



High presence of extended-spectrum β -lactamases and resistance to quinolones in clinical isolates of *Escherichia coli*

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Summary

A study was conducted to detect the presence of extended-spectrum β -lactamases (ESBLs) in 706 isolates of *Escherichia coli*, largely from outpatients (75.2%). The Clinical and Laboratory Standards Institute (formerly NCCLS)-recommended disk diffusion procedure was used to detect ESBL presence; the VITEK 2 system (bioMérieux, Marcy L'Etoile, France) was used to determine the susceptibility to antibiotics of clinical interest, and the ESBLs were characterized by biochemical study, determining the isoelectric point, and by molecular study with PCR. Clonal distribution was studied in eight hospital isolates. There were 115 ESBL-producing isolates (16.3%), with a predominance of CTX-M9 type (58.3%). We draw attention to the high resistance to quinolones (>70%) in CTX-M9 and SHV enzyme producing isolates and the lower aminoglycoside activity in the latter.

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Introduction

Extended spectrum β -lactamases (ESBLs) confer resistance to all penicillins, first-to-fourth generation cephalosporins and monobactams but

are not active against cefoxitin, carbapenems or β -lactamase inhibitors (Bradford, 2001). These enzymes are encoded by plasmid genes and are often located within transposons and integrons, facilitating their association with other transferable genetic determinants of resistance to cotrimoxazole, aminoglycosides, or tetracyclines, among others (Lautenbach et al., 2001a).

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TEM- and SHV-type ESBLs derive by point mutations from classic plasmid β -lactamases designated group 2b in the classification by Bush et al. (1995). Besides giving rise to ESBL phenotypes, these mutations are also responsible for changes in their isoelectric point (pI). CTX-M-type ESBLs are characterized by a better hydrolyzation of cefuroxime, cefotaxime and cefepime than of ceftazidime. All of the above ESBLs are characterized by a greater susceptibility to tazobactam compared with other inhibitors (Bradford, 2001). They are phylogenetically related to the chromosomal β -lactamase of *Kluyvera ascorbata* (Klu-1 and Klu-2) (Humeniuk et al., 2002). More than 60 SHV-type, 135 TEM-type (<http://www.lahey.org/studies/webt.asp>), and more than 40 CTX-M-type ESBLs have been described to date.

OXA-type β -lactamases, another group of ESBLs, belong to functional class 2d of the Bush classification and have mainly been reported in *Acinetobacter* spp. and *Pseudomonas aeruginosa*, initially in Turkey and France (Danel et al., 1999). It has not been possible to classify within the above groups some other ESBLs (BES-1, PER-1, PER-2, VEB-1, etc.) that are similar to chromosomal cephalosporinases of *Bacteroides* spp. (Rossolini et al., 1999).

ESBL-producing microorganisms in Spain were first described in 1988, although retrospective studies of bacteria isolated in Madrid between 1985 and 1987 identified susceptibility phenotypes compatible with ESBL production (Baquero et al., 1988). Later reports included studies of an outbreak at the Bellvitge Hospital in Barcelona from 1993 to 1995, when ESBL-producing isolates of *Klebsiella pneumoniae* represented 35% of all isolates of this species. Among these isolates was a predominant clone producing an ESBL with a pI of 7.6 (Peña et al., 1998).

ESBL types detected in Spain include TEM-4, TEM-10, TEM-12, TEM-24, TEM-27, TEM-54, SHV-2, SHV-5a, SHV-12, CTX-M1, CTX-M3, CTX-M7, CTX-M9, CTX-M10, CTX-M14, and CTX-M15 (Valverde et al., 2004). Reports of CTX-M-derived ESBLs in isolates of *Escherichia coli* have been especially frequent (Sabaté et al., 2000; Oliver et al., 2001).

Until recently, studies of ESBL-producing clinical isolates focused on the hospital setting and on *K. pneumoniae*, and there are considerably less data on ESBLs in *E. coli* in the outpatient setting (Mirelis et al., 2003). The present study was designed to determine the presence and type of ESBLs in clinical isolates of *E. coli* and their susceptibility to commonly used antibiotics and to investigate the clonal relationship among isolates of hospital origin.

