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Diversity of Extended-Spectrum and Plasmid-Mediated AmpC β-Lactamases in *Enterobacteriaceae* Isolates from Portuguese Health Care Facilities

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A group of 124 *Enterobacteriaceae* isolates resistant to third generation cephalosporins, and collected in distinct health care facilities of different Portuguese regions was analysed. The great majority of the isolates were also resistant to fourth generation cephalosporins (83.9%), monobactam (96%), amoxicillin plus clavulanic acid (85.5%), and piperacillin plus tazobactam (66.9%). Overall, 84.7% (105/124) were multidrug resistant. Molecular methods enabled us to identify 86.3% (107/124) extended-spectrum β-lactamases (ESBL) producers, revealing a diversity of class A β-lactamases from different families, like TEM (TEM-1, TEM-10, TEM-24, and TEM-52), SHV (SHV-1, SHV-12, and SHV-28), CTX-M (CTX-M-1, CTX-M-9, CTX-M-14, CTX-M-15, and CTX-M-32), and GES (GES-1). We have also detected class C enzymes like plasmid-mediated AmpC β-lactamases (PMAβs, DHA-1, and CMY-2) and chromosomal AmpCs in *Enterobacter* and *Citrobacter* spp. The PMAβ genetic context mapping suggests association with mobile elements, plasmid importation and the potential emergence of these β-lactamases. The most prevalent β-lactamase detected was CTX-M-15 (66.1%) and in 41.1% of the isolates it was associated with TEM-, OXA-type β-lactamases and Aac(6)'-Ib-cr, which might indicate that the respective genotype has settled in our country. Indeed, CTX-M-15 was distributed amongst distinct clinical settings of several health care facilities (93.5%) from various regions. We provide evidence of a concerning clinical situation that includes vast occurrence of ESBLs, the settling of CTX-M β-lactamases, and the report of plasmidic and chromosomal AmpC in Portugal.

Keywords: diversity, ESBL, PMAβ, health care facilities

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Introduction

Antibiotic resistance is a global and emerging problem and β-lactamase production is the most representative bacterial resistance mechanism in *Enterobacteriaceae* isolates (Livermore, 2009). Throughout the years, this situation led to a questioning of the effectiveness of β-lactams, especially in Portugal, where they constitute the most commonly used therapy for this type of infections (Adriaenssens *et al.*, 2011). The so called "new β-lactamases", like extended-spectrum β-lactamases (ESBL, class A), plasmid-mediated AmpC β-lactamases (PMAβ, class C) and carbapenemases (class A, B, and D) seem to be the focus on this concerning issue, since they compromise the efficacy of a great range of β-lactams. Although ESBLs can be detected by the synergy between a β-lactam and clavulanic acid, AmpC enzymes are resistant to β-lactamase inhibitors and cephamycins as well (Jacoby, 2009; Livermore, 2009). On the other hand, carbapenemases are the most feared β-lactamases , since they hydrolyse carpabenems, which constitute the last stand on β-lactam therapy (Queenan and Bush, 2007; Jacoby, 2009; Livermore, 2009).

Nowadays, little is known about the transferable AmpC enzymes in Portugal, where the rate of ESBLs is considerably high (Mendonça *et al.*, 2007, 2009; EARS-Net, 2012). Concerning carbapenemases, their prevalence is still uncommon in Portugal (Grundmann *et al.*, 2010). The mobilization and consequent spread of β-lactamase-encoding genes may occur through several genetic elements, including plasmids, transposons, insertion sequences, and integrons, which constitute reliable markers in the study of the dissemination of these resistance determinants (Cambray *et al.*, 2010; Toleman and Walsh, 2011).

Resistance to β-lactams due to β-lactamase production is frequently associated with resistance to several other groups of antibiotics (e.g. fluoroquinolones, aminoglycosides, sulfamethoxazole and tetracycline) and this association confines the therapeutic options available (Philippon *et al.*, 2002; Paterson and Bonomo, 2005).

In this study we aimed to evaluate the diversity of β-lactamases produced by a group of *Enterobacteriaceae* isolates, collected during five years, in distinct clinical settings in Portugal, to evaluate and explain the emerging rate of resistance to expanded-spectrum cephalosporins.

Materials and Methods

Bacterial isolates

One hundred and twenty four *Enterobacteriaceae* isolates, collected over a five year period (2004 to 2008), from diverse clinical specimens, were selected from the Reference Laboratory of Antimicrobial Resistance collection, at the National Institute of Health, in Lisbon. These isolates were recovered from patients that attended twelve distinct Portuguese health care facilities, distributed between four Portuguese regions (north, center, tagus valley, and south). The bacterial identification of these isolates was performed by automated methods (ATB G-5, VITEK 1, VITEK 2, and Phoenix). The selection criteria of the isolates were the presence of non-susceptibility to, at least, one third generation cephalosporin and the inexistence of duplicated strains. We also conducted an in-depth study of one of the health care facilities (G), as an ulterior objective of this investigation.

