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Enteropathogenic *Escherichia coli* Strains Carrying Genes Encoding the PER-2 and TEM-116 Extended-Spectrum β-Lactamases Isolated from Children with Diarrhea in Uruguay

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We studied 13 extended-spectrum β-lactamase (ESBL)-producing enteropathogenic *Escherichia coli* isolates from children suffering acute diarrhea in Uruguay. ESBL characterization in crude extracts showed a single band at pI 5.4. PCR amplification and sequencing data allowed identification of bla_{PER-2} and $bla_{TEM-116}$. Retrospective analysis suggests that these strains were disseminated in the community, even if unnoticed, prior to their access to the hospital environment more than a decade ago.

The emergence of new resistance mechanisms is usually recognized when their corresponding genes reach hospital pathogens, by mobilization or capture of plasmids, transposons, or integrons. These resistance mechanisms are evidenced in the hospital environment only after sudden, and sometimes remarkable, changes in the resistance profile of nosocomial microorganisms. Therefore, it is difficult to define how the corresponding genes gain access to the hospital environment, as resistance is followed primarily in disease-causing microorganisms.

Even if commensally microorganisms play a role in disseminating resistance genes and represent a route for gaining access to pathogenic bacteria, they do not necessarily constitute their ultimate origin. A continuous relationship of those microorganisms with humans may complicate the evaluation of their contribution to resistance, as they may have been submitted to the same antibiotic pressure forces affecting pathogenic bacteria and thus may have gained resistance genes from other microorganisms. Moreover, even environmental microorganisms obtained from most sources may have been in previous contact with bacteria of human origin.

It is accepted that most plasmidic β-lactamase-encoding genes (of prevalent broad and extended-spectrum β-lactamases and recently carbapenemases) derive from chromosomally located genes of commensal or environmental microorganisms. However, most of these sources are not known, even for the most promiscuous genes, such as bla_{TEM}. *Kluyvera* species, such as *Kluyvera ascorbat*a, *K. cryocrescens*, and *K. georgiana*, have been suggested, for example, as reservoirs of one of the emergent groups of extended-spectrum β-lactamases (ESBLs) worldwide: the CTX-M β-lactamases (4, 6, 9, 18).

These enzymes appear to be natural ESBLs or "born oxyimino-cephalosporinases" (21), for whose expression there is no need for any mutational shift leading to a modification of the spectrum of activity. The only requisite is to find the right promoter, copy number, or even genetic environment, allowing the proper expression of the β-lactamase-encoding gene, and therefore the expected resistance levels for an ESBL. Spread is due to their recruitment or mobilization to more promiscuous elements, such as plasmids harboring transposons or integrons.

Other worldwide prevalent ESBLs, especially TEM- and SHV-derived enzymes, emerge from mutations in genes coding for narrower-spectrum enzymes, already located in transferable elements, resulting in β-lactamases with a wider hydrolytic activity (5). Surprisingly, TEM-derived ESBLs have been described only recently in this region (south of Brazil, Argentina, Paraguay, and Uruguay) (16). Additionally, PER-2, the second most prevalent ESBL (20) in isolates from Argentina, has never been reported in any other country.

Acute diarrheal disease is still an important cause of infectious morbidity in Uruguayan children (as well as in many other developing countries), enteropathogenic *Escherichia coli* (EPEC) being the most frequently found bacterial pathogen. It is usually associated with infants from the poorer social groups; the most prevalent serogroups are O111, O119, and O55 (in decreasing frequency) (23).

Antimicrobial treatment is not recommended for diarrheal diseases caused by enteropathogenic bacteria other than shigellae (i.e., EPEC). Therefore, antibiotic resistance genes harbored by these microorganisms can remain unnoticed and may be silently transferred between different bacterial species in the community or among patients.

We previously reported EPEC isolates resistant to several antibiotics, including 13 oxyimino-cephalosporin-resistant strains, from 68 EPEC strains originally isolated between October 1990 and April 1993. Some characteristics are shown in Table 1. They were obtained from diarrheal cultures of hospi-

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TABLE 1. Main characteristics of 68 EPEC isolates[a]

Serogroup	*n*	No. of isolates resistant to:									
		AMP	CEF	CXM	TGC	GEN	AMK	SXT	CHL	NAL	TET
O119	27	27	26	16	11	11	2	9	10	0	6
O111	25	25	13	1	0	0	0	10	2	0	14
O55	14	13	11	4	0	2	3	6	3	0	5
O142	2	2	2	2	2	2	0	0	0	0	0
Total	68	67 (99)[b]	52 (76)	23 (34)	13 (19)	15 (22)	5 (7)	25 (37)	15 (22)	0	25 (37)

[a] *n*, number of isolates; AMP, ampicillin; CEF, cephalothin; CXM, cefuroxime; TGC, extended-spectrum cephalosporins (ceftazidime or ceftriaxone); GEN, gentamicin; AMK, amikacin; SXT, trimethoprim-sulfamethoxazole; CHL, chloramphenicol; NAL, nalidixic acid; TET, tetracycline.
[b] Percentages of isolates are in parentheses.

talized children under 30 months old at one pediatric ward of Hospital de Niños "Pereira Rossell" in Montevideo, Uruguay (23).