Material and methods

Clinical isolates were studied at the Microbiology lab of our hospital during two periods, from March 2000 to September 2002 and from October to November 2002.

During the first study period, isolates were obtained in a randomized fashion. Every day, a colony with morphology compatible with enterobacteria, grown in McConkey agar medium (bioMérieux) and not previously identified was selected at random. Each enterobacteria was subsequently identified by means of biochemical tests (Murray et al., 2003) using the Api20E gallery (bioMérieux). Only enterobacteria identified as *E. coli* that were isolated from samples from different patients were selected.

During the second period, the isolates were obtained in a systematic and prospective manner from among all enterobacteria identified by our hospital laboratory in isolates from patients with a range of infections. This identification was performed using our routine laboratory procedure (WIDER System, Francisco Soria Melguizo S.A., Spain) (Cantón et al., 2000). Only enterobacteria identified as *E. coli* were selected for analysis.

All isolates were kept at -40°C until their analysis. Culture purity was tested in blood and McConkey agar and suspensions were prepared to a 0.5 McFarland turbidity standard. Suspensions were then inoculated onto Mueller-Hinton agar plates (bioMérieux) for phenotypic study of the presence of ESBLs using the Clinical and Laboratory Standards Institute (formerly NCCLS) disk diffusion method (NCCLS, 2004), and for study of their susceptibility to common antibiotics using the VITEK 2 automated system (bioMérieux) according to the manufacturer's instructions (Sorlózano et al., 2005). The VITEK 2 system includes an Advanced Expert System for interpreting the resistance phenotype from the reading of MICs obtained for each bacteria identified. Thus, when there is an inappropriate combination between resistance phenotype and species or the supposed mechanism implies the appearance of resistance to active antibiotics in vitro, the expert system changes the clinical category of the antibiotic from susceptible to non-susceptible.

Phenotypic confirmation of the presence of ESBLs in isolates was followed by biochemical and molecular study of the β -lactamases found. In the second study period, the clonal distribution in hospital isolates was also studied.

Reference strains *K. pneumoniae* ATCC 700603 and *E. coli* ATCC 25922 were used in all assays.

Determination of β -lactamases

The procedure of Sorlózano et al. (2004a) was used. In brief, in order to determine the pI of the enzyme, bacteria exponentially growing at 37 °C in Luria Bertani medium were harvested and cell-free lysates were prepared by sonication. Isoelectric focussing was performed by applying the crude extract to Phast gels (pH gradient, 3–9) in a Phast System apparatus (Amersham Pharmacia Biotech, Uppsala, Sweden). β -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 focussed in parallel as controls. Gels were stained with 500 mg of Nitrocefin (Oxoid, United Kingdom) per ml to identify β -lactamase bands.

Subsequently, Polymerase Chain Reaction (PCR) of the gene of interest was performed. For CTX-M-9, primers CTX-F (5'-GTG-ACA-AAG-AGA-GTG-CAACGG-3') and CTX-R (5'-ATG-ATT-CTC-GCC-GCT-GAAGCC-3') were used, and for SHV-types, 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.

Pulsed-field gel electrophoresis (PFGE)

PFGE was performed as described by Maslow et al. (1993). Organisms were lysed in situ, the chromosomal DNA was digested with *Xba*I, and slices of the plugs were loaded into the wells of a gel. DNA fragments were separated on 1.2% agarose gel in 0.5 × Tris-Borate-EDTA buffer at 14 °C by using a contour-clamped homogeneous electric field apparatus (CHEF Mapper, Bio-Rad, Hercules, CA). The electrophoresis conditions were 6 v/cm for 22 h with initial and final pulse times of 2 and 54 s, respectively. Gel was stained with ethidium bromide and photographed under ultraviolet light. Fragment patterns were interpreted as described by Tenover et al. (1995). Lambda Ladder PFG Marker (BioLabs, Beverly, MA) was used as molecular size standards (effective size range: 48.5 kb to 1018.5 kb).

