Extended-Spectrum β-Lactamases in *Enterobacteriaceae* in Buenos Aires, Argentina, Public Hospitals

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Resistance to extended-spectrum cephalosporins is often associated with plasmid encoded extended spectrum β-lactamases (ESBL). In order to evaluate the prevalence and diversity of ESBLs in enterobacteria in our city, a 1-month-period survey was carried out from April to May 2000. Extended-spectrum-cephalosporin-resistant strains, isolated from inpatient clinical specimens other than stools, were collected among 17 participating hospitals. From a total of 427 enterobacterial strains that were collected during this period, 39 were extended-spectrum cephalosporin resistant. The National Committee for Clinical Laboratory Standards' Screening and Confirmatory Tests for ESBL production were performed using cefotaxime and ceftazidime; cefepime and cefepime-clavulanic acid-containing disks were included. β-Lactamases were characterized by isoelectric focusing and PCR amplification using specific primers. Three different ESBLs were detected: SHV-related (4 isolates), PER-2-type (9 isolates), and CTX-M-2-related (26 isolates). Sequencing of the corresponding genes confirmed CTX-M-2 in 19 of 21 and CTX-M-31 (an allelic variant) in the remaining 2 of 21. CTX-M-2 (or its variant) was detected in all *Escherichia coli*, *Enterobacter aerogenes*, *Serratia marcescens*, *Proteus mirabilis*, and *Providencia stuartii* strains, while PER-2 was detected in *Enterobacter cloacae*, *E. aerogenes*, and *Klebsiella pneumoniae*; SHV-related ESBL were found only in *K. pneumoniae*. These results clearly show that CTX-M-2 is the most prevalent ESBL produced by enterobacterial species isolated from public hospitals in Buenos Aires.

Antimicrobial resistance among *Enterobacteriaceae* has become a growing problem in our country (1, 15, 16). Resistance to extended-spectrum cephalosporins is often associated with plasmid encoded extended-spectrum beta-lactamases (ESBL). Although elsewhere many of these enzymes are derived through a single amino acid substitution or a few amino acid substitutions from the parental enzymes TEM-1, TEM-2, and SHV-1, other emerging class A enzymes, such as CTX-M and PER enzymes (3-7, 10), have been reported as long as a decade ago in our region. Hyperproduction of inducible chromosomally encoded beta-lactamases in some species of *Enterobacteriaceae* may also contribute to resistance in those species (9, 11)

Since its first detection in our country (2, 4, 18), CTX-M-2 has been identified not only in all oxyiminocephalosporin-resistant members of *Enterobacteriaceae* (15–17) but also in *Vibrio cholerae* and *Aeromonas hydrophila* (14, 19) (M. Quinteros, M. Radice, P. Power, et al., Abstr. 9th Int. Congr. Infect. Dis., abstr. 15884, 2000). PER-2 has been reported in *Klebsiella pneumoniae*, *Enterobacter cloacae*, and *Escherichia coli* among others. Although frequently stated in national and international meetings as prevalent (in particular, CTX-M-2 has been repeatedly mentioned for different enterobacterial species), no

reliable epidemiological data are available currently in our region.

In 1999, a network of microbiology laboratories was established within the metropolitan Public Hospitals under administration by the Buenos Aires City Government to optimize resource utilization and to establish rules for different methodologies and diagnostic criteria. Detection and classification of ESBL in public hospitals was one of the multicentric studies proposed by this group.

To evaluate the prevalence of diverse ESBLs in our hospitals, a 1-month survey was carried out from April to May of 2000 in Metropolitan Public Hospitals of Buenos Aires City. Epidemiological data both on antibiotic resistance and on the involved resistance mechanisms should be useful for public health workers in evaluating the current recommendations for antibiotic usage.