We focused our attention on the 13 oxyimino-cephalosporin-resistant EPEC isolates, 11 of them belonging to serogroup O119 and the remaining 2 belonging to serogroup O142. Some of their features are displayed in Table 2. All strains were confirmed as *eaeA* and *bfpA* positive by PCR (7, 8). MICs, with and without clavulanic acid (4 μg/ml), of ceftriaxone (CRO), ceftazidime (CAZ), and cefepime (FEP) were determined as specified by the NCCLS (13) for isolates showing resistance to CAZ or cefotaxime in disk diffusion tests. Studies of EPEC strain susceptibility to a wide variety of antibiotics were performed by disk diffusion tests following NCCLS guidelines (14).

Antimicrobial susceptibility data suggested a more efficient hydrolysis of CAZ compared to CRO or FEP. Data on gentamicin and trimethoprim-sulfamethoxazole resistance were also compared, rendering three different antibiotypes, as shown in Table 2.

Crude extracts from late-log-phase cultures were obtained from all 13 oxyimino-cephalosporin-resistant EPEC isolates. ESBL characterization was performed by isoelectric focusing (IEF) in broad-range (pH 3 to 10) polyacrylamide gels as previously described (11). All crude extracts displayed only one β-lactam-hydrolyzing band (pI 5.4) when revealed with an iodometric overlay system (19) using CRO (1,000 μg/ml) as the substrate.

Guided by IEF results, the presence of $bla_{\text{PER-2}}$ (using primers PER-2F [5′-TGT GTT TTC ACC GCT TCT GCT CTG-3′] and PER-2R [5′-AGC TCA AAC TGA TAA GCC GCT TG-3′]) and bla_{TEM} genes (using primers OTR 3 [5′-ATG AGT ATT CAA CAT TTC CG-3′] and OTR 4 [5′-CCA ATG CTT AAT CAG TGA GG-3′]) could also be inferred by PCR in each isolate. Plasmid DNA was extracted by alkaline lysis (3). The reaction mixture contained 2 μl of plasmid DNA, and 1.25 U of *Taq* DNA polymerase (Gibco/BRL) was added to a total volume of 50 μl containing 0.2 mM deoxynucleoside triphosphate, 20 mM Tris-HCl (pH 8.4), 1.5 mM $MgCl_2$, 50 mM KCl, and 0.5 μM primers. The reaction protocol required an initial step of 5 min at 95°C, followed by 30 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 20 min. PCR products were automatically sequenced in both strands by primer extension with the same primers, using an ABI PRISM 3700 DNA sequencer. The sequences showed strict identity with $bla_{\text{PER-2}}$ (2) and $bla_{\text{TEM-116}}$ (10).

Plasmid DNA was also used as the template in PCR screening for bla_{SHV} and $bla_{\text{CTX-M}}$ using specific primers (1, 19), all results being negative.

PER-2 was first described by Bauernfeind et al. after being sequenced from an Argentinean *Salmonella enterica* serovar Typhimurium isolate (2). Other microorganisms harboring the same enzyme have been circulating in Buenos Aires since at least 1989; the enzyme was named, at that time, ARG-1 (A. Rossi, M. Quinteros, E. Couto, et al., Abstr. XI Congr. Latinoam. Microbiol., abstr. B49, 1991; M. A. Rossi, G. Gutkind, M. Quinteros, et al., Abstr. 31st Intersci. Conf. Antimicrob. Agents Chemother., abstr. 939, 1991). The gene was also recognized in other enterobacteria and related pathogens, including a 1993 *Vibrio cholerae* isolate (17, 20).

This study represents the first report of PER-2 in a microorganism outside Argentina (however, in a neighboring country, Uruguay).

In addition, we found that TEM-derived ESBLs were already present in Uruguay in the early 1990s. This is the second worldwide TEM-116 clinical report. This enzyme was reported very recently in Korea (10). An interesting coincidence in apparent pIs makes resolution of these two enzymes by IEF analysis very unlikely, perhaps masking one of them just by supposing, from previous data, the existence of the other in the region.

The first $bla_{\text{PER-2}}$-$bla_{\text{TEM-116}}$-carrying-EPEC isolate was obtained in November 1991 during a programmed survey. By the time the epidemiological work was concluded (April 1993) (23), two more groups of isolates carrying the same enzymes but showing different resistance profiles (3 and 1, respectively) were obtained (see details in Table 2). In all three profiles, the initial isolate corresponded to one child stool sample obtained at admission or within 24 h of admission. Other cultures were obtained from hospitalized patients and may correspond to dissemination of these EPEC strains in the ward. However, the three index case cultures are clear indicators of community circulation of the corresponding resistance genes before accessing the hospital. These cultures are probably part of the initial burst of plasmid-borne $bla_{\text{PER-2}}$ diffusion that later involved many bacterial species.

