	>16	>2	5.4, 8.9	$bla_{\text{TEM-1b}}$, $bla_{\text{CTX-M-15}}$
M35-6	>128	64	>128	32	>128	8	>512	>512	16	>128	≤4	>16	≥4/76	>64	>16	>64	8	≤16	≤1	5.4, 8.0	$bla_{\text{TEM-1b}}$, $bla_{\text{CTX-M-2}}$
C52-5	>128	64	>128	128	>128	4	>512	>512	16	>128	>8	>16	≥4/76	>64	>16	>64	8	≤16	≤1	5.4, 8.0	$bla_{\text{TEM-1b}}$, $bla_{\text{CTX-M-2}}$
V55-6	>128	32	>128	128	>128	8	>512	>512	16	>128	>8	>16	≥4/76	>64	>16	>64	8	≤16	≤1	5.4, 8.0	$bla_{\text{TEM-1b}}$, $bla_{\text{CTX-M-2}}$
Transconjugants																					
MKD-Y23	>64	ND	ND	ND	ND	<2	0.12	ND	ND	ND	0.06	>16	≤2/38	>64	>16	>64	>64	≤16	≤1	5.4	$bla_{\text{TEM-1b}}$
MKD-M35	>64	ND	ND	ND	ND	<2	>64	>64	4	32	0.06	16	≤2/38	>64	>16	>64	8	ND	ND	5.4, 8.0	$bla_{\text{TEM-1b}}$, $bla_{\text{CTX-M-2}}$
MKD-C52	>64	ND	ND	ND	ND	<2	>64	>64	4	>64	4	16	≤2/38	>64	>16	>64	8	ND	ND	5.4, 8.0	$bla_{\text{TEM-1b}}$, $bla_{\text{CTX-M-2}}$
MKD-V55	>64	ND	ND	ND	ND	<2	>64	>64	8	>64	4	16	≤2/38	>64	>16	>64	8	ND	ND	5.4, 8.0	$bla_{\text{TEM-1b}}$, $bla_{\text{CTX-M-2}}$
MKD-135	1	ND	ND	ND	ND	1	≤0.03	≤0.03	≤0.03	0.03	0.06	0.25	1	0.12	0.25	2	0.25	0.5	≤0.03	ND	

[a] The susceptibility of *E. coli* MKD-135 is also shown for comparison.

[b] Abbreviations: AMP, ampicillin; AMC, amoxicillin-clavulanic acid; PIP, piperacillin; TZP, tazobactam-piperacillin; CEF, cefepime; FOX, cefoxitin; CTX, cefotaxime; CRO, ceftriaxone; CAZ, ceftazidime; ATM, aztreonam; TET, tetracycline; CHL, chloramphenicol; SXT, sulfamethoxazole-trimethoprim; GEN, gentamicin; TOB, tobramycin; KAN, kanamycin; AMK, amikacin; NAL, nalidixic acid; CIP, ciprofloxacin; ND, not determined.

presence of the β-lactamase bands with alkaline pIs observed in the four isolates (see above).

PCR analysis with primers TEMp-for and TEM-rev, designed for amplification of bla_{TEM} genes and some of the upstream region, followed by sequencing of the amplification products, revealed the presence of a bla_{TEM-1b} determinant in each of the four isolates (Table 1). These results were consistent with the presence of the β-lactamase bands of pI 5.4 observed in the four isolates.

Analysis of the four *E. coli* isolates by a multiplex PCR procedure that allows distinction of *E. coli* into phylogenetic groups (13) revealed that the CTX-M-2-producing isolates (isolates M35-6, C52-5, and V55-6) belonged to three different phylogenetic groups (groups D, A, and B2, respectively), while the CTX-M-15-producing isolate (isolate Y23-8) belonged to phylogenetic group D.

Transferability of CTX-M determinants. Conjugational transfer of cefotaxime resistance was observed at a frequency of approximately 10^{-3} transconjugants/recipient for the three isolates producing CTX-M-2. The transconjugants exhibited decreased susceptibilities to ampicillin, oxyimino-cephalosporins, aztreonam, chloramphenicol, aminoglycosides (gentamicin, tobramycin, and kanamycin), and, except for the transconjugant derived from isolate M35-6, tetracycline (Table 1). A double-disk diffusion test revealed synergistic activity between clavulanate or tazobactam and cefotaxime, ceftazidime, cefepime, and aztreonam against all transconjugants (data not shown). IEF analysis of the transconjugants revealed, in all cases, a pattern of β-lactamase bands that was apparently identical to that of the donors. PCR analysis of the transconjugants with primers CTX-MU1 and CTX-MU2 (followed by analysis of the restriction profiles after digestion with PstI and PvuII) and primers TEMp-for and TEM-rev (followed by sequencing of the amplification products) yielded the same results obtained with the respective donors, confirming that both the CTX-M-2 and the TEM-1 determinants had been transferred. Plasmid DNA was detectable in all the CTX-M-2-positive transconjugants. Analysis of the plasmids carried by the transconjugants after digestion with PstI revealed three different plasmid profiles. Plasmids transferred from the Bolivian isolates (isolates C52-5 and V55-6) were apparently similar to each other, although not identical, while the plasmid transferred from the Peruvian isolate (isolate M35-6) exhibited a different restriction profile, although some bands were apparently similar (Fig. 1). In a Southern blot hybridization, a bla_{CTX-M}-specific probe recognized a 2.2-kb PstI fragment in all three conjugative plasmids (Fig. 1), suggesting that in all cases the genetic environment of the $bla_{CTX-M-2}$ determinants was similar to that of In35 and InS21 (2, 14).

