Occurrence of Extended-Spectrum Beta-lactamases in *Enterobacteriaceae* Isolated from Hospitalized Patients in Curitiba, southern Brazil

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Production of extended-spectrum beta-lactamases (ESBL) by enterobacteria is an important resistance mechanism against antimicrobial beta-lactamics. We tested 498 bacterial strains isolated from two tertiary-care teaching hospitals for ESBL production, using screening breakpoints for aztreonam and third generation cephalosporins, according to CLSI recommendations. Among these isolates, 155 were positive for the ESBL screening test, and 121 (78%) were confirmed by the clavulanic acid combination disk method. We found a high frequency of ESBL (24%) among *Enterobacteriaceae*, with a frequency of 57.4% for *Klebsiella pneumoniae*, 21.4% for *Klebsiella oxytoca*, and 7.2% for *E. coli*. In other members of *Enterobacteriaceae,* non-*Klebsiella* and non-*E. coli,* the prevalence was 21.6%. Ceftriaxone and cefotaxime showed a higher sensitivity in the screening test (99.2%) when compared to ceftazidime, aztreonam and cefpodoxime. However, cefotaxime/cefotaxime plus clavulanic acid showed a higher sensitivity in the confirmatory test (96.7%).
Key Words: ESBL, beta-lactamases, *Enterobacteriaceae*.

Extended-spectrum beta-lactamases (ESBL) were initially described by Knothe et al. (1983) [1]; they have contributed to the recently-observed large increase in beta-lactamic antimicrobial resistance. These enzymes are capable of hydrolyzing penicillins, cephalosporins (including wide spectrum ones) and monobactamics, except for the cefamicines and the carbapenemics [2]. The ESBLs are sensitive to inhibitors of beta-lactamases, such as clavulanic acid, classified in group 2 of Bush et al. [3].

Many of the ESBLs arise from point mutations in genes that encode narrow-spectrum beta-lactamases, provided by plasmids such as TEM and SHV [2]. However, members of a newly-emerging ESBL group, CTX-M, derived from class A chromosomal beta-lactamases, have been identified recently [4]. Routine antimicrobial susceptibility tests do not always detect ESBLs, thus requiring special detection tests [5].

Plasmid genes are easily transferred among enterobacteria, contributing to ESBL dissemination [6]. Plasmids that carry beta-lactamase genes frequently harbor resistance genes to other antimicrobials, such as trimethopim-sulfamethoxazole and aminoglycosides [7]. Therefore, the detection of ESBL-producing isolates is critical to assure appropriate therapy and to provide necessary information to prevent their dissemination.

The Clinical Standard Laboratory Institute (CLSI) recommends that screening and confirmatory tests should be routinely undertaken for *K. pneumoniae, K. oxytoca, E. coli* and *Proteus mirabilis.* The screening tests are based on the detection of an increase in the minimum inhibitory concentration (MIC) or a reduction in the susceptibility zone diameter for one or more of five antimicrobial agents (cefpodoxime, aztreonam, ceftriaxone, cefotaxime and ceftazidime). Confirmatory tests are based on the increased susceptibility to antimicrobial agents tested in combination with clavulanic acid *versus* susceptibility when tested alone.

According to Schawber et al. [8], the identification of ESBLs in non-*E.coli* and non-*Klebsiella* spp. enterobacteria and the applicability of the CLSI criteria for their detection remain uncertain and inconclusive. *Proteus mirabilis* was the first non-classic ESBL-producing species included in CLSI 2005 for screening and confirmatory tests of isolates from clinically-relevant samples (e.g., blood samples).

We analyzed the occurrence of ESBL in different enterobacteria species and determined the best enzyme substrate to detect ESBL in two university hospitals. We also studied the pattern of susceptibility of the isolates to other clinically-relevant antimicrobials.

Material and Methods

Samples

Four-hundred-ninety-eight isolates of enterobacteria were obtained prospectively from hospitalized patients. The strains were derived from urine, blood, cerebrospinal fluid, sputum, tracheal aspirate, bronchoalveolar lavage and surgical wounds. Among these, 438 isolates were obtained from the Clinical Hospital of the Federal University of Paraná (HC-UFPR) from August 2003 to August 2004. Sixty isolates were obtained from patients at the Hospital of the Catholic University of Paraná (HUC-PUC), from January to August 2004**.** The preliminary identification of *Enterobacteriaceae* was accomplished using the Vitek ® (bioMérieux) automated system and afterwards confirmed by a panel of standard biochemical tests recommended for enterobacteria identification [9-14].