Isolates included several species, specifically *Escherichia coli* (n=80), *Klebsiella pneumoniae* (n=32), *Enterobacter cloacae* (n=5), *Enterobacter aerogenes* (n=5), *Klebsiella oxytoca* (n=1), and *Citrobacter freundii* (n=1). Control strains were used to validate the susceptibility testing, isoelectric focusing, and molecular characterization results.

Antimicrobial susceptibility

Susceptibility testing of clinical isolates was performed by standard disk diffusion method, according to the Antibiogram Committee of the French Society of Microbiology (CA-SFM, http://www.sfm-microbiologie.org). Moreover, a disk of amoxicillin plus clavulanic acid (20 μg+10 μg) was placed 20 mm apart from cefotaxime (30 μg), ceftazidime (30 μg), and cefpodoxime (10 μg), for ESBL and GES detection; an imipinem disk with EDTA (750 μg) was placed adjacent to imipinem and meropenem (10 μg) disks for metallo-β-lactamase detection.

Isolates that presented reduced susceptibility to third generation cephalosporins restored by clavulanic acid, were assumed as presumptively producers of ESBL enzymes, while those with resistance to ceftazidime, but susceptibility to cefotaxime were presumptively producers of GES β-lactamases.

In isolates non-susceptible to cefoxitin, three cefoxitin disks (30 μg), three ceftazidime disks (30 μg) and one cefotaxime disk (30 μg) were placed 20 mm apart from each other, where cefotaxime was placed in the middle of a previously inoculated Mueller-Hinton agar plate; then, 600 μg of boronic acid and 750 μg of cloxacillin were applied to two of the cefoxitin and ceftazidime disks, respectively. where cefotaxime was placed in the middle of a previously inoculated Mueller-Hinton agar plate; here, the detection of synergy between any of the antibiotics mentioned and both boronic acid (≥4 mm) and cloxacillin (≥5 mm) suggested the production of a PMAβ and/or a chromosomal AmpC β-lactamase.

Finally, isolates were considered multidrug resistant if they presented reduced susceptibility to three or more structurally unrelated antibiotics.

Isoelectric focusing (IEF)

Isoelectric point (pI) was determined by IEF, as previously described (Caniça *et al.*, 1997), in order to search for the presence and expression of chromosomal and plasmidic AmpC β-lactamases in the isolates where the susceptibility methods suggested their production.

Molecular characterization

Detection of TEM, SHV, OXA, CTX-M, and GES encoding genes was carried out by PCR in all isolates screened by phenotypic methods as putative ESBL or GES producers, as previously described (Mendonça *et al.*, 2007). PMAβ encoding genes (bla_{MOX}, bla_{CMY}, bla_{FOX}, bla_{DHA}, bla_{ACC}, bla_{ACT}, and bla_{MIR}) were also detected by PCR and identified as reported elsewhere (Manageiro *et al.*, 2012). All amplified products were purified with ExoSAP IT (USB Corporation, USA) and sequenced directly on both strands, as previously described (Mendonça *et al.*, 2007). TEM and SHV encoding genes were only identified for CTX-M and GES negative isolates. All isolates were also subjected to the search of Aac(6')-Ib-cr-encoding gene, as described elsewhere (Robicsek *et al.*, 2006).

Transferability and genetic context of PMAβ-encoding genes

Transferability of the bla_{CMY-2} and bla_{DHA-1} genes was performed by mixing equal volumes of donor (INSRA 7609 and INSRA 6331, respectively) and recipient strains (*E. coli* C600 Rif^R and *E. coli* J53 $NaN3^R$) on Brain Heart Infusion broth. β-Lactam resistant *E. coli* were then selected on MacConkey agar containing either cefoxitin (10 μg/ml) or ceftazidime (2 μg/ml) plus rifampicin (250 μg/ml) or sodium azide (200 μg/ml). Then, the genetic context of those β-lactamase-encoding genes was obtained by PCR mapping assays based on known sequences. Plasmids obtained from both parental and transconjugant strains were assigned to incompatibility groups by PCR-based replicon typing (PBRT), using previously described conditions (Carattoli *et al.*, 2005). Detection of class 1 integrons and respective 5'CS-3'CS content was assessed, using specific primers (Leverstein-van Hall *et al.*, 2002), as well as class 2 (Jones-Dias *et al.*, 2013) and 3 integrons (5′-ATGGTGACGGTGTTCGG-3′ and 5′-CTAG GCATGATCTAACCCTC-3′).

Results

Characterization of clinical isolates

Seventy three isolates were collected from urine (58.9%), 20 from blood (16.1%), 13 from secretions (10.5%), 5 from sputum (4.0%), 4 from catheters (3.2%), 2 from cerebrospinal fluid (1.6%) and 7 from other locations (5.6%), among patients attending to 12 distinct health care facilities. Regarding clinical department, the great majority of the isolates were collected in the internal medicine ward (31.5%), followed by the emergency room (16.9%). The remaining isolates were collected in other wards. Of the 67/124 (54.0%) strains isolated from women, 48 were from patients over 65 years old, 17 from patients between 19 and 64 years old and

2 from patients under 18 years of age. Of the 56/124 (45.2%) isolates from men, 34 were from patients over 65 years old, 20 from patients between 19 and 64 years old and 2 from patients under 18 years. Information regarding the patient age and gender was lacking for one of the isolates.