Information about ESBLs in EPEC infections is not readily available. This can be due, at least in part, to the fact that laboratories usually do not devote resources to the identifica-

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TABLE 2. Main features of EPEC strains carrying PER-2–TEM-116 ESBLs

Strain[c]	Serogroup	Presence of virulence genes subtype		pI	Presence of ESBL gene		Resistance phenotype[b]	MIC (μg/ml)[a]					Admission date	Date of isolation
		eae	*bfp*		bla_{PER-2}	$bla_{TEM-116}$		CAZ	CAZ-CLA	CRO	CRO-CLA	FEP	FEP-CLA	
IH105^{PD}	O119	+/β2	+/β	5.4	+	+	1	>256	0.5	4–8	<0.125	4–8	<0.125	11/21/1991	11/21/1991
IH116^{PD}	O119	+/β2	+/β	5.4	+	+	2	>256	0.5	8–16	<0.125	8–16	<0.125	12/05/1991	12/11/1991
IH117^{PD}	O119	+/β2	+/β	5.4	+	+	2	>256	0.5	8–16	<0.125	8–16	<0.125	12/10/1991	12/19/1991
IH118^{PD}	O119	+/β2	+/β	5.4	+	+	2	>256	0.5	8–16	<0.125	8–16	<0.125	12/12/1991	12/18/1991
IH121^{PD}	O119	+/β2	+/β	5.4	+	+	2	>256	0.5	8–16	<0.125	8–16	<0.125	12/19/1991	12/19/1991
IH122^{PD}	O119	+/β2	+/β	5.4	+	+	2	>256	0.5	8–16	<0.125	8–16	<0.125	12/19/1991	12/19/1991
IH123^{PD}	O119	+/β2	+/β	5.4	+	+	2	>256	0.5	8–16	<0.125	8–16	<0.125	12/23/1991	12/23/1991
IH178^{PD}	O142	+/α1	+/β	5.4	+	+	1	>256	0.5	4–8	<0.125	4–8	<0.125	05/19/1992	05/28/1992
IH179^{AD}	O142	+/α1	+/β	5.4	+	+	1	>256	0.5	4–8	<0.125	4–8	<0.125	05/26/1992	05/26/1992
IH193^{PD}	O119	+/β2	+/β	5.4	+	+	1	>256	0.5	4–8	<0.125	4–8	<0.125	11/16/1992	11/16/1992
IH226^{PD}	O119	+/β2	+/β	5.4	+	+	3	>256	0.5	4–8	<0.125	4–8	<0.125	03/08/1993	03/23/1993
IH235^{AD}	O119	+/β2	+/β	5.4	+	+	3	>256	0.5	4–8	<0.125	4–8	<0.125	03/22/1993	03/22/1993
IH239^{PD}	O119	+/β2	+/β	5.4	+	+	2	>256	0.5	4–8	<0.125	4–8	<0.125	04/12/1993	04/12/1993

[a] CAZ, ceftazidime; CLA, clavulanic acid; CRO, ceftriaxone; FEP, cefepime.
[b] One Caz^r Gen^s Chl^s Sxt^r strain, two Caz^r Gen^r Chl^r Sxt^s strains, and three Caz^r Gen^r Chl^s Sxt^s strains.
[c] PD, persistent diarrhea; AD, acute diarrhea.

tion of EPEC isolates and their antimicrobial susceptibility profile because antibiotic treatment is not indicated for EPEC diarrhea. Thus, data on the resistance of these strains is not routinely produced, as for other bacterial pathogens. Moreover, the attention of microbiologists usually focuses on pathogenic microorganisms but not on related bacteria of indigenous flora that may also develop resistance in treated patients under sustained selective pressure of misguided excessive antibiotic use.

Under these circumstances, EPEC strains are microbial pathogens that can cause in our country, and probably in others, sporadic or epidemic infections that are not easily recognized by most clinical microbiology laboratories (12). These microorganisms can contribute to build an enteric reservoir of unnoticed transmissible factors of antibiotic resistance that may precede its recognition in routinely searched for pathogens. As an example, in 1970 to 1974, a protracted outbreak of a diarrhea-causing, multiple-drug-resistant *S. enterica* serovar Typhimurium strain at the "Hospital de Niños" (Montevideo) was preceded by an increasing prevalence of resistance that was observed in EPEC isolates at the same hospital (22).

Thus, the importance of studying these microorganisms from an epidemiological standpoint should be stressed. Efforts must be made to improve the training of technical personnel in antigenic typing techniques of EPEC and to organize regional reference centers capable of performing molecular confirmatory identification.

The antibiotic resistance of these bacteria has to be monitored. The World Health Organization and the Pan American Health Organization have promoted a surveillance program to improve regional knowledge in this field, considering that most major antibiotics may soon be no longer active against bacterial agents responsible for the two main infectious causes of death in children younger than 5 years old in the region: acute respiratory illnesses and diarrhea (15).

Nucleotide sequence accession numbers. The sequences determined in this study were deposited into the EMBL database under accession numbers AJ786366, AJ847362, AJ847363, and AJ847364.

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