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A preliminary study on the presence of extended-spectrum beta-lactamases (ESBL) in clinical isolates of *Escherichia coli* in Granada (Spain)

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Abstract - A preliminary study was conducted to detect extended spectrum beta-lactamases (ESBLs) and to describe ESBL types in clinical isolates of *Escherichia coli* from Granada (Spain). Sixty-two isolates were examined using the VITEK 2 system, disk diffusion method and Epsilon test. Fourteen randomly selected isolates were subjected to genetic analysis in order to detect the ESBL type (CTX-M-10, CTX-M-9 and SHV). ESBL isoelectric points were determined by isoelectric focusing using crude extracts from sonicated cells. Out of the 62 isolates with ESBLs detected by the VITEK 2 system, 61 (98.4%) were confirmed to contain ESBLs by disk diffusion and/or Epsilon test. The prevalent ESBL type in *E. coli* was CTX-M-9.

Key words: *Enterobacteriaceae*, beta-lactamases, antimicrobial agent, *Escherichia coli*.

INTRODUCTION

Extended spectrum beta-lactamases (ESBLs) are plasmid-mediated enzymes able to hydrolyse aztreonam and to extend the substrate specificity to cephalosporins *in vivo.* However, isolates harbouring ESBL enzymes remain susceptible to methoxy-beta-lactams (cefoxitin) and carbapenems (imipenem and meropenem). Moreover, in the absence of a permeability mechanism, ESBL-producing isolates are susceptible to beta-lactam-beta-lactamase inhibitor combinations (Livermore, 1995). ESBLs were first described in enterobacteria in 1983 (Knothe *et al.*, 1983) and were found in Andalusia (Spain) in 2001 (Daza *et al.*, 2001). ESBLs include numerous enzymes. To date, TEM-, SHV-, and OXA- derived beta-lactamases have been characterized. In addition, a heterogeneous group designated CTX-M type ESBLs, related to chromosomal class A beta-lactamases of *Kluyvera ascorbata,* are also considered as ESBL enzymes. ESBLs are largely found in *Escherichia coli* and *Klebsiella pneumoniae*,

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although they have also been reported in other species of *Enterobacteriaceae* and *Pseudomonas aeruginosa* (Bradford, 2001).

Automated systems used in bacteriology laboratories should be able to detect their presence. Most manufacturers claim to have provided this capability through the incorporation of "intelligent" systems that interpret results obtained *in vitro*. However, these systems appear to be prone to produce false positives. Thus, the presence of ESBLs is indicated by a very small increase in the minimal inhibitory concentration (MIC) of third-generation cephalosporins (VITEK 2 system, bioMérieux, Marcy l'Etoile, France) (Sanders *et al*., 1996), or by a very small reduction in the MIC in association with clavulanic acid (Wider system, Soria Melguizo, Spain) (Cantón *et al*., 2000). The high sensitivity of these systems evidently responds to the need to avoid therapeutic failures in severely ill patients. On the other hand, the predisposition for certain cephalosporins to be degraded *in vitro* makes precise knowledge of the distribution of ESBL types in a given geographic region an important issue.

Several methods have been proposed to characterize ESBL, including phenotypic, biochemical and molecular methodologies. Isoelectric point determination by isoelectric focusing can provide an initial approach to the identity of the enzyme. PCR technology has also been used to detect these enzymes and to identify specific point mutations after sequencing the PCR amplification product.

With this background, the present study aimed to determine the ESBL types among clinical isolates of *Escherichia coli*, in order to assist system manufacturers in the development of phenotypic methods that are more closely related to reality. The clonality of the isolates used was not identified.

MATERIAL AND METHODS

A study was conducted of 62 consecutive isolates of *E. coli* gathered from single patients at our hospital. They were identified as ESBL-producers by means of the VITEK 2 system. All were frozen at –40 ºC until their phenotypic study for the presence of ESBL, using the disk diffusion method and the Epsilon-test as reference tests. The presence of ESBL was considered to be confirmed if either test gave a positive result. Fourteen isolates were randomly selected for ESBL type analysis from among all those with phenotypic confirmation of the presence of ESBLs. The analysis was carried out at the Microbiology Service of University Hospital Ramón y Cajal in Madrid, Spain. Reference strains *Klebsiella pneumoniae* ATCC 700603 and *Escherichia coli* ATCC 25922 were used as controls.

