Extended-spectrum β-lactamases from the North of Portugal in the boundaries to Spain: emergence of high resistance to 4th generation cephalosporins

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ABSTRACT

During the past 15 years, emergence and dissemination of third-generation cephalosporins resistance in nosocomial Enterobacteriaceae became a serious problem worldwide, due to the production of extended-spectrum-β-lactamases (ESBLs). The aim of this study was to investigate among the presence of ESBL-producing enterobacteria among Portuguese clinical isolates nearby Spain, to investigate the antimicrobial susceptibility patterns and to compare the two countries. The β-lactamases genes, bla_{TEM}, bla_{SHV} and $bla_{\text{CTX-M}}$ were detected by molecular methods. Among the ESBL-producing isolates it was found extraordinary levels (98.9%) of resistance to the fourth-generation cephalosporin Cefepime. These findings point to the need of reevaluate the definition of ESBL.

Nos últimos15 anos, a emergência e disseminação da resistência das enterobactérias nosocomiais às cefalosporinas de teceira geração devido à produção de β-lactamases de espectro ampliado (ESBL) tem-se tornado uma preocupação a nível mundial. O objectivo deste estudo é determinar a presença de ESBL em isolados clínicos de enterobactérias, o perfil de susceptibilidade aos antimicrobianos e comparar a situação portuguesa com a espanhola. Os genes bla_{TEM}, bla_{SHV} e $bla_{\text{CTX-M}}$ das β-lactamases foram detectados por métodos moleculares. Nas estirpes portuguesas produtoras de ESBL foram detectados níveis preocupantes de resistência à Cefepima (4ª geração), 98,9%, levando-nos a questionar sobre a necessidade de redefinir ESBL.

1. INTRODUCTION

Extended-spectrum β-lactamases (ESBLs) are enzymes that confer resistance to Aztreonam, Cefotaxime, Ceftazidime, and related oxyimino-β-lactams as well as to older penicillins and cephalosporins but are inhibited by Clavulanic acid (Bush et al., 1995). ESBL-producing *Enterobacteriaceae* were first reported in Europe in the 1980s and have since become a worldwide problem (Paterson and Bonomo, 2005). This has resulted in increased morbidity, mortality and cost in treating the infections they cause (Paterson et al., 2001). The first ESBL were mutants of the TEM and SHV plasmid-mediated penicillinases with one or more aminoacid substitutions. The mutations conferred resistance to all oxyimino-cephalosporins but not α-methoxy-cephalosporins (cephamycins) or carbapenems by causing enlargement of ESBL active site, which allowed the deflection of the oxyimino group diminishing the attack efficiency on the β-lactam ring (Livermore and Woodford, 2006). TEM and SHV present to date over 200 members known (http://www.lahey.org/studies). Another ESBL group, include the CTX-M enzymes, that are organized in five major CTX-M groups: 1, 2, 8, 9, and 25 (Bonnet, 2004). The CTX-M comprise a rapidly growing family distributed both over wide geographic areas among a wide range of bacteria of clinical significance and are becoming more prevalent than its ancestors TEM and SHV in several countries in Europe (Livermore *et al.*, 2009). Several studies have been reported in Iberian Peninsula describing the genetic and clinical environments of ESBL occurrence (Rodríguez-Baño *et al.*, 2004; Hernández *et al.*, 2005; Machado *et al.*, 2006; Mendonça *et al.*, 2007, Amador *et al.* 2009, Fernandes *et al.* 2009).

Here we report the molecular and antimicrobial susceptibility profile of the ESBLs found in the Portuguese occidental cost in the boundaries between the two countries, Portugal and Spain. For this task it were used methods of molecular typing that have been developed for the identification of the β-lactamases *bla* genes in *Enterobacteriaceae* bacteria from clinical hospitalized and non-hospitalized patients for a period of two years.