Phenotypic characterization

All isolates presented diminished susceptibility to amoxicillin, ticarcillin and piperacillin, as well as reduced susceptibility to, at least, one of the third generation cephalosporins tested (cefotaxime, ceftriaxone, ceftazidime, cefpodoxime, and cefixime), as this was one of the major criteria adopted in this study. Regarding fourth generation cephalosporins and monobactam, the great majority of the isolates also exhibited diminished susceptibility (83.9% and 96.0%, respectively). Only 14.5% of the isolates revealed to be susceptible to amoxicillin plus clavulanic acid and 33.1% to piperacillin plus tazobactam. In respect to cefoxitin, 24.2% of the isolates were non-susceptible and all revealed to be susceptible to carbapenems. Overall, 84.7% of the isolates were multidrug resistant, mostly due to diminished susceptibility to quinolones (83.9%, 104/124), and aminoglycosides (76.6%, 95/124) (Tables 1 and 2). In contrast, fosfomycin (99.2%) and nitrofurantoin (99.2%) were the antibiotics to which the isolates were more susceptible.

Molecular characterization of *bla* genes

The presence of bla_{ESBL} genes was confirmed in 86.3% (107/124) of the isolates (Table 1); genes encoding β-lactamases belonged to bla_{TEM} (n=80), bla_{SHV} (n=33), bla_{OXA} (n=70), $bla_{CTX\text{-}M}$ (n=100), and bla_{GES} (n=1) families. Amongst bla_{TEM} gene family, three bla_{ESBL} variants were identified: $bla_{TEM\text{-}10}$ (n=1), $bla_{TEM\text{-}24}$ (n=2), $bla_{TEM\text{-}52}$ (n=1). Within bla_{SHV} family, $bla_{SHV\text{-}12}$ (n=4) was the unique bla_{ESBL} gene encountered, since the remaining coded for the penicillinases SHV-1 (n=8), SHV-28 (n=2), and SHV-type (n=19). Amongst $bla_{CTX\text{-}M}$ family, 87 genes from group 1 were detected, mainly $bla_{CTX\text{-}M\text{-}15}$ (n=82), followed by $bla_{CTX\text{-}M\text{-}1}$ (n=3) and $bla_{CTX\text{-}M\text{-}32}$ (n=2). Among the remaining $bla_{CTX\text{-}M}$ genes, 13 were from group 9, predominantly $bla_{CTX\text{-}M\text{-}14}$ (n=12) and $bla_{CTX\text{-}M\text{-}9}$ (n=1). The representative of the bla_{GES} family was the $bla_{GES\text{-}1}$ gene. Moreover, two PMAβ-encoding genes were detected, $bla_{CMY\text{-}2}$ and $bla_{DHA\text{-}1}$. The majority of the ESBL-producing isolates expressed multiple β-lactamases, predominantly CTX-M-15 and OXA-type plus TEM-type enzymes (Tables 1 and 3). Overall, 94.3% of the multidrug resistant isolates were ESBL producers.

Expression of class C β-lactamases

All *Enterobacter*, *Citrobacter*, *K. oxytoca*, and also one *E. coli* isolate presented reduced susceptibility to cefoxitin, and were presumptively producers of AmpC enzymes (Table 2). This was confirmed for all *Enterobacter* and *Citrobacter* spp. isolates, as they all showed enzymes with a range of pI ≥ 8.0 (Table 2), promptly attributed to chromosomal AmpC β-lactamase production. In *K. oxytoca* and *E. coli* we have also noticed pIs of 7.8 and 9.0, that were attributed to PMAβ DHA-1 and CMY-2, respectively (Table 2), which was in agreement with the phenotypic methods. Additionally, mo-

Table 1. Antimicrobial resistance profile, presence of Aac(6')-Ib-cr, and health care facility distribution of 113 *Enterobacteriaceae* isolates (*E. coli*, *K. pneumonia*, and *K. oxytoca*) expressing, at least, one β-lactamase and lacking chromosomal AmpC

Antimicrobial resistance pattern[a]	ESBL and PMAβ (Nb. of isolates)	Penicillinases (Nb. of isolates)	Aac(6')-Ib-cr (Nb. of isolates)	Health care facility code	Total Nb. of isolates
P C1 C2 C3 C4 M A	CTX-M-14 (2)	-	Positive (1)	G	2
P C1 C2 C3 C4 M F (I) Q (A) (S) (FT)	CTX-M-15 (9) CTX-M-14 (6) DHA-1 (1) CMY-2 (1) TEM-24 (1)	SHV-28 (1) TEM-type (12) OXA-type (6) SHV-type (3)	Positive (9)	A,C,D,F,G,M	17
P C1 C2 C3 C4 M (I) (Q) (A) (S)	CTX-M-1 (1) CTX-M-9 (1) CTX-M-14 (3) CTX-M-15 (4)	TEM-type (5) SHV-type (3)	Positive (4)	A,C,D,E,G	9
P C1 C2 C3 C4 M I Q A (S) (FS)	CTX-M-14 (1) CTX-M-15 (66) CTX-M-32 (1)	TEM-type (53) OXA-type (62) SHV-type (13)	Positive (61)	A,B,C,D,F,G,J,L,M	67
P C1 C2 C3 C4 M (Q) (S)	CTX-M-1 (2) CTX-M-15 (1) CTX-M-32 (1)	TEM-type (2) OXA-type (1)	Positive (1)	G,H	4
P C1 C2 C3 (M) (I) (Q) (S)	GES-1 (1)	SHV-type (7) TEM-type (1)	Positive (2)	G, J	8
P C1 C2 C3 M (I) Q (A) (S)	TEM-10 (1) SHV-12 (3) TEM-52 (1)	TEM-type (1) SHV-28 (1) SHV-type (1)	Positive (2)	B,G,H,I,J	6

