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Jacobs Journal of Microbiology and Pathology

Research Article

Molecular Characterization of *Escherichia coli* and *Klebsiella pneumoniae* Producing Extended-Spectrum Beta-Lactamases (ESBL's) Isolated in Two Hospitals in Murcia (Spain)

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Received: 11-20-2014

Accepted: 01-20-2015

Published: 03-24-2015

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Abstract

Objectives: We collected prospectively and stored at -20ºC all ESBL-producing *Escherichia coli* and *Klebsiella pneumoniae* strains isolated in two hospitals of Murcia during one month in the year 2011. Once we had all the strains we analyzed them together.

Methods: The characterization of the different enzymes was performed by PCR and sequencing. Clinical and epidemiological data of patients and also antimicrobial susceptibility of the strains were collected.

Results: In total, 83 strains were ESBL producers (72 isolates of *E. coli* and 11 of *K. pneumoniae*). The prevalence of ESBL-producing *E. coli* and *K. pneumoniae* strains were 9.3% and 10%, respectively. The most common enzyme was CTX-M (81.9%).

Conclusion: In one hospital, all the strains ESBL-producing *K. pneumoniae* were clonally related whereas the strains ESBL-producing *E. coli* did not show high clonal relationship. The existence of one possible single clone of *K. pneumoniae* and, furthermore, of small outbreaks of *E. coli* highlights the importance of implementing genetic studies to improve nosocomial infection control.

Keywords: Molecular characterization; Extended spectrum beta lactamases (ESBL´s); Enterobacteria

Introduction

The increase in clinical isolation of extended-spectrum β-lactamases (ESBLs) in *Enterobacteriaceae* entails an important worldwide problem because they are resistant to a large number of beta-lactams, but also because they are frequently found in genetic mobile elements in coexistence with genes that confer resistance to other important groups of antimicrobial drugs, thus leading to the restriction in therapeutic options [1].

At the present time the most commonly detected enzyme among ESBLs is CTX-M [2]. The main difference of CTX-M with those previously described [3-5] is that it appears frequently in strains of *Escherichia coli* causing, mainly, community-acquired infections and showing greater clonal diversity. Until recently, few outbreaks caused by a single ESBL-producing *E. coli* strain had been published. However, a clonal group of multiresistant *E .coli* producing CTX-M-15, *E. coli* O25:H4-ST131, can be contributing to the spread of ESBL-mediated resistance [6-7].

In Spain, the published studies describing the characterization of ESBLs are not updated [8-9], and specially, data from the region of Murcia are scarce and not representative. The aims of the present study were: (1) to determine the prevalence of ESBL-producing *E. coli* and *K. pneumoniae* isolated in two hospitals from Murcia, (2) to describe both epidemiological and clinical characteristics of the patients, (3) to study susceptibility to other antimicrobial agents and compare it to non-ESBL-producers, (4) to describe the molecular characterization of the enzymes and (5) to study the clonal relationship among the different isolated strains.

Material and Methods

Bacterial strains: All ESBL-producing *E. coli* and *K. pneumoniae* strains isolated during one month in 2011 from clinical samples in the Virgen de la Arrixaca University Hospital (HUVA, reference hospital) and José María Morales Meseguer University Hospital (HMM, tertiary hospital), Murcia, Spain, were included in our study. ESBL-producing *E. coli* and *K. pneumoniae* isolated from different samples but of the same patient were excluded when the antimicrobial susceptibility testing did not show phenotypic differences.

Demographic and clinical characteristics: We collected data on demographic and clinical characteristics of all the patients included in the study. The "acquisition of infection" was classified as "community-acquired or nosocomial-acquired infection" as defined by the Centers for Disease Control and Prevention (CDC) [10]. The criteria to include "healthcare associated infections" (HAI) were based on someone else's definition [11].

Antimicrobial susceptibility testing: All isolates were tested with the routine automated methods used in each of the centers, according to the Clinical and Laboratory Standards Institute (CLSI) breakpoints and criteria [12]. Both hospitals, HUVA and HMM, used VITEK2® (Biomerieux) and MicroScan® (Siemens), respectively. ESBL production was confirmed by double-disk synergy test according to the CLSI guidelines.