2. MATERIALS AND METHODS

2.1. Bacterial strains, identification and susceptibility

A total of 7529 clinical strains were included in the study. All isolates were gently provided from Clinical Pathology Laboratories and belong to patients samples recovered from September 2007 to August 2009 in the northern occidental coast of Portuguese territory (Minho). This area comprises several populations of the northern of Portugal and some at the boundaries with Galicia, Spain. Bacteria identification and preliminary antimicrobial susceptibility were determined in accordance with the guidelines of the Clinical and Laboratory Standards Institute (2007). ESBL production was confirmed by the elipsoid method, using two E-test Strips (AB Biodisk, Sweden). Each strip contained several concentrations of a third generation cephalosporin, Ceftazidime (TZ) and Cefotaxime (CT) alone and with Clavulanic acid (TZL, CTL). The ESBL strip contained CT gradient at one end (0.25-16 mg/L) and a gradient of Cefotaxime (0.016-1 mg/L) plus 4 mg/L of Clavulanic acid (L), an inhibitor of ESBL, at the other end (CTL). The other ESBL strip contained TZ gradient at one end (0.5-32 mg/L) and a gradient of TZ (0.064-4 mg/mL) plus L (4 mg/L) at other end (TZL). According to manufacturer an ESBL is considered present whenever the E-test attends at least 8-fold MIC decrease on TZL or CTL.

2.2. Conjugation experiments

Transmissibility of resistance was test by matting clinical isolates to F^- strains of azide-resistant *E. coli* J53 Azi^R on trypticase soy broth (TSB) according with Amador (2010).

2.3. Analytical isoelectric focusing (IEF)

Crude preparations of β-lactamases from clinical strains transconjugants were obtained by sonicating the cells in phosphate buffer, pH 7.0 as described in previously by Fernandes & Prudêncio (2010). Briefly crude extracts were concentrated and crude protein concentrated extract was added to a nitrocefin solution. The sample run in a IEF mini-gels, pH 3 to 10 for 30 min using both anode and cathode electrode buffers. β-lactamases isoelectric point (pI) was determined by pouring molten 3% agarose containing nitrocefin over the gel and comparing the bands to standards run on the same gel. The β-lactamase standards used were extracts of strains containing TEM-1, pI 5.4, SHV-5, pI 8.2 and CTX-M-14, pI 8.1.

2.4. Genetic molecular characterization of bla genes and typing

A single colony of each transconjugant was left to grow for 16h on MacConkey agar and was placed in 200 μL sterile water in a 1.5 mL microtube. Each tube was heated in a microwave oven at 600–700 W for 2 min to burst the cells and release their DNA. PCR was performed according to Fernandes *et al.* (2010). The recovered bands from agarose were subcloned for further sequencing. The nucleotide sequences of both ends of the insert were determined with M13 sequencing primers specific for the cloning vector (Naiemi NA et al., 2005). ERIC profiles were analyzed with software, FPQuest™ version 4.5, Fingerprinting II (Bio-Rad Laboratories, CA, USA).

3. RESULTS

The prevalence of ESBL-producing strains in the north Portuguese territory was 2.6% (n=193). The most frequent ESBL-producing organism was *E. coli* (67.9%, n=131), followed by *K. pneumoniae* (30.6%, n=59), *K. oxytoca* (0,5%, n=1), *E. aerogenes* (0,5%, n=1) and *C. freundii* (0,5%, n=1). The ESBL-producing strains were isolated from urine (n=127), sputum (n=42), bronchoalveolar lavage (n=14), blood (n=7) and ascitic fluid (n=3). The types of ESBL detected in the present study appear in the table 1 and were in percents as follows. TEM types were detected in 40.9%, CTX-M types in 37.3% and SHV types in 23.3%. Several isolates harboring also broad-spectrum β-lactamases, such as TEM-1 (pI=5.4), TEM-2 (pI=5.6) and SHV-1 (pI 7.6) types, but are not presented in this study.