Statistical analysis

The Fisher's exact test for $r \times s$ tables was used to compare the distribution of VITEK 2-determined MICs and clinical categories (susceptible or non-susceptible) between the two types of ESBL-producing isolates for each antibiotic assayed. The presence of a difference between the types in these two variables was the alternative hypothesis (H_1).

Results

During the first and second study periods, 349 and 357 isolates of *E. coli* were obtained, respectively. The isolates were from urine (89%), surgical wound (5%), ulcer or crust (4%) and other samples including sputum, sterile liquid, catheter or vaginal secretion (2%). A majority of samples were obtained from females (68.8%); 75.2% were outpatient samples and 24.8% were inpatient samples. The Clinical and Laboratory Standards Institute disk diffusion test identified 115 ESBL-producing isolates (16.3%). The isoelectric focusing assay identified 23 isolates containing enzymes with isoelectric point (pI) of 8.1, 44 isolates containing enzymes with pI of 8.1+5.4, 9 isolates containing enzymes with pI of 8.2, and 39 isolates containing enzymes with pI of 8.2+5.4. Subsequent PCR study confirmed that a pI of 8.1 corresponded to CTX-M9 (67 isolates) and a pI of 8.2 (48 isolates) to SHV. In *E. coli*, a pI of 5.4 corresponds to TEM-1.

Out of the 115 ESBL-producing isolates, 86.1% derived from urine samples (58.6% of CTX-M9), and 77.4% were outpatient samples (55% of CTX-M9). Among the inpatient samples, 69.2% were CTX-M9; 41.7% of all isolates studied were CTX-M9-producing outpatient urine samples, whereas 31.3% were SHV-producing outpatient urine samples.

Table 1 lists the results obtained using the disk diffusion method, including the mean, standard deviation, and range of the zone diameters of bacterial growth inhibition in the isolates with the two types of ESBL identified. Table 2 shows the MIC₅₀ and MIC₉₀ values and percentage of resistance to each antibiotic for each type of ESBL found, before the correction made by the AES of VITEK 2.

Tables 3 and 4 show comparisons of the VITEK 2 measured MIC activity of different antibiotics between the CTX-M9- and SHV-producing isolates. According to the MIC values and clinical categories found (susceptible or non-susceptible), ceftazidime, gentamicin and tobramycin had a significantly lower activity in SHV-producing than in CTX-M9-producing isolates. According to the MIC values, piperacillin, cefuroxime and cefepime were less active in CTX-M9-producing isolates, and amikacin was less active in SHV-producing isolates. The remaining antibiotic assays showed no significant differences between the ESBL types.

The clonal distribution among eight clinical isolates was studied by pulsed field gel electrophoresis and no clonal relationship was observed among them, with a different PFGE pattern obtained in each one (Fig. 1). These eight isolates alone were selected because they were the only isolates that fulfilled two indispensable

Table 1. Mean, standard deviation and range of zone diameter (in mm) obtained by the disk diffusion method (NCCLS, 2004) in 115 ESBL-producing *E. coli* isolates

	CAZ	CD02	CTX	CD03	CPD	CD01	ESBL type
Mean	24.5	30	15.5	29.7	6.2	20.1	CTX-M9
Standard deviation	3.87	2.9	3.34	2.97	0.99	2.87	
Range	12–32	23–36	8–22	24–36	6–12	13–26	
Mean	19.8	28.8	16.1	29.9	7.1	21.5	SHV
Standard deviation	6.9	3.2	4.4	3.1	2.3	3.4	
Range	6–27	20–34	6–26	23–36	6–14	16–30	

CAZ: Ceftazidime (30 µg), CD02: Ceftazidime plus clavulanic acid (30/10 µg), CTX: Cefotaxime (30 µg), CD03: Cefotaxime plus clavulanic acid (30/10 µg), CPD: Cefpodoxime (10 µg), CD01: Cefpodoxime plus clavulanic acid (10/1 µg).