[a] P, penicillins (amoxicillin, piperacillin, ticarcillin); C1, first-generation cephalosporin (cephalothin); C2, second-generation cephalosporin (cefuroxime); C3, third-generation cephalosporin (cefotaxime, ceftriaxone, ceftazidime, cefpodoxime, cefixime); C4, fourth-generation cephalosporin (cefepime); F, cefoxitin; M, monobactam (aztreonam); I, β-lactamase inhibitors (amoxicillin plus clavulanic acid and piperacillin plus tazobactam); Q, Quinolones (nalidixic acid, pefloxacin, norfloxacin, ciprofloxacin); A, aminoglycosides (amikacin, gentamicin, kanamycin); S, folate pathway inhibitors (trimethoprim/sulfamethoxazole, trimethoprim); FS, Fosfomycin; FT, Nitrofurantoin.
Variable presence of non-susceptibility phenotype is indicated by parentheses.

Table 2. Characteristics of 13 cefoxitin non-susceptible AmpC β-lactamase-producing-isolates: susceptibility profile, β-lactamases, pI's and Aac(6')-Ib-cr expression

Species	Code	Non-susceptibility profile	β-Lactamases[a]	pI	Aac(6')-Ib-cr	Health care facility code
E. aerogenes	INSRA6172	PC1C2C3MFIQAS	TEM-24, AmpC	6.5, ≥8.0	-	M
	INSRA7606	PC1C2C3MFI	AmpC	≥8,0	-	G
	INSRA7610	PC1C2C3MFIA	AmpC	≥8,0	-	G
	INSRA7611	PC1C2C3MFI	AmpC	≥8,0	-	G
	INSRA7614	PC1C2C3MFI	AmpC	≥8,0	-	G
E. clocae	INSRA7581	PC1C2C3MFI	AmpC	≥8,0	-	G
	INSRA7601	PC1C2C3MFIS	AmpC	≥8,0	-	G
	INSRA7626	PC1C2C3C4MFIQAS	TEM-1, OXA-1, AmpC, CTX-M-15	5.4 7.4 ≥8,0 8.9	+	G
	INSRA7629	PC1C2C3MFI	AmpC	≥8,0	-	G
	INSRA7736	PC1C2C3C4MF	AmpC, CTX-M-15	≥8.0 8.9	+	G
C. freundii	INSRA7598	PC1C2C3MFIQAS	TEM-1, SHV-12, AmpC	5.4, 8.2, ≥8,0	+	G
E. coli	INSRA7609	PC1C2C3C4MFIQAS	TEM-1, OXA-1, CTX-M-15, CMY-2	5.4, 7.4, 8.9, 9.0,	-	G
K. oxytoca	INSRA6331	PC1C2C3C4MFIQAS	DHA-1, SHV-1, CTX-M-14	7.8 8.1 8.2	-	D

[a] AmpC β-lactamases and the respective pI are underlined.
P, penicillins (amoxicillin, piperacillin, ticarcillin); C1, first-generation cephalosporin (cephalothin); C2, second-generation cephalosporin(cefuroxime); C3, third-generation cephalosporin (cefotaxime, ceftazidime, cefpodoxime, ceftriaxone, cefixime); C4, fourth-generation cephalosporin (cefepime); F, cefoxitin; M, monobactam (aztreonam); I, β-lactamase inhibitors (amoxicillin plus clavulanic acid and piperacillin plus tazobactam) Q, Quinolones (nalidixic acid, pefloxacin, norfloxacin, ciprofloxacin); A, aminoglycosides (amikacin, gentamicin, kanamycin); S, folate pathway inhibitors (trimethoprim/sulfamethoxazole, trimethoprim).

lecular methods allowed us to identify the ESBLs CTX-M-14, CTX-M-15, TEM-24, and SHV-12 enzymes, among other β-lactamases (TEM-, OXA-, and SHV-type).