Table 1: *Characterization ESBL-producing strains*

ESBL	IEF (pI)	Microorganism (no. of isolates)	Conju-gation (% positive)	Resistance phenotype (% Non-susceptible: I+R)					ERIC types patterns
				β-lactams		Non β-lactams			
				CEP	FOX	CIP	GEN	Bactrim	
TEM-4	5.9	*E. coli* (2)	100	100	50	100	50	100	A
		K. pneumoniae (1)	100	100	0	100	100	0	L
TEM-10	6.0	*E. coli* (8)	87.5	100	25	87,5	0	75	B,D,H
TEM-24	6.5	*E. coli* (12)	83.3	100	50	75	8.3	8.3	C,G,H,I
		K. pneumoniae (11)	81.8	100	20	72.7	50	63.6	K,L,O
		K. oxytoca (1)	100	100	100	100	0	100	n.a.
		E. aerogenes (1)	100	100	0	0	100	100	n.a.
TEM-52	6.0	*E. coli* (27)	81.4	100	36.4	84.6	53.8	42.3	A,B,E,F,J
		K. pneumoniae (12)	100	100	8.4	91.6	83.3	33.3	N,O
TEM-116	5.4	*E. coli* (n=4)	100	100	50	75	75	0	F
SHV-2	7.6	*K. pneumoniae* (3)	100	33.3	33.3	66.7	66.7	100	L
		E. coli (1)	100	0	0	100	100	100	A
SHV-5	8.2	*K. pneumoniae* (11)	72.7	100	27.3	63.6	72.7	27.3	L,N
		E. coli (6)	83.3	100	66.7	100	100	0	
SHV-12	8.2	*K. pneumoniae* (18)	83.3	100	27.7	61.1	94.4	77.8	K,M,N,O
		E. coli (6)	100	100	33.3	100	100	66.7	C,D,E
CTX-M-1	6.3	*E. coli* (1)	100	100	25	100	100	0	E
CTX-M-3	8.4	*C. freundii* (1)	0	100	0	0	100	0	n.a.
CTX-M-9	8.1	*E. coli* (26)	92.3	100	26.9	96.2	84.6	3.8	A,C,D,G,I,J
CTX-M-14	8.1	*E. coli* (16)	68.8	100	12.5	100	75	0	D,F,H, J
CTX-M-15	8.6	*E. coli* (21)	85.7	100	38.1	100	90.5	14.3	A,C,E,I,J
		K. pneumoniae (3)	100	100	100	100	100	100	N
CTX-M-32	9.0	*E. coli* (1)	100	100	0	100	100	0	D

ESBL: extended-spectrum β-lactamase; IEF: isoelectric focusing; pI: isoelectric point; I: Intermediate; R: Resistant; n.a.: Not applicable. Antimicrobials: CEP: Cefepime; FOX: Cefoxitin; CIP: Ciprofloxacin; Gen: Gentamicin; Bactrim: combination of Trimethoprim and Sulfamethoxazole;

MIC tests shown that isolates producing TEM, SHV and CTX-M ESBL types also appears in Table 1and were mostly resistant to Cefepime (98.9%) and susceptible to Carbapenems (100%) and Amikacin (99.5%). Nevertheless, some SHV-producing strains, shown an important reduced susceptibility to other member of Aminoglycosides, namely Gentamicin (11.1%). ESBL-producing strains from this sample also present reduced susceptibility to Quinolones (14.6%) in the generality, being more conspicuous in the members of the CTX-M family (1.9%) rather than TEM and SHV members (19.2% and 27.9% respectively).

Regarding interspecific genetic similarity, it was observed a high genetic diversity. It was possible to define 10 clusters for *E. coli* based on Pearson's correlation coefficient in PCR-ERIC based profile (data not shown). For *K. pneumoniae* it was defined 5 different clusters for the PCR-ERIC (data not shown).

4. DISCUSSION

As reported in previous Portuguese (Machado *et al.*, 2006; Mendonça *et al.*, 2007; Costa *et al.*, 2004, Costa *et al.*, 2008, Fernandes *et al.*, 2008, Amador *et al.*, 2009, Fernandes *et al.*, 2009, Amador *et al.*, 2010, Fernandes & Prudêncio 2010), Spanish (Brinãs et al., 2002; Coque et al., 2008; Hernández, et al., 2005; Miró et al., 2005) and other European (Livermore *et al.*, 2009) studies *E. coli* and *K. pneumonia* are the species where ESBL is the most frequently identified. In this study *E. coli* were the most frequent (n=131) organism expressing ESBL phenotypes, more than two fold of the *K. pneumonia* (n=59), the second most frequent.