Table 2. MIC₅₀ and MIC₉₀ (in µg/ml) and percentage of resistance to studied antibiotics measured by VITEK 2 system before correction by Advanced Expert System

Antibiotics assayed	CTX-M9 (n = 67)			SHV (n = 48)			p
	MIC ₅₀	MIC ₉₀	% non-susceptible isolates	MIC ₅₀	MIC ₉₀	% non-susceptible isolates	
Ampicillin	≥32	≥32	100%	≥32	≥32	100%	1.000
Amoxicillin-clavulanic	4/2	16/8	11.9%	4/2	16/8	20.8%	0.298
Piperacillin	≥128	≥128	100%	64	≥128	95.8%	0.172
Piperacillin-tazobactam	≤4/4	≤4/4	3%	≤4/4	8/4	2.1%	1.000
Cefalotin	≥64	≥64	100%	≥64	≥64	100%	1.000
Cefuroxime	≥64	≥64	100%	32	≥64	93.8%	0.070
Cefoxitin	≤4	16	20.9%	≤4	16	10.4%	0.203
Cefotaxime	16	≥64	56.7%	8	≥64	29.2%	0.004
Cefpodoxime	≥8	≥8	100%	≥8	≥8	97.9%	0.417
Ceftazidime	≤1	2	4.5%	16	≥64	77.1%	<0.001
Cefepime	2	2	7.5%	≤1	8	2.1%	0.245
Meropenem	≤0.25	≤0.25	0	≤0.25	≤0.25	0	1.000
Amikacin	≤2	≤2	0	≤2	4	0	1.000
Gentamicin	≤1	4	9%	≤1	≥16	27.1%	0.012
Tobramycin	≤1	2	7.5%	≤1	≥16	27.1%	0.008
Ciprofloxacin	≥4	≥4	73.1%	≥4	≥4	72.9%	1.000
Norfloxacin	≥16	≥16	70.1%	≥16	≥16	70.8%	1.000
Ofloxacin	≥8	≥8	74.6%	≥8	≥8	72.9%	1.000
Nitrofurantoin	32	64	31.3%	≤16	64	25%	0.533
Cotrimoxazole	≥16/304	≥16/304	59.7%	≤1/19	≥16/304	45.8%	0.184

conditions for this analysis: they were obtained during the second period, when the collection was systematic and there was consequently no risk of missing an isolate; and they were all from inpatient samples and therefore had the same origin (although they did not all come from the same hospital department). Table 5 shows the ESBL type and the hospital departments from which these isolates derived.

Discussion

This study disclosed an elevated frequency of ESBL presence in *E. coli*, as previously reported in Spain by our group and other authors (Hernández et al., 2003; Mirelis et al., 2003; Sorlózano et al., 2004b).

Two types of ESBL were detected in our series, types CTX-M9 and SHV, with a predominance

Table 3. Relationship between CTX-M9 and SHV-producing isolates and distribution of MICs (in µg/ml) of antibiotics studied with the VITEK 2 system