Genetic context of PMAβ-encoding genes

The bla_{CMY-2} gene carried by the *E. coli* isolate INSRA7609 was located on a conjugative plasmid that was promptly transferred to an isogenic system, along with a TEM-1 β-lactamase, and classified as IncA/C type. The combination of conventional and long distance PCR assays revealed a 11 kb region bearing a composite genetic structure: besides the bla_{CMY-2}-*blc-sugE-ΔecnR* originated from the *C. freundii* chromosome, bla_{CMY-2} was flanked upstream by a copy of an IS*Ecp1* element and downstream by four open reading frames (*orfs*) of unknown meaning, just before *dsbc* and *traC* genes at the 3'-end (Fig. 1A). The bla_{TEM-1} gene that was also present in this IncA/C plasmid was located uptream of a truncated *mer* operon. We were not able to transfer the bla_{DHA-1} bearing plasmid, but we identified that DHA-1-producing *K. oxytoca* isolate INSRA6331 also carried an IncA/C plasmid. The PCR mapping assay showed that this isolate harboured an 8kb fragment encompassing a complex integron backbone that resulted in the following structure: *intI1-aadA1-qacΔE1-sul1*-IS*CR1*-bla_{DHA-1}-*ampR-qacΔE1-sul1* (Fig. 1B). Both the CMY-2-producing *E. coli* and the DHA-1-producing *K. oxytoca* were negative for the production of class 2 and 3 integrons, but they both carried class 1 integrons (Fig. 1A and 1B).

Distribution and association of *aac(6')-Ib-cr* genes with other antibiotic resistance determinants

The *aac(6')-Ib-cr* genes were detected in 66.1% (82/124) of the isolates, being 98.8% (81/82) of them co-expressed with ESBL β-lactamases. We observed a striking association of CTX-M-15 with TEM and OXA-type β-lactamases, as well as to Aac(6')-Ib-cr, being 41.1% (51/124) of total isolates co-producers of these four resistance determinants (Tables 1 and 2). This profile was observed in 8 health care facilities distributed among the 4 regions of the country.

Distribution of ESBL-producing isolates by clinical department

Considering the clinical importance of TEM, SHV, CTX-M, and GES β-lactamase families, the distribution of these enzymes in all of the health care facilities included in this study and respective wards was analyzed. Table 3 shows that internal medicine and emergency room wards were the richest in ESBL-producing isolates (29.3% and 15.4%, respectively), especially CTX-M-15 (66.1%), which was present in 15 out of 16 (93.5%) clinical departments, namely in hospital G (50.9%, 27/53). In other hospitals, such as hospital D (n=8), we identified enzymes like CTX-M-14 (25.0%) and CTX-

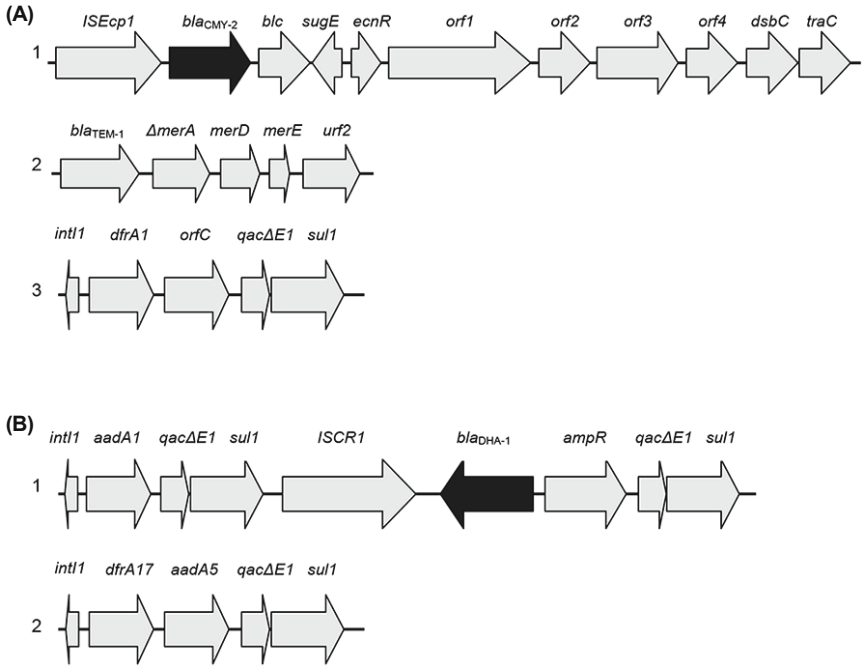

Fig. 1. **(A) Genetic organization of the environment of *bla*$_{CMY-2}$ gene and characterization of other genetic structures present in *E. coli* INSRA7609 isolate.** 1, Genetic context of the *bla*$_{CMY-2}$ gene; 2, *bla*$_{TEM-1}$ gene located nearby the *mer* operon; 3, Gene array contained in the class 1 integron. (B) Genetic organization of the environment of *bla*$_{DHA-1}$ gene and characterization of other genetic structures present in *K. oxytoca* INSRA6331 isolate. 1, Genetic context of the *bla*$_{DHA-1}$ gene; 2, Gene array contained in the class 1 integron.