MATERIALS AND METHODS

Bacterial strains. During April to May 2000, 17 Public Hospitals of Buenos Aires City (see Acknowledgments) collected all consecutive and nonrepetitive enterobacterial isolates from inpatients. Due to the expected high number (and supposed resistance levels as reported in reference 1 and by Whonet (www.paho .org) of *E. coli* and *K. pneumoniae* isolates, only those corresponding to 1 week (within this period) were fully analyzed (see Table 1). All species were identified using both conventional techniques and automatized assays (MicroScan; Vitek). Isolates not involved in the primary infection according to the Centers for Disease Control and Prevention criteria and those from stools were excluded (8). All cefotaxime (CTX)-resistant and/or ceftazidime (CAZ)-resistant isolates (independently of bacterial species, with inhibition zone of ≤27 mm for CTX or ≤22 mm for CAZ) were further analyzed for their ESBL presence by microbiological, biochemical, and genetic analysis.

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TABLE 1. Percentage of resistance within each bacterial species

Bacterial species	Total no. of isolates analyzed	No. (%) of resistant isolates
E. coli	186[a]	5 (3)
K. pneumoniae	29[a]	10 (34)
P. mirabilis	100	7 (7)
E. aerogenes	17	3 (18)
E. cloacae	44	7 (16)
S. marcescens	21	3 (14)
C. freundii	19	1 (5)
Providencia spp.	11	3 (3)

[a] Corresponding to 1-week period.

Susceptibility tests and detection of ESBL producers. Susceptibility tests were performed in each laboratory by disk diffusion assays according to National Committee for Clinical Laboratory Standards recommendations (13) and confirmed using MicroScan Neg Urine Combo 5 panel. MicroScan panels were tested using the AutoScan-4 instrument with v22.26 of MS Data Management System. Screening and Confirmatory Tests for ESBL production were assayed accordingly to National Committee for Clinical Laboratory Standards recommendations for E. coli and Klebsiella spp. (independently of bacterial species). CTX (30 μg)- and CAZ (30 μg)-containing disks and their combination with clavulanic acid (10 μg) were included (13). An increment in the inhibition zone of at least 5 mm was considered as ESBL production. We also included cefepime (FEP) (30 μg)- and FEP-clavulanic acid (30 and 10 μg)-containing disks for their proposed better discriminatory power between ESBL production and AmpC hyperproduction (21).

Isoelectric focusing analysis and enzyme inhibition assay. Soluble crude β-lactamase extracts obtained by ultrasonic disruption followed by centrifugation $(27,000 \times g, 10 \text{ min}, 4°C)$ were subjected to isoelectric focusing by the method of Mathew et al. on broad-range gels (pH, 3 to 10), using an LKB Multiphor apparatus (Pharmacia Sweden) (12). β-Lactamase activity was revealed by the agar iodometric system using ampicillin (500 μg/ml) and ceftriaxone (1,000 μg/ml) as substrates (18). The pIs of the β-lactamases were estimated from the pIs of known enzymes and commercial pI markers (Pharmacia). Inhibition assays were carried out after preincubating the gels in a clavulanic acid solution (20 mM) for 15 min, with activity revealed as previously mentioned.

Molecular detection of bla genes. Plasmid DNA was prepared by a modified Birnboim and Doly alkaline lysis method (20). bla genes were amplified using the following oligonucleotide primers (5 min at 95°C, 30 cycles [1 min at 94°C, 1 min of annealing at 52°C, 1.5-min extension at 72°C] followed by a 10-min final extension at 72°C): bla_{TEM} (5′ ATAAAATTCTTGAAGACGAAA 3′, 5′ GAC AGTTACCAATGCTTAATCA 3′), bla_{SHV} (5′ TCGGGCCGCGTAGGCA TGAT 3′, 5′ AGCAGGGCGACAATCCCGCG 3′), bla_{CTX-M} (5′ TTAATGA TGACTCAGAGCATTC 3′, 5′ GATACCTCGCTCCATTTATTG 3′), and bla

$_{PER-2}$ (5′ TGTGTTTTCACCGCTTCTGCTCTG 3′, 5′ CAGCTCAAACTGAT AAGCCGCTTG 3′).

Sequencing of CTX-M-positive PCR products. PCR products were automatically sequenced in both strands by primer extension with the same primers, using an ABI PRISM 3700 DNA.