Disk-Diffusion Screening (DD) and ESBL confirmatory test

The disk-diffusion screening and ESBL confirmatory test was performed as described by Clinical Laboratory Standard International [14,15].

Determination of the Minimum Inhibitory Concentration (MIC)

A MIC assay was performed on all strains that showed zone reduction for one or more of the antimicrobials used in the ESBL screening test. The agar micro-dilution technique was performed as described by the National Committee for Clinical Laboratory Standard [16], using amikacin, aztreonam, cefepime, cefotaxime, ceftazidime and ceftriaxone (Sigma Chemical CO, St. Louis, MO, USA), ciprofloxacin (ICN Biomedicals Inc. Dawn, OH, USA), imipenem and ertapenem (Merck Sharp & Dohme Ltda., Campinas, SP, Brazil). *Pseudomonas aeruginosa* ATCC 27853 and *E. coli* ATCC 25922 [17] were used as quality-control strains.

Results

Among the 498 strains of enterobacteria that were isolated, 155 presented decreased susceptibility to third-generation cephalosporins and aztreonam and were considered possible ESBL producers. Among these, 121 isolates were confirmed by the combined disk method. Table 1 presents the number of isolates of each species and the distribution of positive samples by screening and confirmatory tests. The largest isolate numbers from species confirmed as ESBL positive were *K. pneumoniae* (67), *E. cloacae* (20) and *E. coli* (14). *Serratia marcescens, Enterobacter aerogenes* and *K. oxytoca* provided three or four confirmed bacterial isolates, while other species provided one or two, except for *Enterobacter gergoviae,* which did not provide any ESBL isolates.

The prevalence of ESBL-positive isolates within the group of enterobacteria was 24.3%. Among the species considered classic ESBL producers, *K. pneumoniae* was the species with the highest isolate occurrence (57.4%), followed by *K. oxytoca* (21.4%), and *E. coli* (7.2%), with a 21.6% positive frequency for other species isolates. *Proteus mirabilis*, which recently was included in the CLSI 2005 among species recommended for ESBL testing generated a positive isolate frequency of 8% in our study. By grouping the affected isolates by genus, a high positive percentage for *Klebsiella* (54.3%), *Enterobacter* (27.1%), and *Serratia* (20.9%) was observed. The occurrence of *Citrobacter, Morganella, Proteus* and *Escherichia* was also striking, varying from 7.3% to 14.3%. The genera *Providencia* and *Pantoea* were not included in this analysis, due to the small number of isolates. Among the positive enterobacteria identified by the screening test, 20 isolates of *Enterobacter* (23.5%), five isolates of *Klebsiella* (4.0%), three isolates of *Serratia* (12.5%), three isolates of *Morganella* (17.6%) and one isolate of *Citrobacter* (7.1%) provided inconclusive results by the clavulanic acid inhibition test.

Sixty-four (52.9%) of 121 ESBL isolates were resistant to cefoxitine. The sum of the frequency of resistant samples to cefoxitine in non-*E. coli* and non-*Klebsiella* spp. species were higher than those found in classic ESBL producers. Among the classic producers, the highest frequency of resistance to cefoxitine was found in the *K. pneumoniae* isolates, followed by *K.oxytoca* and *E.coli*. Among non-*E.coli* and non-*Klebsiella* spp. isolates, *E. cloacae* had 18 of 20 ESBL positive isolates resistant to cefoxitine. Out of four ESBL-positive *S. marcescens* isolates, three were resistant to cefoxitine. The species *S. liquefaciens, P. rettgerii, P. mirabilis, P.agglomerans, M. morganii, E. gergoviae, E. aerogenes, C.amalonaticus* and *C. freundii* each included only one isolate resistant to cefoxitine as well as ESBL positive, while *P. vulgaris* had none.

The antimicrobials used for ESBL testing were analyzed for estimation of sensitivity and specificity in the detection of ESBL by comparing the isolates from the screen test with those positive in the confirmatory test. The greatest sensitivity was obtained with ceftriaxone and the smallest with ceftazidime. The specificity was low for all the antimicrobials (Table 2).