M-15 (75.0%); in this hospital, CTX-M-15 was also present in three clinical departments. The same situation was observed in Hospital C (n=18), where SHV-28 (5.6%), TEM-1 (5.6%), CTX-M-14 (33.3%), and CTX-M-15-producing isolates (61.1%) were detected in different wards, where this last CTX-M-type enzymes were also present in more than one clinical department. Moreover, the 10 isolates from hospital J were producers of SHV-1 (10.0%), TEM-52 (10.0%), CTX-M-15 (80.0%), and CTX-M-32 (10.0%) and nearly all of them were isolated from patients belonging to internal medicine department. All the isolates from hospitals A (n=9) and F (n=14) were producers of CTX-M-15 and were distributed in several distinct clinical departments. Additionally, two of the four isolates from hospital M were TEM-24-producers and detected in cardiology and internal medicine clinical departments (Table 3).

Discussion

Antibiotic resistant *Enterobacteriaceae* isolates are frequently implicated in human infections. ESBL- and PMAβ-producing

Table 3. **Distribution of Class A β-lactamase-producing isolates by ward in twelve health care facilities**

Ward	β-Lactamases												
	SHV-1	SHV-12	SHV-28	TEM-1	TEM-10	TEM-24	TEM-52	CTX-M-1	CTX-M-9	CTX-M-14	CTX-M-15	CTX-M-32	GES-1
Cardiology						M							
Emergency room	G		B		B				G	C,G	A,C,D,F,G		
Gastroenterology	G										G		
Intensive care unit	G										G		
Infecciology		G		G							B,G	G	
Internal Medicine	J		C	C		M	J			C,G	A,C,D,F,G,J,L,M	J	
Nephrology		H		H				G			B,E,G,H		G
Neurology											G		
Observation service										C	A,F,G,M		
Obstetrics	G										F,G		
Oncology											G		
Otorhinolaringology		G									G		
Outpatient		I								G,D	A,G,F		
Pneumology										G	C,D		
Surgery	G										C, F, J		
Urology								G		D	G		

Hospital A, n=9; Hospital B, n=3; Hospital C, n=18; Hospital D, n=8; Hospital E, n=1; Hospital F, n=14; Hospital G, n=53; Hospital H, n=2; Hospital I, n=1; Hospital J, n=10; Hospital L, n=1; Hospital M, n=4. North region: C, J, M; Center region: B, I; Tagus Valley region: D, E, F, G, H, L; South region: A.

Enterobacteriaceae are nowadays frequently associated with this type of clinical scenario, compromising the therapeutic use of third generation cephalosporins, amongst others (Bradford, 2001; Philippon *et al.*, 2002; Jacoby, 2009).

Until the early 90's, only TEM and SHV-type β-lactamases were described in Europe. However, CTX-M β-lactamases are currently predominant over all other enzyme families (Livermore *et al.*, 2007; Nicolas-Chanoine *et al.*, 2008).

In this study, we highlight the predominance of CTX-M-15 β-lactamase (82/124, 66.1%), that was the most abundant variant detected among all CTX-M-producers (99/124, 79.8% considering that one isolate harboured two CTX-M variants, CTX-M-15 plus CTX-M-32), with exception of hospital I.

Indeed, CTX-M enzymes have proved to be the most successful ESBLs worldwide, being widely disseminated through community and clinical settings; recent studies have even described a 5 to 12% faecal carriage rate of CTX-M-producing isolates, in populations from Europe, Asia and America, which constitutes an additional global concern (D'Andrea *et al.*, 2013).

Moreover, we were able to detect a high proportion of other resistant determinants, associated with CTX-M-15 production; the $bla_{\text{CTX-M-15}}$ + $bla_{\text{TEM-type}}$ + $bla_{\text{OXA-type}}$ + *aac(6')-Ib-cr* genotype, was detected in 51 isolates (41.5%) of several species (*Enterobacter* spp., *E. coli*, and *K. pneumoniae*), which indicates that the referred genotype seems to have settled in our country, highly contributing to multidrug resistance (Machado *et al.*, 2006; Mendonça *et al.*, 2006). The success of this particular genotype, that has already been described in many countries in all five continents, is unprecedented and seems to be a combination of several factors that include association of $bla_{\text{CTX-M}}$ with highly efficient mobile elements, vertical dissemination of virulent clones and elevated selective pressure caused by extensive use of cephalosporins and fluoroquinolones in both clinical and veterinary environments (Briales *et al.*, 2012; Alouache *et al.*, 2013; Silva-Sánchez *et al.*, 2013; Yano *et al.*, 2013).

This panorama also allowed the substitution of TEM and SHV β-lactamases in the hospital settings towards CTX-M family (Livermore *et al.*, 2007; Nicolas-Chanoine *et al.*, 2008).