Disk diffusion method. The methods proposed by Jarlier *et al*. (1988) were followed, using 30 µg disks of cefotaxime (CTX), ceftazidime (CAZ), cefpodoxime (CPD) or aztreonam (AZT) (BBL, Becton Dickinson, USA) at a distance of 30 mm from a disk of amoxicillin/clavulanic acid (AMC) (20/10 µg). Positive synergy and presence of ESBLs were defined by an increase of 5 mm or more in the inhibition halo of a cephalosporin or AZT. If no such increase was shown, the test was repeated using a distance of 20 mm, in order to increase its sensitivity (Bradford, 2001).

Epsilon test. Paper strips containing antibiotic (AB Biodisk, Solna, Sweden) were used for this test. The Etest ESBL strip carries two gradients: on the one end, ceftazidime (TZ) (32 to 0.5 µg/ml), and on the opposite end, ceftazidime (from 4 to 0.064 µg/ml) plus a fixed concentration of clavulanic acid (4 µg/ml) (TZL). In the case of cefotaxime, the Etest ESBL strip carries two gradients: on the one end, cefotaxime (CT) (16 to 0.25 µg/ml), and on the opposite end, cefotaxime (1 to 0.016 µg/ml) plus a fixed concentration of clavulanic acid (4 µg/ml). Positive synergy with clavulanic acid and presence of ESBLs were defined by a reduction in ceftazidime or cefotaxime MIC of three or more dilutions in the presence of clavulanic acid (Bradford, 2001). The assays were performed in duplicate.

Determination of beta-lactamases. The procedure of Canton *et al*. (2002) was used, with slight modifications in the temperature cycles. The isoelectric point of the enzyme was determined, and PCR of the gene of interest was then performed.

Isoelectric focusing (IEF). Bacteria exponentially growing at 37 ºC in Luria-Bertani medium were harvested, and cell-free lysates were prepared by sonication. IEF was performed by applying the crude extract to Phast gels (pH gradient, 3 to 9) in a Phast System apparatus (Amersham Pharmacia Biotech, Uppsala, Sweden). Beta-lactamases with known pIs (TEM-1, pI 5.4; TEM-4, pI 5.9; SHV-2, pI 7.6; CTX-M-10, pI 8.1 and SHV-5, pI 8.2) were focused in parallel as controls. Gels were stained with 500 mg of Nitrocefin (Oxoid, United Kingdom) per ml to identify betalactamases bands.

PCR amplification of ESBLs. Bacterial DNA was obtained by boiling a suspension of one or two fresh colonies in distilled water for 10 min. Ten microlitres of supernatant were added to a master mix containing PCR buffer (1x), $MgCl_2$ (2 mM), dNTPs (200 µM), primer (0.5 µM) and Taq polymerase (2 U). An Eppendorf thermal cycler was used for amplification. For CTX-M-10, the primers CTX-M-F8 (5'-CCG-CGC-TAC-ACT-TTG-TGG-C-3') and CTX-M-R3 (5'-TTA-CAA-ACC-GTT-GGT-GAC-G-3') were used, cycling conditions were 35 cycles of 94 ºC for 1 min, 59 ºC for 1 min and 72 ºC for 2 min, with a final period of extension at 72 ºC for 10 min. For CTX-M-9, the primers CTX-F (5'-GTG-ACA-AAG-AGA-GTG-CAA-CGG-3') and CTX-R (5'-ATG-ATT-CTC-GCC-GCT-GAA-GCC-3') were used, cycling conditions were 35 cycles of 94 ºC for 1 min, 62 ºC for 1 min and 72 ºC for 1 min, with a final period of extension at 72 ºC for 10 min. For SHV-types, the primers SHV-3FT (5'-GGG-TTA-TTC-TTA-TTT-GTC-GC –3') and SHV-5FT (5'-TTA-GCG-TTG-CCA-GTG-CTC –3') were used, cycling conditions were 35 cycles of 94 ºC for 1 min, 56 ºC for 1 min and 72 ºC for 1 min, with a final period of extension at 72 ºC for 10 min. The PCR products were separated in 0.8% agarose gel and visualised under UV light after staining with ethidium bromide. To avoid false positive amplifications, procedures recommended to prevent contamination were meticulously observed.

TABLE 1 – MIC_{50} and MIC_{90} (μg/ml) for ceftazidime and cefotaxime alone and in combination with clavulanic acid (CA), obtained with the Etest procedure, with range, mean and standard deviation of diameters (in mm) of halos obtained by the disk diffusion method for each antibiotic against the 62 isolates

Antibiotic	MIC_{50}	MIC_{90}	Range	X ± DS
Ceftazidime	0.75	>32	30 - 0	20.18 ± 7.81
Ceftazidime + CA	0.125	0.38		
Cefotaxime	>16	>16	29 - 8	16.09 ± 4.66
Cefotaxime + CA	0.064	0.19		
Cefpodoxime			30 - 0	10.76 ± 5.66
Aztreonam			30 - 0	20.71 ± 7.93