When only *E. coli* and *Klebsiella* spp. were analyzed, the results of sensitivity and specificity were similar, and the sensitivity to ceftazidime was lower for the ESBL detection of non-*E. coli* and non *Klebsiella* spp. species. Moreover, in the confirmatory test for the production of ESBL, a higher sensitivity was observed with cefotaxime (CTX) and cefotaxime-clavulanic acid (CTX+AC). The specificity was 100% for all combinations (Table 2). This was seen only when *E. coli* and *Klebsiella spp*. were evaluated. However, the sensitivity for all combinations of non-*E.coli* and non-*Klebsiella* spp. species was lower. When evaluating the two combinations used simultaneously, the sensitivity for cefpodoxime and cefpodoxime together with clavulanic acid (CPD/CPD+AC) against ceftazidime and ceftazidime combined

Table 1. Frequency of ESBL-positive screen and confirmatory tests distributed by species and genus

Species	Isolate	ST (+)	CT (+)	Genus	Isolate	ST (+)	%	CI	CT (+)	%	CI
Citrobacter amalonaticus	1	1	1	*Citrobacter*	14	3	21.4	5.7-51.2	2	14.3	2.5-43.8
Citrobacter freundii	13	2	1								
Enterobacter aerogenes	14	7	3	*Enterobacter*	85	43	50.6	39.6-61.5	23	27.1	18.3-37.9
Enterobacter cloacae	67	34	20								
Enterobacter gergoviae	4	2	0								
Escherichia coli	193	14	14	*Escherichia*	193	14	7.2	4.2-12.1	14	7.3	4.2-12.1
Klebsiella oxytoca	14	3	3	*Klebsiella*	129	75	58.1	49.1-66.7	70	54.3	45.3-63.0
Klebsiella pneumoniae	115	72	67								
Morganella morgani	17	5	2	*Morganella*	17	5	29.4	11.38-56.0	2	11.8	2.1-37.7
Pantoea agglomerans	2	1	1	*Pantoea*	2	1	*	*	1	*	*
Proteus mirabilis	25	4	2	*Proteus*	33	5	15.2	5.7-32.7	3	9.1	2.3-25.5
Proteus vulgaris	8	1	1								
Providencia rettgeriii	1	1	1	*Providencia*	1	1	*	*	1	*	*
Serratia liquefaciens	4	1	1	*Serratia*	24	8	33.3	16.4-55.3	5	20.9	7.9-42.7
Serratia marcescens	20	7	4								
Total	498	155	121		498	155	31.1		121	24.3	

ST= screen test; CT=confirmatory test; CI=confidence interval.

Table 2. Sensitivity and specificity of the antimicrobials used for screening and confirmatory tests to detect ESBL producers

Test	Antibiotics	Sensitivity (%)	Specificity (%)
Screening	Aztreonam	94,2	26,5
	Ceftriaxone	99,2	11,8
	Cefpodoxime	95,9	11,8
	Ceftazidime	85,1	32,3
	Cefotaxime	99,2	11,8
Confirmatory	Cefpodoxime + clavulanic acid	76,9	100
	Ceftazidime + clavulanic acid	70,2	100
	Cefotaxime + clavulanic acid	96,7	100

Table 3. Potency (MIC50), activity (MIC90) and sensitivity of antimicrobials assayed for 155 ESBL producer isolates

Antibiotic	MIC50	MIC90	Sensitivity (%)
ATM	32	>256	16.5
COM	16	64	46.1
CAZ	32	>128	32.9
CTX	128	256	7.4
CRO	256	>256	9.1
IPM	0.5	2	100
AMI	8	32	86.6
CIP	16	>16	33.8
ERT	0.12	2	100

MIC50 = minimum concentration capable to inhibit 50% of the isolates; MIC90 = minimum concentration capable to inhibit 90%.

with clavulanic acid (CAZ/CAZ+AC) was 89.2%. A comparison of CPD/CPD+AC with cefotaxime and cefotaxime combined with clavulanic acid (CTX/CTX+AC) yielded 97.3% sensitivity. CAZ/CAZ+AC and CTX/CTX+AC was effective for 99.2% of

the isolates tested. Total sensitivity was only reached when the three combinations were used.

The sensitivity for antimicrobials tested by the agar dilution method for ESBL positive isolates is shown in Table 3. Ertapenem was the antibiotic with the highest potency, followed by imipenem, although they presented the same sensitivity.

Discussion

ESBLs are the main cause of resistance to beta-lactam antibiotics in members of the *Enterobacteriaceae*; their biochemistry, genetics and epidemiological characterization have been extensively studied. Due to the clinical importance of the detection of ESBLs, screening and confirmatory methods have been routinely used to investigate the production of these enzymes in *E. coli* and *Klebsiella* spp. As their occurrence in other enterobacterias has been increasing, it becomes essential to evaluate their occurrence in this population.