TEM-52 and TEM-24 were the most frequent TEM types, 20.2% (n=39) and 12.9% (n=25) respectively. Members of the TEM-10 (n=8) and TEM-116 (n=4) were also detected. Within CTX-M family, the CTX-M-9 group is more prevalent than the CTX-M-1 group (58.3% against 41.6%). CTX-M-9 group was represented by CTX-M-9 (n=26, 36.1%) and CTX-M-14 (n=16, 22.2%). In the CTX-M-1 group, CTX-M-15 was most frequent type (n=24, 33.3%), followed by CTX-M-1 (n=4, 5.5%), CTX-M-3 (n=1, 1.3%) and CTX-M-32 (n=1, 1.3%). The SHV enzymes occurred only in 23.3% of all ESBL- producing organisms. Within this type, the most frequent was the SHV-12 (n=24, 53.3%), followed by SHV-5 (n=17, 37.8%) and finally SHV-2 (n=4, 8.8%). Some isolates co-produced more than one ESBL type: TEM-52/CTX-M-14 (n=1), TEM-116/CTX-M-14 (n=1) and TEM-116/CTX-M-15 (n=1).

Regarding the high diversity ESBL types obtained in our study, we find interesting to compare with neighbor regions, such as Douro Litoral located at south of Minho, Trás-os-Montes e Alto Douro, eastern and the Spanish province of Galiza, located at north of Minho (Figure 1). From this comparison and regarding to TEM types ESBL, TEM-10, TEM-20, TEM-26 and TEM-116 in *E. coli* and TEM-4 in *K. pneumoniae* were present only in this region but have been reported in other locations of Iberian Peninsula. Isolates of *E. coli* producing SHV-2 and SHV-5 were also found only in this region. Nevertheless, it has been described previously in the Iberian Peninsula (Hernandez et al., 2005). Regarding CTX-M types, it seems that CTX-M-14 is widespread among the northwestern Iberian Peninsula. *K. pneumoniae* harboring a CTX-M-15 was described for the first time in Portugal in 2005 (Conceição et al., 2005) in Lisbon area but it is also found in the north of Portugal in this study and Douro (Machado et al., 2006). Other ESBL-producing species non-*E.coli* and non-*K. pneumonia* were also found. It has been the first time that an *E. aerogenes* is described in this country as producer of a TEM-24 and the first time that is found a *C. freundii* as a producer of a CTX-M-32.

In what concerns to the clonally studies, it seems clear the high genetic diversity among the strains in study. Nevertheless, some considerations are needed. K cluster for *K. pneumoniae* was represented by TEM-24 producing members only. Nevertheless, it may be due to a coincidence. The two strains presenting the tightest resemblance were only 74% genetically related. The antibiogram for the four strains were also different between all them (data not shown). Moreover, they belong to four patients, two males and two females, interned in different clinical services. In the opposite situation, the CTX-M-15 producing *K. pneumoniae*, bellowing to N cluster, presented the exactly antibiogram profile and the strain less related to the other two, shared a 93% of genetic similarity and the most related two of them, presented a 98% of genetic similarity. The patients, three females with ages between 76 and 86 years old, shared the same clinical service, Medicine Females, during the same period, from October to November 2007.

Cefepime presents, in this study a surprisingly low activity against ESBL-producing microrganisms. In our sample only two *K. pneumoniae* harboring SHV-2 ESBL were susceptible to Cefepime. All the other

clinical isolates, 98.9% (n=191) expressing the ESBL phenotype were resistant to Cefepime. It seems interesting that a recent study showed that Cefepime was successfully administrated to three patients (two females and one male) with ages between 47 and 87 years old carrying a gram-negative ESBL positive strain (Bhavnani *et al.*, 2006). Nevertheless Other studies worldwide start to describe the emergence of high resistance among ESBL gram-negative producers (Grover *et al.*, 2006; Sader *et al.*, 2007).