Antibiotic	ESBL	≤0.25	0.5	1	2	4	8	16	32	64	≥128	p
Ampicillin	CTX-M9	0	0	0	0	0	0	0	100% ¹			1.000
	SHV	0	0	0	0	0	0	0	100% ¹			
Piperacillin	CTX-M9	0	0	0	0	0	0	0	16.4%	13.4%	70.1%	0.035
	SHV	0	0	0	0	0	0	4.2%	31.3%	16.7%	47.9%	
Cefalotin	CTX-M9	0	0	0	0	0	0	0	0	100% ²		1.000
	SHV	0	0	0	0	0	0	0	0	100% ²		
Cefuroxime	CTX-M9	0	0	0	0	0	0	1.5%	0	98.5% ²		<0.001
	SHV	0	0	0	0	2.1%	4.2%	29.2%	18.8%	45.8% ²		
Cefoxitin	CTX-M9	58.2% ³						20.9%	13.4%	6%	1.5% ²	0.565
	SHV	70.8% ³						18.8%	8.3%	2.1%	0	
Cefotaxime	CTX-M9	0			3%	6%	34.3%	14.9%	4.5%	37.3% ²		<0.001
	SHV	6.3% ⁴			8.3%	31.3%	25%	4.2%	6.3%	18.8% ²		
Cefpodoxime	CTX-M9	0	0	0	0	3%	97% ⁵					0.751
	SHV	2.1%	0	0	0	2.1%	95.8% ⁵					
Ceftazidime	CTX-M9	86.6% ⁴			6%	3%	0	4.5%	0	0		<0.001
	SHV	14.6% ⁴			2.1%	4.2%	2.1%	54.2%	2.1%	20.8% ²		
Cefepime	CTX-M9	38.8% ⁴			53.7%	0	0	1.5%	4.5%	1.5% ²		<0.001
	SHV	70.8% ⁴			16.7%	2.1%	8.3%	0	0	2.1% ²		
Meropenem	CTX-M9	100%	0	0	0	0	0	0	0	0	0	1.000
	SHV	100%	0	0	0	0	0	0	0	0	0	
Amikacin	CTX-M9	100% ⁶				0	0	0	0	0		0.011
	SHV	89.6% ⁶				2.1%	6.3%	2.1%	0			
Gentamicin	CTX-M9	88.1% ⁴				0	3%	0	9% ⁷			0.023
	SHV	68.8% ⁴				0	4.2%	6.3%	20.8% ⁷			
Tobramycin	CTX-M9	88.1% ⁴				4.5%	0	4.5%	3% ⁷			0.024
	SHV	70.8% ⁴				2.1%	0	10.4%	16.7% ⁷			
Ciprofloxacin	CTX-M9	16.4%	1.5%	9%	4.5%	68.7% ⁸						0.364
	SHV	25%	0	2.1%	2.1%	70.8% ⁸						
Norfloxacin	CTX-M9	13.4% ⁹		4.5%	10.4%	1.5%	7.5%	62.7% ⁷				0.676
	SHV	19.8% ⁹		2.1%	8.3%	0	2.1%	68.8% ⁷				
Ofloxacin	CTX-M9	11.9%	0	3%	10.4%	4.5%	70.1% ⁵					0.757
	SHV	18.8%	0	2.1%	6.3%	2.1%	70.8% ⁵					

¹MIC ≥ 32 µg/ml; ²MIC ≥ 64 µg/ml; ³MIC ≤ 4 µg/ml; ⁴MIC ≤ 1 µg/ml; ⁵MIC ≥ 8 µg/ml; ⁶MIC ≤ 2 µg/ml; ⁷MIC ≥ 16 µg/ml; ⁸MIC ≥ 4 µg/ml; ⁹MIC ≤ 0.5 µg/ml.

(58.3%) of the former. In Spain, CTX-M9 has always been the most frequently identified ESBL type, reported in 70% (Sabaté et al., 2002) and 64% (Rodríguez-Baño et al., 2004) of *E. coli* species, which were gathered from outpatient isolates in the latter study. CTX-M type enzymes have also been associated with focal outbreaks in Italy (Pagani et al., 2003) (types 1, 2 and 15), Eastern Europe (Edelstein et al., 2003) (types 1 and 2) and China (Munday et al., 2004) (types 3 and 14), among others.

Certain difficulties in the detection of CTX-M-type ESBLs have been reported, especially when ceftazidime is used as sole marker (Sabaté et al., 2000). Results obtained with the disk diffusion method (Table 1) show the lower capacity of ceftazidime to detect CTX-M9-type ESBLs, given that 74.6% of the isolates that produce them

proved to be sensitive, exceeding the NCCLS zone diameter breakpoint value (mean 24.5 mm, range 12–32 mm).

The mean percentages of resistance to fluoroquinolones measured by the VITEK 2 system in the 115 isolates were 70.4% to norfloxacin, 73% to ciprofloxacin and 73.9% to ofloxacin, with no significant differences between the two types of enzyme. However, in a previous study of non-ESBL-producing isolates at the same laboratory only 22% of isolates were found to be resistant to ciprofloxacin (Daza et al., 2001). The association between ESBL production and resistance to these antibiotics was already described (Lautenbach et al., 2001b; Valverde et al., 2004) and may be in part due to joint transference of the two mechanisms via plasmids (Martinez-Martinez et al., 1998). The elevated resistance observed