Molecular characterization of ESBL: Sixty-one of eighty-three ESBL producers were studied. The presence of bla_{TEM}, bla_{SHV} y bla_{CTX-M} genes was detected by PCR with specific primers (Table 1) [13]. Amplicons were purified with an enzymatic method (ExoSAP-IT®, USB) and were then sequenced. Sequencing was performed in an external laboratory equipped with an ABIPrism 337-DNA sequencer (Applied Biosystem). Later, the sequences were analyzed with the Chromas application and the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequences were compared with other sequences using the public database GenBank NCBI (National Center for Biotechnology Information).

Molecular typing: Clonal relationships were determined by REP-PCR (*repetitive extragenic palindromic-PCR*) and PFGE (*pulsed-field gel electrophoresis*). All strains of hospitalized patients were studied by REP-PCR using REP-1 and REP-2 oligonucleotides (Table 1) [14].

Table 1. Specific primers used for the characterization of ESBL enzymes (blaSHV, blaCTX-M, blaTEM) and for molecular typing by REP-PCR.

Gene		Primers (5´→ 3´)	Size (bp)
bla_{SHV}	bla_{SHV}F	ATGCGTTATATTCGCCTGTG	850
	bla_{SHV}R	TTAGCGTTGCCAGTGCTCT	
bla_{CTM}	bla_{CTX-M}F	SCSATGTGCAGYACCAGTAA	550
	bla_{CTX-M}R	CCGCRATATGRTTGGTGGTG	
bla_{TEM}	bla_{TEM}F	ATGAGTATTCAACATTTCCGTG	850
	bla_{TEM}R	TTACCAATGCTTAATCAGTGAG	
REP-1		IIIGCGCCGICATCAGGC	
REP-2		ACGTCTTATCAGGCCTAC	

Additionally, strains of ESBL-producing *E. coli* isolated in HMM were typed by PFGE using a restriction endonuclease digestion (*SpeI*), following the procedure described by CDC [15] in a CHEF-DR® II system (Bio-Rad). DNA band patterns were performed following criteria established by Tenover [16].

Statistic analysis: Data were analyzed using statistic program SPSS v15.0. Differences in proportions among categorical data were calculated by the chi-square test. A *p* value less than 0.05 was considered statistically significant.

Results

Global data and characteristics of patients.

A total of 774 strains of *E. coli* were isolated over the reference month in two hospitals (436 in HUVA and 338 in HMM). Total prevalence of strains producing ESBL enzymes was 9.3% (72/774). The prevalence was 8.5% (37 strains) in HUVA and 10.3% (35 strains) in HMM. In the same period, 111 strains of *K. pneumoniae* were isolated: 67 in HUVA and 44 in HMM. Of these, 11 ESBL-producing strains (9.9%) were detected: 8 strains (12%) in HUVA and 3 (7%) in HMM.

The demographic and epidemiological characteristics of the patients with ESBL-producing *E. coli* and *K. pneumoniae* isolates are described in Table 2. The average age of patients was 60 years old and more than half were women (60%). Most ESBL-producing strains were recovered from urine samples (85%). The other 15% were wounds and abscesses, abdominal samples and blood. Hence, ESBL producers were found mainly in urinary tract infections (85%), followed by skin and soft tissue infections (8%). According to the acquisition, 42 cases (50.6%) were HAIs, 22 (26.5%) cases were community-acquired, and 19 (23%) were nosocomial infections. Differences were found in the type of infection acquisition depending on which bacteria caused the infection. In total, 30.6% (22/72) of ESBL-producing *E. coli* infections were acquired in the community, 18% (13/72) in the hospital and 51% (37/72) were HAI. None of the ESBL-

producing *K. pneumoniae* infections had their origin in the community. Instead, 54.5% (6/11) of the cases were nosocomial and 45.5% (5/11) were health-care associated.

Table 2. Demographic, epidemiologic and clinical data obtained from the patients with infection with ESBL- producing *E. coli* or *K. pneumoniae.*