In our study 121 (24%) of all the samples and 78% of previously-screened isolates (as scored by sensitivity to third-generation cephalosporins and aztreonam) were ESBL positive for ESBL by the clavulanic acid inhibition diffusion test. This value is considered high when compared to other reports [8], but is similar to other results found in Brazil, with 29% of isolates exhibiting ESBLs among all *Enterobacteriaceae* [18]. This highlights the importance of the investigation of resistance mechanisms in hospitals. The proportion of isolated/ confirmed ESBL producers (78%) that we found reinforces the need for a confirmatory test in order to distinguish ESBLs from other resistance mechanisms to beta-lactamics.

Klebsiella pneumoniae (67 ESBL positive isolates) was the most common ESBL producer, as described [2]. However *E. cloacae* (20 ESBL positive isolates) had a higher prevalence than other classic producers (*E. coli*, 14; *K. oxytoca*, 3). The same observation was made by Mendes et al. [18]. ESBL positive isolates were found in all species, except for *E. gergoviae*, and the finding of ESBL in several enterobacteria species has already been reported by others [8,19,20].

The occurrence of ESBL in non-*E. coli* and non-*Klebsiella* spp. isolates was 21.5%. This is a lower frequency than found for *K. pneumoniae*, which is regarded as the classic ESVL producer. This frequency was similar to that of *K. oxytoca* (21.4%), and higher than *E. coli* (7.2%). *Proteus mirabilis* ESBL production was found at a similar frequency to *E. coli* production (8%). These results are higher than those found in the USA [2, 8], but similar to those obtained in several Latin American countries for *Klebsiella* spp. and *E. coli* [21]. Therefore, we conclude that the frequency of ESBL is higher in our geographic region.

Among non-*E. coli* and non-*Klebsiella* spp. species, the highest percentage of ESBL-positive isolates was found in *Enterobacter* (27.1%), *Serratia* (20.9%), *Citrobacter* (14.3%) and *Morganella* (11.8%). All of the aforementioned harbor chromosomal-inducible AmpC type beta-lactamases. Decreased bacterial membrane permeability to the antimicrobials, associated with the production of other inducible beta-lactamases can mark the presence of ESBL [8,22-24]. Since these genera also accounted for a high number of inconclusive samples, it is reasonable to suppose that the occurrence may have been even greater, which points to the necessity of evaluation by molecular biological techniques.

The high number of ESBL isolates resistant to cefoxitine (53%) can be explained by the high number of isolates among the ESBL positives that harbored chromosomal AmpC, since ESBLs do not confer resistance to that antibiotic. Also, cefoxitine cannot be used alone to distinguish ESBL from AmpC or from other beta-lactamases, because other mechanisms can confer that phenotype, such as mutations in porines alone, or associated with beta-lactamase production [25].

Classic ESBL producers also had positive ESBL isolates resistant to cefoxitine (28 *K. pneumoniae*, 5 *E. coli*, 2 *K.*

oxytoca) and *K. pneumoniae* had five inconclusive samples. The recent dissemination of AmpC plasmidial enzymes in these species could explain these findings, in addition to a possible mutation in porines [25,26]. Moreover, enzymes that mask the presence of ESBL in *Enterobacteriaceae* may involve beta-lactamases sensitive to clavulanic acid, and when they are produced in high concentrations they may be capable of reducing the sensitivity to third-generation cephalosporins and aztreonam [3,8,27].

Phenotypic differences were used to distinguish microorganisms that are sensitive to non-ESBL inhibitors from ESBL. In the case of the K1 enzyme of *K. oxytoca*, the property of not conferring resistance to ceftazidime was employed for purposes of distinction. Moreover, K1 only produces resistance to third-generation cephalosporins when it is present in high concentrations, while the concentration of clavulanic acid in the detection test is insufficient to inhibit the enzyme in an effective way [2]. The chromosomal enzymes of *Proteus vulgaris* were used to differentiate non-ESBL isolates that are sensitive to inhibitors from ESBLs, because they do not confer resistance to aztreonam. The enzymes belonging to group 2f found in *E. cloacae* and *S. marcescens* are differentiated from ESBL producers by their capability to degrade carbapenems [3,29].