In contrast, two types of class C β-lactamases were detected: the chromosomal AmpC in *Enterobacter* and *Citrobacter* spp. and PMAβs in one *E. coli* and *K. oxytoca* isolates. PMAβ producers were much less frequent (0.8%) than ESBL producers, although their prevalence seems to be increasing worldwide. Although this constitutes one of the few reports of PMAβs in Portugal, CMY-2 and DHA-1 display a worrying widespread distribution that includes countries such as Algiers, Canada, China, France for CMY-2 and Belgium, China, Spain and Japan for DHA-1, all of which associated with relevant hospital outbreaks (Jacoby, 2009; Mata *et al.*, 2012; Freitas *et al.*, 2013). In this work, the two PMAβ-producing isolates also expressed CTX-M-15, TEM-1, and OXA-1 β-lactamases, or CTX-M-14 and SHV-1 enzymes, usually encoded on plasmids that are normally linked with resistance to several classes of antibiotics, as the level of multidrug resistance detected in our work also suggests. The genetic environment of these acquired AmpC β-lactamases allowed us to understand that a complex integron backbone associated with $bla_{\text{DHA-1}}$ reinforces the mobility potential of this antimicrobial resistant determinant. On the other hand it is the first time that this genetic platform is described in association with an *aadA1* gene cassette, which might suggest that this structure is still evolving. Although the plasmid bearing the $bla_{\text{DHA-1}}$ gene was not transferable, the resemblance of this structure with those found in other countries (Verdet *et al.*, 2006; Mata *et al.*, 2011) suggests the importation of this plasmid. We must highlight the importance of the IS*CR1* mobile element, here detected upstream of $bla_{\text{DHA-1}}$ gene, in the movement of PMAβ genes and other resistance determinants (Mata *et al.*, 2012).

The study of the environment of $bla_{\text{CMY-2}}$ gene revealed a complex structure containing the mobile element IS*Ecp1*, that might had been responsible for the mobilization of bla_{CMY} gene from the *C. freundii* chromosome, along with the *blc-sugE-ΔecnR* structure, encoding an outer membrane lipoprotein, a small multidrug resistance protein and a truncated transcriptional regulatory protein, respectively. The 3′ region of the gene contained four *orfs*, *dsbc*, and *traC* genes, also described in *Salmonella* spp. and *E. coli* isolates (Kang *et al.*, 2006), that might be implicated in the spread of this important resistance determinant. Additionally, INSRA7609 and the respective transconjugant also contained two other resistance regions, specifically a TEM-1 β-lactamase nearby a truncated *mer* operon and a class 1 integron encompassing the detected resistance to trimethoprim, through expression of *dfrA1* gene.

The genetic features that might be favouring the dissemination of such important genes, constitute useful markers to follow their path within bacterial populations and across the different geographical locations, specifically to identify plasmid importation and thus detect and prevent their emergence in clinical settings, as previously reported in other countries (Aarestrup *et al.*, 2004; Call *et al.*, 2010; Ho *et al.*, 2013). The production of either chromosomal or plasmid-mediated AmpC β-lactamases is a matter of great concern since they grant resistance to most of the β-lactam antibiotics, excluding only the fourth generation cephalosporins and carbapenems (Maltezou *et al.*, 2009).

Concerning carbapenemases, although their occurrence had already been described worldwide in association with important therapy failure scenarios, we did not find levels of carbapenem susceptibility that suggested their molecular search along with the phenotypic screening (Patel and Bonomo, 2011). Nevertheless, carbapenem susceptibility monitoring is crucial to maintain clinical efficacy of these antibiotics in each country and continent (Castanheira *et al.*, 2011; EARS-Net, 2012).

In summary, we report a worrying multidrug resistance scenario that includes an overwhelming occurrence of ESBLs, the settling of CTX-M β-lactamases, and the report of AmpCs in Portugal. The β-lactamase diversity, along with the internationally disseminated PMAβ-harbouring plasmids, also containing other antibiotic resistance genes and mobile genetic elements, highlights the global spread of such determinants in clinical settings, also empathising the difficulty to treat such life threatening infections and possible dissemination cross borders.

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References

Aarestrup, F.M., Hasman, H., Olsen, I., and Sørensen, G. 2004. International spread of *bla*$_{CMY-2}$-mediated cephalosporin resistance in a multiresistant *Salmonella enterica* serovar Heidelberg isolate stemming from the importation of a boar by Denmark from Canada. *Antimicrob. Agents Chemother*. **48**, 1916–1917.

Adriaenssens, N., Coenen, S., Versporten, A., Muller, A., Vankerckhoven, V., Goossens, H., and Group, E.P. 2011. European surveillance of antimicrobial consumption (ESAC): Quality appraisal of antibiotic use in europe. *J. Antimicrob. Chemother*. **66** Suppl 6, vi71–77.

Alouache, S., Estepa, V., Messai, Y., Ruiz, E., Torres, C., and Bakour, R. 2013. Characterization of ESBLs and associated quinolone resistance in *Escherichia coli* and *Klebsiella pneumoniae* isolates from an urban wastewater treatment plant in Algeria. *Microb. Drug Resist*. **20**, 30–38.

Bradford, P.A. 2001. Extended-spectrum β-lactamases in the 21st century: Characterization, epidemiology, and detection of this important resistance threat. *Clin. Microbiol. Rev.* **14**, 933–951.

Briales, A., Rodríguez-Martínez, J.M., Velasco, C., de Alba, P.D., Rodríguez-Baño, J., Martínez-Martínez, L., and Pascual, A. 2012. Prevalence of plasmid-mediated quinolone resistance determinants *qnr* and *aac(6′)-Ib-cr* in *Escherichia coli* and *Klebsiella pneumoniae* producing extended-spectrum β-lactamases in Spain. *Int. J. Antimicrob. Agents* **39**, 431–434.