	HMM N= 38	HUVA N=45	Total N= 83	*p*
Age (mean±DE)	67± 21.52	54± 28.71	60± 26.24	0,03
Sex				
- Men	17 (44.7%)	16 (35.5%)	33 (40%)	0,4
- Women	21 (55.3%)	29 (64.4%)	50 (60%)	
Outpatient	24 (63%)	25 (55%)	49 (59%)	0,4
Inpatient	14 (37%)	20 (45%)	34 (41%)	
Infection acquisition				
- Community	12 (31.5%)	10 (22%)	22 (26.5%)	0,04
- Healthcare associated	22 (58%)	20 (45%)	42 (50.6%)	
- Nosocomial	4 (10%)	15 (33%)	19 (22.9%)	
Origin of infection				0,5
- Medical ward	24 (63%)	27 (60%)	51 (61.4%)	
- Surgical ward	1 (3%)	4 (9%)	5 (6%)	
- Primary care	13 (34%)	14 (31%)	27 (32.5%)	
Comorbidities (Yes)[1]	13/14 (93%)	20/20 (100%)	33/34 (97%)	
Previous atb use[1]	8/14 (57%)	12/20 (60%)	20/34 (59%)	
Infection site				
- Urinary tract	35 (92%)	35 (78%)	70(85%)	0,16
- Abdominal	-	5 (11%)	5 (6%)	
- Skin and soft tissues	3 (8%)	4 (9%)	7 (8%)	
- Primaria Bacteraemia	-	1 (2%)	1 (1%)	
Outcome				
- Discharge/Recovery	35 (92%)	40 (89%)	75 (90%)	0,6
- Exitus	3 (8%)	5 (11%)	8 (10%)	

[1]Only hospitalized patients.

No statistically significant differences were found in the characteristics of patients between hospitals, except in the age and type of infection acquisition. Patients of HUVA were younger than patients of HMM (54 years old *vs*. 67 years old respectively; *p*= 0.03), probably due to the fact that HUVA attends children whether HMM do not. The nosocomial acquisition also was more frequent in HUVA (33% *vs*. 10% respectively *p*= 0.04).

Clinical data was only obtained from hospitalized patients (n=34). Of these, almost all (97%) had underlying disease and 60% of them had received antimicrobial therapy in the previous 3 months. During admission 8 patients died.

Susceptibility to antimicrobial agents in ESBL and non-ESBL producers.

Susceptibility results of ESBL- and non-ESBL-producing strains are shown in Table 3.

Table 3. Antimicrobial resistance in ESBL-producing strains *vs.* non-ESBL-producing strains isolated in the two hospitals.

Antibiotics		*E. coli* (773)		*K. pneumoniae* (111)	
		ESBL Nr resistant/Nr total strains (%)	Non ESBL Nr resistant/Nr total strains (%)	ESBL Nr resistant/Nr total strains (%)	Non ESBL Nr resistant/Nr total strains (%)
Amoxicillin-clavulanate	HUVA	19/37 (51,4)	85/398 (21,4)	8/8 (100)	4/59 (6,7)
	HMM	12/35 (34,3)	64/303 (21)	3/3 (100)	6/41 (15)
	Total	31/72 (43)	149/701 (21)	11/11 (100)	10/100 (10)
Piperacillin-tazobactam	HUVA	2/24 (8)	4/326 (1)	0	2/56 (4)
	HMM	4/35 (11)	13/303 (4,3)	2/3 (66,7)	3/41 (7)
	Total	6/59 (10)	17/629 (2,7)	2/3 (66,7)	5/97 (5)
Imipenem	HUVA	0	0	0	0
	HMM	0	0	0	1/41 (2,4)
	Total	0	0	0	1/100 (1)
Nalidixic acid	HUVA	32/37 (86)	176/397 (44)	6/8 (75)	6/59 (10)
	HMM	29/32(90)	125/261 (48)	3/3 (100)	41/41 (100)
	Total	61/69 (88)	301/658 (46)	9/11 (82)	47/100 (47)
Ciprofloxacin	HUVA	32/37 (86)	165/398 (41)	6/8 (75)	3/59 (6)
	HMM	26/35 (74)	107/303 (35)	3/3 (100)	4/41 (10)
	Total	58/72 (80,6)	272/701 (38,8)	9/11 (82)	7/100 (7)
Tobramycin	HUVA	10/37 (27)	37/398 (9)	4/8 (50)	5/59 (8,5)
	HMM	NT	0	NT	NT
	Total	10/37 (27)	37/400 (9,3)	4/8 (50)	5/59 (8,5)
Tigecicline	HUVA	0	0	0	0
	HMM	0	0	0	0
	Total	0	0	0	0
Cotrimoxazole	HUVA	27/31 (87)	133/333 (40)	3/8 (37)	9/42 (21)
	HMM	22/35 (62)	99/303 (33)	2/3 (66)	5/41 (12)
	Total	49/66 (74)	232/636 (36,5)	5/11 (45,5)	14/83 (17)
Gentamicin	HUVA	7/37 (19)	39/398 (10)	4/8 (50)	5/59 (8,5)
	HMM	5/35 (14)	25/303 (8)	1/3 (33,3)	0
	Total	12/72 (17)	64/701 (9)	5/11 (45,5)	5/100 (5)
Amikacin	HUVA	2/35 (6)	0	0	0
	HMM	0	0	0	0
	Total	2/40 (5)	0	0	0
Fosfomycin	HUVA	4/31 (13)	11/335 (3)	5/8 (62)	13/42 (31)
	HMM	1/32 (3)	11/261 (4)	1/3 (33)	8/35 (23)
	Total	5/63 (8)	22/596 (3,7)	6/11 (54,5)	21/77 (27)