No conjugational transfer of cefotaxime resistance could be detected from the CTX-M-15-producing isolate (isolate Y23-8). Attempts to transfer the ESBL determinant to *E. coli* DH5α by electroporation by using a plasmid DNA preparation from this strain also failed. On the other hand, the bla_{TEM-1b} gene carried by this isolate could easily be transferred by conjugation to MKD-135 (at a frequency of approximately 10^{-3} transconjugants/recipient) when ampicillin was used for the selection of transconjugants. In this case the transconjugant showed decreased susceptibility to ampicillin, chloramphenicol, gentamicin, tobramycin, kanamycin, and amikacin (Table 1). IEF analysis of the transconjugant yielded a β-lactamase that focused at pI 5.4, consistent with the transfer of the bla_{TEM-1b} determinant, whose presence was also confirmed by PCR and sequencing. A Southern blot analysis of the genomic DNA from isolate Y23-8 with the bla_{CTX-M}-specific probe revealed a single hybridization signal that corresponded to the band of genomic DNA, suggesting a chromosomal location of the $bla_{CTX-M-15}$ gene (data not shown).

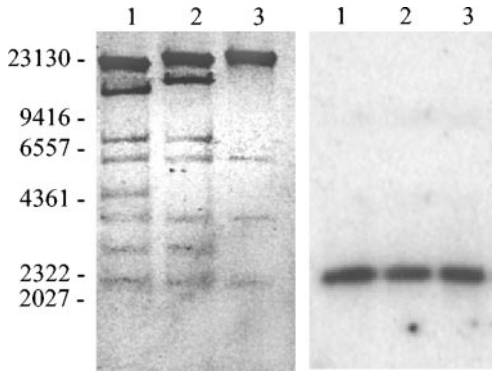

FIG. 1. (Left) PstI restriction profiles of plasmids extracted from the transconjugants obtained with *E. coli* C52-5 (lane 1), V55-6 (lane 2), and M35-6 (lane 3) as donors. (Right) Southern blot hybridization with a bla_{CTX-M}-specific probe. DNA size standards are indicated on the left (in kilobase pairs).

Screening of bla_{CTX-M} determinants in β-lactam-resistant fecal *E. coli* isolates. A sample of 200 ampicillin-resistant but ceftriaxone-susceptible fecal *E. coli* isolates (50 isolates from each area sampled) were randomly selected from among those obtained from children who had yielded ampicillin-resistant but ceftriaxone-susceptible isolates and were tested for the presence of CTX-M-like determinants by colony blot hybridization. The results showed that none of these isolates carried bla_{CTX-M}-related sequences.

DISCUSSION

The ANTRES project is a research project funded by the European Community that deals with antimicrobial use and bacterial resistance in two Latin American countries, Bolivia and Peru. The project monitors the dissemination of microbial drug resistance in the *E. coli* commensal microbiota of healthy children, which has largely been exploited as a useful indicator for similar studies (5, 6, 12, 20, 26). During the initial phase of the study, aimed at determining the rates of antimicrobial resistance in the populations of four different urban settings in the two countries, ceftriaxone-resistant isolates were detected in 4 of 3,208 (0.1%) children studied, and molecular characterization revealed the presence of CTX-M-type β-lactamase genes in the isolates from all four children. Although commensal *E. coli* isolates producing CTX-M-type ESBLs have previously been reported from healthy animals (11), to the best of our knowledge, this is the first report on the presence of similar isolates in the intestinal microbiota of healthy humans.

The CTX-M-type enzymes are now considered the most important "unconventional" ESBLs (i.e., other than TEM- or SHV-type ESBLs) emerging in the family *Enterobacteriaceae* worldwide (10). The finding of similar genes in the commensal

microbiota of humans in each of the settings studied reveals that these genes are widely distributed in those areas, although at low frequencies. The low prevalence could be related to the fact that, due to their cost, expanded-spectrum cephalosporins are still of limited use in those areas. However, under similar conditions, a tendency toward a rapid recruitment in the clinical setting might be expected upon an increase in selective pressure generated by the use of extended-spectrum β-lactams.

CTX-M-2 was the most common type of β-lactamase detected. This was also the first type of CTX-M-type β-lactamase detected in Argentina (and then also in Paraguay and Uruguay), where it is the ESBL most frequently found (75% of ESBLs) in the *Enterobacteriaceae* (10). Analysis of the genetic environment of the $bla_{\text{CTX-M-2}}$ determinants described in this study revealed that they were associated with open reading frame *orf513* and that they were presumably located within a genetic context, similar to In35 and InS21 (2, 14). The location of the $bla_{\text{CTX-M-2}}$ genes on conjugative plasmids with apparently related structures in *E. coli* strains belonging to different phylogenetic groups suggests that the plasmid-mediated dissemination of these resistance genes plays a relevant role.

More recently, CTX-M-type enzymes have also been reported in other Latin American countries, such as Brazil (CTX-M-2, CTX-M-8, and CTX-M-9 groups) and Colombia (CTX-M-12) (10, 36). The finding of $bla_{\text{CTX-M}}$ determinants in Bolivia and Peru as well confirms the wide distribution of these resistance genes in Latin America. This is also the first description of the $bla_{\text{CTX-M-15}}$ variant in Latin America.

The potential role of the resistant isolates from the commensal microbiota as human pathogens remains to be established, and it would be interesting to investigate these strains for the presence of virulence factors or pathogenicity islands. However, the findings of the present study underscore the role that commensal *E. coli* isolates could play as potential reservoirs of these clinically relevant resistance determinants.

ACKNOWLEDGMENT

This work was supported by the European Commission within the ANTRES project (INCO-DEV contract no. ICA4-CT-2001-10014).

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