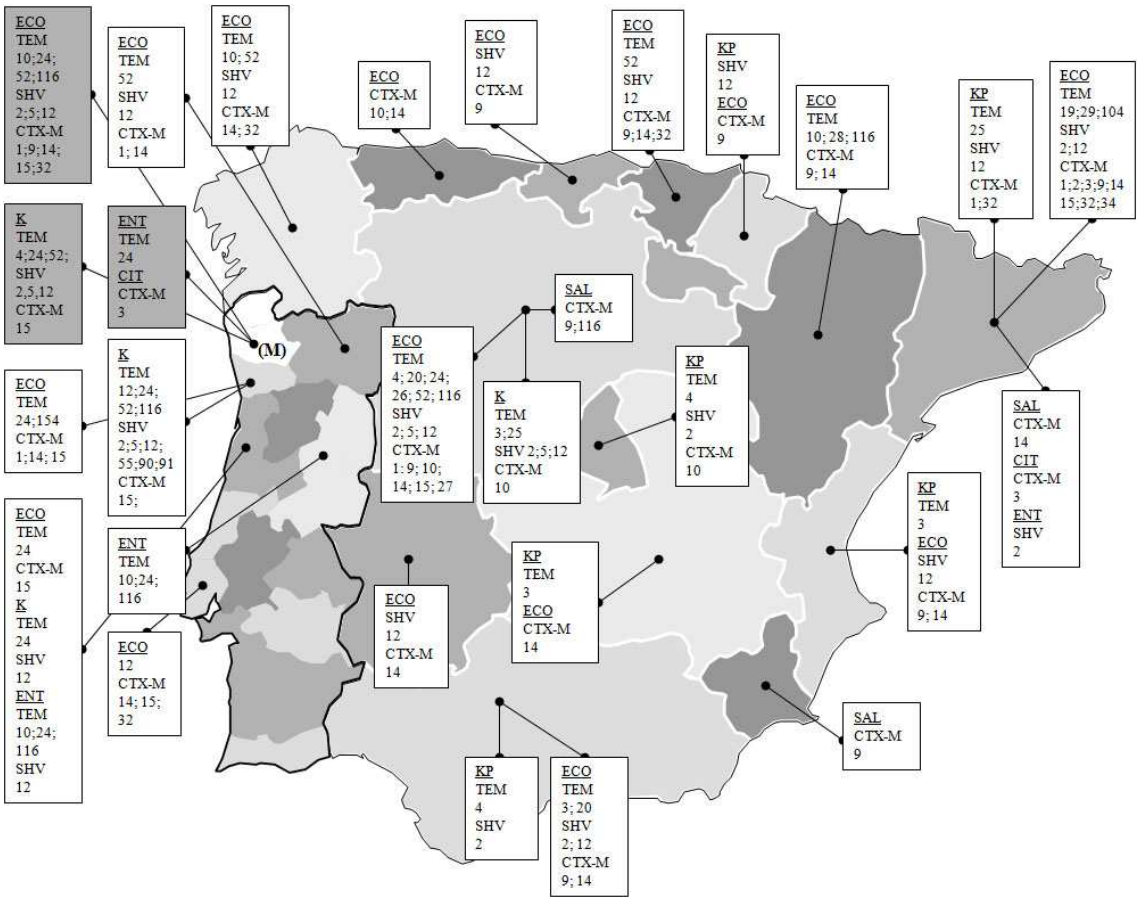

Figure 1. *Occurrence of ESBL-TEM, ESBL-SHV and ESBL-CTX-M type in the north of Portugal. The present study territory, Minho (M), is represented at the map in white. Other regions either from Portugal or Spain, are represented with different gray tones. Data was collected from this study and from several studies in these two countries namely by Amador et al. (2009,2010), Briñas et al. (2002,2005), Coque et al. (2000,2008), Costa et al. (2002,2008,2009), Conceição et al. (2005), Fernandes et al. (2008,2009), Fernandes & Prudêncio (2010), Fernández et al. (2007), Hernández et al. (2005), Machado et al. (2006) and Mendonça et al. (2007).*

In summary, this work showed the high diversity of ESBL-enzymes occurring in Portugal. In this country, the most prevalent type is still the TEM-type but CTX-M is growing rapidly. The ERIC analysis shows to be efficient in the clonally relationships between the different *Enterobacteriaceae* isolates, and it was possible to detect an episode of nosocomial infection. Nevertheless, it was obtained indistinguishable patterns among ESBL-producing strains. The emergence of ESBL producers resistant to Cefepime in Portugal is a matter of concern. We believe that the uncontrolled use of cephalosporins may have an important role in the acquisition of resistance mechanisms, specially the production of ESBL enzymes. It seems to be urgent the establishment of policies to monitor drug delivery in hospital and ambulatory pharmacies and the implementation of public health defense strategies towards health promotion and drug resistance prevention.

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