NT: No tested

Overall resistance rates in isolates from HUVA were higher than HMM ones, although the differences were not statistically significant, except for fosfomycin (23.1% HUVA *vs*. 5.7% HMM; *p*= 0.03). When comparing non-ESBL and ESBL producers, we found substantial differences in the percentages of resistance, with the higher rates being found in those producing ESBL. Furthermore, the differences were statistically significant in nalidixic acid, ciprofloxacin and cotrimoxazole. In the case of ESBL-producing *E. coli*, 88% of the strains were resistant to nalidixic acid, 81% to ciprofloxacin and 74.2% to cotrimoxazole in contrast with 46%, 39% and 36.5% of resistance, respectively, in non-ESBL producing strains ($p < 0,001$ for all). For ESBL-producing K. *pneumoniae*, 82% showed resistance to nalidixic acid and ciprofloxacin, and 45.5% to cotrimoxazole, while for non-ESBL producing strains the resistances were of 47%, 7% and 17%, respectively ($p < 0,001$). All ESBL-producing K. *pneunoniae*

strains and 43% of ESBL-producing *E. coli* were resistant to amoxicillin-clavulanate. The rates of resistance to piperacillin-tazobactam were 10.4% and 66% in ESBL-producing *E. coli* and *K. pneumoniae*, respectively. The antimicrobials that showed greater activity against ESBL producers were tigecycline, imipenem and amikacin: all strains were susceptible to the three drugs, except one *K. pneumoniae* strain that was resistant to imipenem and two *E. coli* strains that were not susceptible to amikacin.

Molecular characterization of ESBLs.

Molecular characterization of ESBLs was performed in the 61 strains of hospitalized patients (Table 4). The ESBL type most frequently detected was CTX-M (50 strains; 81.9%), followed by SHV (8 strains; 13.1%). One strain of ESBL-producing *K. pneumoniae* carried an enzyme from the TEM group.

Table 4. ESBL types produced by the *E. coli* and *K. pneumoniae* studied strains (n=61).

BLEE	*E. coli* (%)	*K. pneumoniae* (%)	TOTAL (%)
CTX-M	42/52 (80.7)	8/9 (88.8)	50/61 (81.9)
-CTX-M1	8 (15.3)	6 (66.6)	14/61 (22.9
-CTX-M14	28 (53.8)	0 (0)	28/61 (45.9)
-CTX-M15	5 (9.6)	1(11.1)	6/61 (9.8)
-Others	1 (1.9)	1 (11.1)	2/61 (3.3)
SHV	7/52 (13.4)	0/9 (0)	7/61 (11.4)
-SHV- 12	6 (11.5)	0 (0)	6/61 (9.8)
-SHV-50	1 (1.9)	0 (0)	1/61 (1.6)
TEM	0/52 (0)	1/9 (11.1%)	1/61 (1.6)
-TEM-139	0	1 (11.1)	1/61 (1.6)

The characterization was not possible to perform in three strains. Within the CTX-M group, the most commonly detected enzyme type was CTX-M-14, obtained in 28 strains (45.9%), followed by CTX-M-1 and CTX-M-15 which represented 22.9% and 9.8%, respectively. Most of the ESBLs belonging to the SHV group were SHV-12 (9.8%), and only one strain of *E. coli* was found to carry SHV-50. The only ESBL of the TEM type was TEM-139, derived from one strain of *K. pneumoniae*. We detected more than one type of beta-lactamase in 21 strains, presenting all of them a CTX-M ESBL and a non-ESBL TEM type. No differences were found in relation to the enzyme types and the two studied species between the two hospitals. Nor nosocomial or community origin of infection influenced the type of ESBL (Table 5).