RESULTS

Distribution of clinical isolates and resistance. From 427 enterobacteria recovered within this period, 9% of them (39 isolates) were considered resistant to oxyiminocephalosporins. No carbapenem-resistant isolates were detected in this period. They included 5 E. coli isolates, 10 K. pneumoniae isolates, 7 Proteus mirabilis isolates, 7 E. cloacae isolates, 3 Enterobacter aerogenes isolates, 3 Serratia marcescens isolates, 3 Providencia stuartii isolates, and 1 Citrobacter freundii isolate. The percentage of resistance in each bacterial species is shown in Table 1. Not a single CAZ-resistant, CTX-sensitive microorganism was detected.

A single S. marcescens strain reported in one hospital was not submitted for characterization and was not included in these numbers.

Detection of ESBL producers. CTX–CTX-clavulanic acid disks confirmed the presence of ESBL in E. coli, K. pneumoniae, and P. mirabilis isolates but failed to confirm ESBL production in four of six E. cloacae isolates, one of three S. marcescens isolates, and two of three Providencia spp. CAZ–CAZ-clavulanic acid-containing disks displayed less ESBL detection ability, as they failed to detect ESBL production in one E. coli isolate, all P. mirabilis isolates, four E. cloacae isolates, two S. marcescens isolates, and two Providencia spp. The use of FEP–FEP-clavulanic acid improved noticeably the detection of ESBL, as only one ESBL-producing Providencia spp. was not detected. ESBL distribution among species is shown in Table 2.

Prevalence of ESBL in enterobacterial strains. An extended-spectrum β-lactamase with a pI of 5.4 was detected in all isolates included in this study. PCR amplification using specific primers for bla TEM was positive in all cases.

Of the 39 total strains, almost all of them (37 isolates) were ESBL producers.

TABLE 2. NCCLS' ESBL confirmatory tests with ESBL-harboring enterobacteria

Microorganism (n^a) or group	No. of positives/total no. (% positive)[b]		
	CTX/CTX-Cla	CAZ/CAZ-Cla	FEP/FEP-Cla
E. coli (5)	5/5	4/5	5/5
K. pneumoniae (10)	10/10	10/10	10/10
P. mirabilis (7)	7/7	0/7	7/7
All noninducible AmpC producers (22)	22/22 (100)	14/22 (64)	22/22 (100)
E. aerogenes (3)	3/3	3/3	3/3
E. cloacae (6)	2/6	2/6	6/6
S. marcescens (3)	2/3	1/3	3/3
Providencia spp. (3)	2/3	2/3	2/3
All inducible AmpC producers (15)	9/15 (60)	8/15 (53)	14/15 (93)
Total (37)	31/37 (84)	22/37 (59)	36/37 (97)

[a] n, no of isolates.
[b] CTX/CTX/Cla, cefotaxime (30 μg) plus cefotaxime-clavulanic acid (30 μg/10 μg). CAZ/CAZ/Cla, ceftazidime (30 μg) plus ceftazidime-clavulanic acid (30 μg/10 μg). FEP/FEP/Cla, cefepime (30 μg) plus cefepime-clavulanic acid (30 μg/10 μg).

TABLE 3. Distribution of ESBL in different enterobacterial species

Species (n^a)	ESBL pI (n)	Presence of ESBL *bla* gene[c]		
		$bla_{\text{CTX-M}}$	bla_{PER}	bla_{SHV}
E. coli (5)	8.2 (4)	X		
	5.4 (1)		X	
K. pneumoniae (10)	8.2 (5)	X		
	7.6 (3)			X
	5.4 (2)		X	
P. mirabilis (7)	8.2 (7)	X		
E. aerogenes (3)	8.2 (2)	X		
	5.4 (1)		X	
E. cloacae (7^b)	8.2 (3)	X		
	5.4 (3)		X	
S. marcescens (3)	8.2 (3)	X		
Providencia spp. (3)	8.2 (2)	X		
	7.6 (1)			X

[a] *n*, no. of isolates.
[b] One AmpC hyperproduction.
[c] X indicates that the gene is present.

Two isolates, one *E. cloacae* and the other *C. freundii*, were characterized as AmpC hyperproducers, corresponding to 5% of the resistant strains.