RESULTS AND DISCUSSION

Table 1 lists MIC_{50} and MIC_{90} values for CAZ and CTX when tested either alone or with clavulanic acid, and shows the range, mean and standard deviation of the diameters of haloes obtained. It also shows the range, mean and standard deviation of the diameters of the haloes of CPD and AZT with the disk diffusion method. The presence of ESBLs was confirmed by the disk diffusion method (30 mm distance) and the Epsilon-test in 54 (87%) of the 62 isolates with ESBLs detected by the VITEK 2 system. Neither method detected ESBLs in the remaining 8 isolates. When diffusion was repeated with the disk at 20 mm, synergy was observed in a further seven cases. Thus, the presence of ESBLs was confirmed in a total of 61 (98.4%) out of the 62 isolates identified by the VITEK 2 system. These results for VITEK 2 are better than those reported by Sanders et al. (2000) (84.2% agreement) and Leverstein-van Hall *et al.* (2002) (87.9% agreement) but similar to the findings of Livermore *et al.* (2002) (92% agreement).

Table 2 exhibits the results of ESBL type analysis. According to the PCR results they were distributed as follows: 9 CTX-M-9 type enzymes (64%) and 5 SHV-type (36%), in agreement with the phenotypic study. The CTX-M-9 enzymes showed an elevated MIC of CTX that was reduced in presence of clavulanic acid. The SHV enzymes additionally showed an elevated MIC of CAZ that was reduced in presence of clavulanic acid (Table 1). These results are in line with ESBL type studies in other regions of Spain (Hernández *et al*, 2003; Sabaté *et al*., 2002).

In conclusion, CTX-M-9 ESBLs predominate in our setting.

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TABLE 2 – Results of isoelectric focusing and PCR for the 14 isolates studied

Isolates	pI	PCR	VITEK 2	Inhibition Halo (with Clavulanic Acid)				MIC TZ	MIC TZL	MIC CT	MIC CTL	RATIO TZ/TL	RATIO CT/CTL
				CAZ	CTX	CPD	ATZ						
1	8.1 + 5.4	CTX-M9	+	32	10 (S)	10 (S)	26 (S)	1	0.38	>16	0.032	2.6	>500
16	8.2 + 5.4	SHV	+	12 (S)	21 (S)	20	14 (S)	>32	0.25	0.5	0.032	>128	15.6
27	8.1 + 5.4	CTX-M9	+	26	19 (S)	16 (S)	28	<0.50	<0.064	2	0.016	ND	125
42	8.1 + 5.4	CTX-M9	+	19 (S)	16 (S)	16 (S)	30	<0.50	0.125	12	0.047	<4	255
51	8.1	CTX-M9	+	28	20 (S)	14 (S)	27 (S)	0.75	0.064	>16	0.016	11.7	>1000
56	8.1 + 5.4	CTX-M9	+	30	20 (S)	16 (S)	30	0.75	0.064	>16	0.032	11.7	>500
101	8.2 + 5.4	SHV	+	0 (S)	14 (S)	0 (S)	0 (S)	>32	0.125	>16	0.023	>256	>695
112	8.1 + 5.4	CTX-M9	+	26	10 (S)	0 (S)	24 (S)	1	<0.064	>16	0.016	>15.63	>1000
121	8.2 + 5.4	SHV	+	10 (S)	20 (S)	14 (S)	10 (S)	>32	0.19	>16	0.023	>168	>695
124	8.1 + 5.4	CTX-M9	+	26	19 (S)	13 (S)	26 (S)	0.75	0.094	>16	0.016	7.9	>1000
135	8.1 + 5.4	CTX-M9	+	18 (S)	12 (S)	12 (S)	16 (S)	4	0.125	>16	0.047	32	>340
141	8.1 + 5.4	CTX-M9	+	26	14 (S)	12 (S)	26 (S)	<0.50	0.094	>16	0.094	<5.3	>170
150	8.2	SHV	+	14 (S)	22 (S)	20	16 (S)	12	0.094	1	0.064	127.7	15.6
162	8.2 + 5.4	SHV	+	8 (S)	20 (S)	14 (S)	8 (S)	>32	0.19	>16	0.094	>168	>170

+: ESBL-producers according to the Vitek 2 system; (S) synergy with clavulanic acid; CAZ: Ceftazidime; CTX: Cefotaxime; CPD: Cefpodoxime; ATZ: Aztreonam; TZ: Ceftazidime Etest; TZL: Ceftazidime/clavulanic acid Etest; CT: Cefotaxime Etest; CTL: Cefotaxime/clavulanic acid Etest.

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