	ESBL		
	CTX-M	OTHER	*p*
Hospitals			
- HUVA	35/38 (92.1)	4/38 (10.5)	0.6
- HMM	15/20 (75)	4/20 (20)	
Microorganism			
- *E. coli*	42/49 (85.7)	7/49 (14.2)	1.0
- *K. pneumoniae*	8/9 (88.8)	1/9 (11.1)	
Infection acquisition			
- Community	9/58 (15.5)	5/58 (8.6)	0.05
- Nosocomial	14/58 (24.1)	1/58 (1.72)	
- Healthcare associated	27/58 (46.5)	3/58 (3.44)	

Table 5. ESBL types according to hospital isolation, carrying microorganism and from acquisition.

Clonal study of the ESBL-producing *E. coli* and *K. pneumoniae* strains.

The clonal relationship was studied in all strains of hospitalized patients using REP-PCR. Additionally, eight strains of hospitalized patients of ESBL-producing *E. coli* isolated in HMM were typed by PFGE. Most of *E. coli* strains did not show clonal relationship by REP-PCR (Figure 1), except 4 strains (2 isolated in HUVA and 2 in HMM).

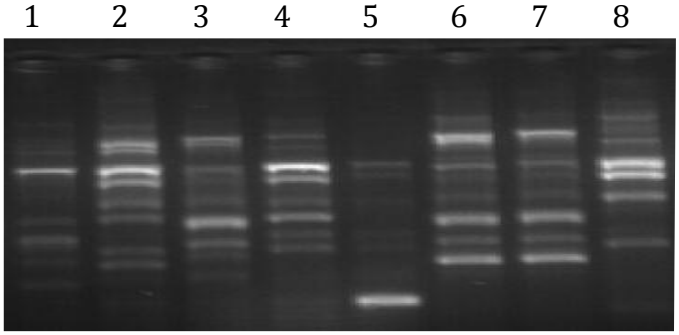

Figure 1. Detected patterns by REP-PCR of the ESBL producing *E. coli* isolated of HMM. Streets 1-8, strains of *E. coli*.

Nevertheless, 5 strains of *K. pneumoniae* isolated in HUVA presented the same bands. Four out of the five strains were from patients admitted in the ICU and the fifth patient was admitted to neonatal ICU. The 8 strains of *E coli* obtained from inpatients of HMM were analyzed also using PFGE, but there was no clonal relationship among them (Figure 2).

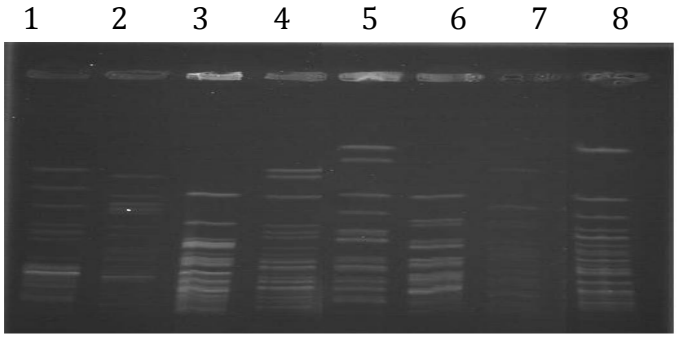

Figure 2. Pulsed-field gel electrophoresis patterns of ESBL producing *E. coli* strains isolated in HMM.

Discussion

The emergence of ESBL-producing in *Enterobacteriaceae* has become a great worldwide problem of public health and, in the last years, their incidence has increased. In our study, we obtained a prevalence of ESBL-producing *E. coli* and *K. pneumoniae* of 9.3% and 9.9%, respectively, in two of the main hospitals of the city of Murcia. We did not find significant differences between the two hospitals. According to data of a Spanish multicenter study performed in 2006 [9], the percentage of ESBL-producing in *E. coli* and *K. pneumoniae* strains was 4.04% and 5.04%, respectively. In Spain, few studies of prevalence have been performed; the most recent published data is found in a European study

of 2008 and percentages of prevalence are 5-10% [17].

Our data can also be compared to the global epidemiology of ESBL-producing *E. coli* and *K. pneumoniae* in Spain and others areas of the world [8-18]. Community-acquired infections by ESBL-producing *E. coli* are rising worldwide, and, if we only consider nosocomial- vs. community-acquired infection, as proposed as CDC [10], our data show a similar trend, with 81.6% (30.6% of "strict" community origin and 51% of HAI) of the strains being of community origin. Anyway, we decided to add to the origin of acquisition another section based on the most recent definition of HAI, since it is closer to what actually happens, resulting in 51% of the non-nosocomial cases (81.6%) related to the healthcare rather to pure community acquisition. Other studies obtained similar data when considering HAI in a separate section of community-acquired infections: Díaz *et al* [9] published 67.2% of *E. coli* strains of community origin, but among these strains, 53.1% could have been considered as HAI.