Isoelectric focusing and gene amplification allowed the characterization of three different ESBL: CTX-M-2-type (26 isolates), PER-2-type (7 isolates), and SHV enzymes (4 isolates). By sequencing of 21 of 26 of the CTX-M-2-type PCR-positive amplicons, it was confirmed that in 19 of 21 it was this gene (100% identity) and not one corresponding to any other known enzyme (CTX-M-4, CTX-M-5, CTX-M-6, CTX-M-7, CTX-M-20, TOHO-1, or KLU-A-1). However, two of them (one *Providencia* sp. and one *E. coli* isolate) were allelic variants (CTX-M-31) with a single nucleotide change, resulting in a Thr-162-Ser substitution.

Confirmation of clavulanic acid activity on each suspected ESBL active band could be achieved by isolectric focusing and preincubation with inhibitors, after which activity was revealed by the iodometric gel overlay.

The presence of two or more ESBLs in the same isolate was not detected. The distribution of different ESBLs in each species is shown in Table 3.

DISCUSSION

In the past, detection of resistance mediated by classical ESBLs was carried out using any of the common extended-spectrum cephalosporins (CAZ or CTX) or aztreonam. Appearance of new resistance mechanisms made mandatory the use of more than one potential substrate and implied also modifications in the associated breakpoints (17). Overall resistance detection abilities of different disks proved, for our epidemiological situation, that CTX and CAZ outperformed CAZ as a single disk, due to the prevalence (suspected and confirmed in this work) of CTX-M-2.

Presumptive and confirmatory detection of ESBLs by meth-

ods based on the inhibitory effects of clavulanic acid are usually precluded for enterobacteria that may posses inducible chromosomal β-lactamases, because of the potential induction effects of clavulanic acid over the chromosomal β-lactamase expression, the final effect being a balance between the induction and the inhibitory effect. Even if in the past it was not critical from a clinical point of view, discrimination between ESBLs and hyperproduction of class C β-lactamases is obviously of epidemiological importance for the hospital environment, and since availability of cefepime may also prove clinically relevant. The use of the FEP–FEP-clavulanic acid-containing disks improved detection of ESBLs in these species to 93% compared with 60 and 53% obtained with CTX and CAZ, respectively.

ESBL production was the main resistance mechanism in all enterobacterial strains, although 5% of resistance was due to AmpC hyperproduction.

CTX-M-2 (and its variant) was the prevalent ESBL in our hospitals, corresponding to 67% of the characterized β-lactamases. CTX-M-2 was ubiquitous in all species, being the only ESBL detected in *P. mirabilis* and *S. marcescens*. PER-2 was detected in 18% of the isolates. SHV enzymes were the third-most-prevalent ESBLs, but they were almost restricted to *K. pneumoniae*.

As previously stated in different reports, no TEM-derived ESBL enzymes were detected as ESBLs.

Even if not formally published, resistance prevalences of different extended-spectrum cephalosporins have been estimated to be, for our region, much higher than in other regions of the world (1) (www.paho.org). However, most of these estimations have been obtained either from surveillance programs or by the analysis of microorganisms submitted for characterization in reference or "resistance analysis-dedicated" laboratories. These estimations may have been biased by the origin of the microorganisms, it not being an easy task to clean up the relevance of strain duplications, or cultivation only after unsuccessful treatments.

Our results, even if they still are higher than those reported for other cities or hospitals around the world, are much closer to the worldwide experience. We cannot discard the possibility, obviously, that just by chance resistance was underrepresented in this period, but it seems improbable, at least given the number of different hospitals involved, each one contributing to the final numbers.

and C. Garbasz, General de Agudos "Dr. Ignacio Pirovano," two isolates; M. T. López Reyes, General de Agudos Donación "Santojanni," two isolates; L. Botto and M. Cervetto, Materno Infantil "Dr. Ramón Sardá," one isolate; M. Hoffman and H. Villar, General de Agudos "Dr. E. Tornú," one isolate; R. Mondino and L. Lobov, General de Agudos "Dr. Abel Zubizarreta," four isolates; H. Lopardo and E. Rubeglio, Nacional de Pediatría "Dr. Juan P. Garrahan," three isolates; O. Sivori, Instituto del Lisiado; M. L. Chavez, Instituto Oncológico, one isolate; and J. Kovensky, de Quemados.

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