Schwaber et al. [8] has reported that there is no need for standardization in the USA for investigation of ESBL in non-*E. coli* and non-*Klebsiella* spp. due to the low prevalence of that phenotype (2.2%). In our study the prevalence was approximately 10 times higher, justifying the determination of ESBL in non-*E. coli* and non-*Klebsiella* spp. species in these hospitals in Curitiba.

Controversies exist regarding the clinical importance of reporting ESBL in chromosomal-inducible beta-lactamase-producing species that would correspond to most of the non-classic ESBLs found in our study. The third-generation cephalosporins are considered resistant to that group. Despite this, cefepime may be a therapeutic option for AmpC-producing microorganisms, while ESBL producers are resistant to fourth-generation cephalosporins [29]. In the case of *P. mirabilis,* detection is as important as in *E. coli* and *K. pneumoniae*, because it does not produce known AmpC chromosomal beta-lactamases [29]. In our study, the incidence of ESBL in *P.mirabilis* was similar to the incidence in *E. coli,* but higher numbers of isolates are needed to understand its contribution to the problem. Its detection is epidemiologically important, since the resistance genes are found in conjugative plasmids [24].

Evaluation of the efficiency of the substrate used in the screening and confirmatory tests for ESBL production showed better sensitivity for the antimicrobials ceftriaxone and cefotaxime. When a hierarchy of species is considered, *E. coli* and *Klebsiella* spp. sensitivities were higher than others. Good results were also found with cefpodoxime and aztreonam, while less satisfactory ones were obtained with ceftazidime,

especially for non-*E. coli* and non-*Klebsiella* species. These results mainly involve the type of enzyme present, which has different affinities for several substrates. In this case, the enzyme has a higher affinity for cefotaxime than for ceftazidime, which is a typical characteristic of the CTX-M beta-lactamases already found in other hospitals in Brazil [28,30]; it is considered the dominant type in South America [31].

The sensitivity analysis derived from the addition of clavulanic acid demonstrated the need to associate at least two different combinations of cephalosporins or cephalosporins combined with clavulanic acid in order to obtain a good result. This occurred mainly for non-*E. coli* and non-*Klebsiella* species. M'Zali et al. [32] also observed an increase in sensitivity for ESBL detection when more than one substrate was used.

Among the antimicrobials evaluated for minimum inhibitory concentration, the carbapenemics presented the best potency (MIC50), with ertapenem (0.12 µg/mL) being more potent than imipenem (0.5 µg/mL). Kholer et al. [33] indicated that this may be attributable to the high affinity of ertapenem for PBP 3. This value is similar to the affinity of PBP 2 for imipenem or ertapenem. Ertapenem has been recommended for the treatment of infections caused by ESBL-producing bacteria. This antibiotic can be administered in a single daily dose, which reduces cost and facilitates ambulatory treatment. It has also been reported that ertapenem does not present good activity against glucose non-fermenters and does not contribute to the selective pressure responsible for the increase in the prevalence of *Pseudomonas* spp. and *Acinetobacter* spp., which are resistant to carbapenemics [34-36].

The reduced sensitivity (33.8%) of ESBL isolates to ciprofloxacin confirms that cross-resistance among beta-lactamics and quinolones is quite common in enterobacteria. This point is reinforced when one considers that an overall sensitivity of 67.3% to ciprofloxacine was found among the bacterial isolates in our study. The reason for this association is not well understood, although it is described that patients with quinolone-resistant isolates frequently share prior intensive antibiotic use [37].

Cross-resistance to amikacine was a less common event. In this study, this aminoglycoside presented high potency and sensitivity, following the carbapenems. These results were in accordance with those of other authors [38], despite the fact that amikacine is an antibiotic with high toxicity and its use is quite restricted [39].

MIC50 and MIC90 were high for all the cephalosporins and aztreonam, as expected. The *in* vitro tests showed sensitivity for some isolates, underlining the need for specific tests for detection of ESBL enzymes to avoid reporting false sensitivity results in routine tests for beta-lactamic antibiotics. That need has been discussed by several authors as essential to guarantee therapy adapted to the patients and yet help in the establishment of adequate control measures [40].

We conclude that there is a necessity to look for ESBL production in non-*E. coli* and non-*Klebsiella* spp., at least in South America. Finally, we confirm that cefotaxime is the best antibiotic to date for the screening and confirmation of ESBL in classic ESBL producing as well as other species of *Enterobacteriae*.

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