ESBL-producing *K. pneumoniae* is considered a nosocomial pathogen almost exclusively due to its epidemiological behavior [19]. In our experience, more than half *K. pneumoniae* infections were nosocomial (54.5%) and the rest were HAI. None of the infections could be strictly related to a community acquisition. Differences were found in the prevalence of this species between the two hospitals studied (HUVA 33% *vs*. HMM 10%). This fact can be explained by the likely existence of one clone which was disseminated in a specific ward of HUVA in the period of study.

Risk factors associated with the acquisition of infections by ESBL-producing strains are diverse and differences are found from one study to another [20-21]. Severe comorbidities are associated with a higher frequency of infection by ESBL-producing *Enterobacteriaceae*. In our work, most of the patients have had previous contact with the healthcare system, thus implying some underlying medical condition. The preceding use of antibiotics is also considered as a predisposing factor for multiresistant strains acquisition. In our study, 60% of the hospitalized patients had received antibiotics in the last three months.

Other important problem is the associated multiresistance which limit therapeutic options [22]. We found significant differences in the activity of some commonly used antimicrobials such as ciprofloxacin and cotrimoxazole between ESBL-producing strains and non ESBL-producing strains, with percentages of resistance to ciprofloxacin of 81% *vs*. 39% and to cotrimoxazole of 74% *vs*. 36.5% in *E. coli*.

Until recently, carbapenems were considered as the first line of treatment in severe infections. Except one strain of *K. pneumoniae*, the rest of isolated strains were susceptible to imipenem. There are doubts about the use of combinations of beta-lactams and beta-lactamase inhibitors like amoxicillin-clavulanate, because though the concept of ESBL includes susceptibility to that combination, actually resistance to it is not infrequent, as well as the emergence of other mechanisms like decreased permeability or ESBL hiperproduction [23]. Our rates of resistance in ESBL-producing *E. coli* are 43%, and in the case of *K. pneumoniae* 100% of the strains were resistant.

Tigecycline can be a therapeutic option in our environment because we did not find resistant strains. However, its use has been only approved for abdominal and soft tissues infections. The fosfomycin shows good results *in vitro* and *in vivo* to treat urinary tract infections caused by ESBL-producing *E. coli* [25-26], nevertheless our study at the HUVA showed high rates of resistance and are discordant compared to the global rates in Spain. One possible explanation is its massive use to treat non-complicated urinary tract infections in the community.

In our study, the predominant family of ESBL was CTX-M (81.9%). This situation is concordant with most of the studies performed in Spain [8-9], however it is important to highlight that the type of ESBL can change from one hospital to another. Differences were not found in the type of ESBL between the two hospitals of the study. We neither found differences in the type of ESBL between producer species: for both *E. coli* and *K. pneumoniae*, the predominant family was CTX-M (87.5% *vs*. 88.8%). Within this family, CTX-M-14 was the predominant in *E. coli* and in *K. pneumoniae*, CTX-M-1. In the family SHV, SHV-12 was the most frequent. Only one strain of *K. pneumoniae* produced a TEM enzyme, which was *aTEM*-139. This distribution is similar to the multicenter study of Díaz *et al* published in 2006 [9].

We did not find clonal relationship between most strains of *E. coli*, except 2 strains isolated in HUVA and two in HMM, which can correspond to small outbreaks. Nevertheless, 5 strains of *K. pneumoniae* isolated in HUVA present the same profile of bands by REP-PCR. We could further confirm the clonal relationship by PFGE, but unfortunately, in this report the work was not carried out. These epidemiological differences between ESBL-producing *E. coli* and *K. pneumoniae* have important consequences in nosocomial infection control [27]. In conclusion, the high rates of ESBL-producing strains in Murcia and high rates of resistance to other betalactamic antibiotics bring about important health problems and confirm the need to continue studying these strains and risk factors that contribute to their diffusion, including genetic factors.

The likely emergence of one clone of *K. pneumoniae* in HUVA and small outbreaks of *E. coli* show the need to keep doing epidemiological studies of molecular typing and characterization of enzymes to implement appropriate measures of control against nosocomial infection.

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