Table 4. Relationship between CTX-M9- and SHV-producing isolates and the distribution of MICs (in µg/ml) of amoxicillin-clavulanic acid, piperacillin-tazobactam, nitrofurantoin and cotrimoxazole, obtained with the VITEK 2 system

	MIC	CTX-M9	SHV	<i>p</i>
Amoxicillin-clavulanic acid	≤2/1	3%	14.6%	0.076
	4/2	64.2%	52.1%	
	8/4	20.9%	12.5%	
	16/8	10.4%	18.8%	
	≥32/16	1.5%	2.1%	
Piperacillin-tazobactam	≤4/4	92.5%	85.4%	0.378
	8/4	4.5%	12.5%	
	32/4	1.5%	0	
	≥64/4	1.5%	2.1%	
Nitrofurantoin	≤16	38.8%	50%	0.846
	32	29.9%	25%	
	64	22.4%	16.7%	
	128	7.5%	6.3%	
	≥256	1.5%	2.1%	
Cotrimoxazole	≤1/19	40.3%	52.1%	0.128
	2/38	0	2.1%	
	4/76	0	0	
	≥16/304	59.7%	45.8%	

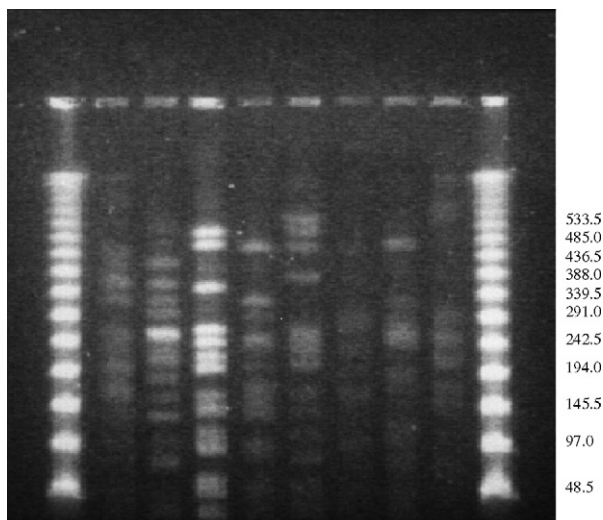


Figure 1. Pulsed field gel electrophoresis. Lanes 1 and 10: Molecular weight standard (effective size range: 48.5–1018.5 kb). Lanes 2 to 9: Isolates 218, 235, 261, 246, 248, 255, 258 and 260, respectively.

may result from a possible increase in the use of fluoroquinolones in infections by ESBL-producing microorganisms that fail to respond to β -lactam treatment.

Table 5. ESBL type and origin of isolates in clonal distribution study

Isolate n°	ESBL type	Origin
218	SHV	ICU
235	CTX-M9	Surgery
246	CTX-M9	Surgery
248	CTX-M9	Oncology
255	SHV	Traumatology
258	CTX-M9	Pediatrics
260	CTX-M9	Internal Medicine
261	SHV	Surgery

The three aminoglycosides assayed using VITEK 2 (amikacin, gentamicin and tobramycin) were active against producers of both types of ESBL, although MIC values were slightly higher in the SHV-producers. Hadziyannis et al. (2000) found no association between presence of ESBLs and resistance to gentamicin. However, Fernández-Rodríguez et al. (1992) found that 84% of a series of SHV- and TEM-type ESBLs were resistant to gentamicin, largely due to the presence of aminoglycoside 3-V-acetyltransferase, aminoglycoside 3'-phosphotransferase, and aminoglycoside 3'-phosphotransferase type-I enzymes. Nicolas et al. (1989) demonstrated the presence of genes that encode for SHV-3 and aminoglycoside 6'-N acetyltransferase enzymes in the same plasmid.

Finally, PFGE study of the eight inpatient isolates of *E. coli* showed no clonal distribution, even among isolates producing the same ESBL. The dissemination of an ESBL may possibly be produced more by exchange of mobile genetic elements that carry it than by clonal dissemination of a given producer strain (Wiener et al., 1999).

In conclusion, an elevated presence of CTX-M9-type ESBLs and a high resistance to quinolones were observed in isolates of *E. coli* in our setting.

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