Call, D.R., Singer, R.S., Meng, D., Broschat, S.L., Orfe, L.H., Anderson, J.M., Herndon, D.R., Kappmeyer, L.S., Daniels, J.B., and Besser, T.E. 2010. *bla*$_{CMY-2}$-positive IncA/C plasmids from *Escherichia coli* and *Salmonella enterica* are a distinct component of a larger lineage of plasmids. *Antimicrob. Agents Chemother*. **54**, 590–596.

Cambray, G., Guerout, A.M., and Mazel, D. 2010. Integrons. *Annu. Rev. Genet*. **44**, 141–166.

Caniça, M.M., Barthélémy, M., Gilly, L., Labia, R., Krishnamoorthy, R., and Paul, G. 1997. Properties of IRT-14 (TEM-45), a newly characterized mutant of TEM-type β-lactamases. *Antimicrob. Agents Chemother*. **41**, 374–378.

Carattoli, A., Bertini, A., Villa, L., Falbo, V., Hopkins, K.L., and Threlfall, E.J. 2005. Identification of plasmids by PCR-based replicon typing. *J. Microbiol. Methods* **63**, 219–228.

Castanheira, M., Mendes, R.E., Woosley, L.N., and Jones, R.N. 2011. Trends in carbapenemase-producing *Escherichia coli* and *Klebsiella* spp. from Europe and the Americas: Report from the sentry antimicrobial surveillance programme (2007-09). *J. Antimicrob. Chemother*. **66**, 1409–1411.

D'Andrea, M.M., Arena, F., Pallecchia, L., and Rossolini, G.M. 2013. CTX-M-type β-lactamases: A successful story of antibiotic resistance. *Int. J. Med. Microbiol*. **303**, 305–317.

EARS-Net. 2012. Antimicrobial resistance surveillance in Europe 2011. Annual report of the European Antimicrobial Resistance Surveillance Network (EARS-Net). European Centre for Disease Prevention and Control (ECDC), Stockholm, Sweden.

Freitas, F., Machado, E., Ribeiro, T.G., Novais, A., and Peixe, L. 2013. Long-term dissemination of acquired AmpC β-lactamases among *Klebsiella* spp. and *Escherichia coli* in Portuguese clinical settings. *Eur. J. Clin. Microbiol. Infect. Dis.* doi:10.1007/s10096-013-1983-9.

Grundmann, H., Livermore, D.M., Giske, C.G., Canton, R., Rossolini, G.M., Campos, J., Vatopoulos, A., Gniadkowski, M., Toth, A., Pfeifer, Y., and *et al.* 2010. Carbapenem-non-susceptible *Enterobacteriaceae* in *Europe*: Conclusions from a meeting of national experts. *Euro Surveill.* **15**.

Ho, P.L., Lo, W.U., Chan, J., Cheung, Y.Y., Chow, K.H., Yam, W.C., Lin, C.H., and Que, T.L. 2013. pIMP-PH114 carrying *bla*$_{IMP-4}$ in a *Klebsiella pneumoniae* strain is closely related to other multidrug-resistant IncA/C2 plasmids. *Curr. Microbiol*. doi: 10.1007/s00284-013-0471-x.

Jacoby, G.A. 2009. Ampc β-lactamases. *Clin. Microbiol. Rev.* **22**, 161–182.

Jones-Dias, D., Manageiro, V., Francisco, A.P., Martins, A.P., Domingues, G., Louro, D., Ferreira, E., and Caniça, M. 2013. Assessing the molecular basis of transferable quinolone resistance in *Escherichia coli* and *Salmonella* spp. from food producing animals and food products. *Vet. Microbiol*. doi: 265 10.1016/j.vetmic.2013.08.010.

Kang, M.S., Besser, T.E., and Call, D.R. 2006. Variability in the region downstream of the *bla*$_{CMY-2}$ β-lactamase gene in *Escherichia coli* and *Salmonella enterica* plasmids. *Antimicrob. Agents Chemother*. **50**, 1590–1593.

Leverstein-van Hall, M.A., Box, A.T., Blok, H.E., Paauw, A., Fluit, A.C., and Verhoef, J. 2002. Evidence of extensive interspecies transfer of integron-mediated antimicrobial resistance genes among multidrug-resistant *Enterobacteriaceae* in a clinical setting. *J. Infect. Dis.* **186**, 49–56.

Livermore, D.M. 2009. β-Lactamases-the threat renews. *Curr. Protein Pept. Sci.* **10**, 397–400.

Livermore, D.M., Canton, R., Gniadkowski, M., Nordmann, P., Rossolini, G.M., Arlet, G., Ayala, J., Coque, T.M., Kern-Zdanowicz, I., Luzzaro, F., and *et al.* 2007. CTX-M: Changing the face of ESBLs in Europe. *J. Antimicrob. Chemother*. **59**, 165–174.

Machado, E., Coque, T.M., Cantón, R., Baquero, F., Sousa, J.C., Peixe, L., and the Portuguese Resistance Study Group. 2006. Dissemination in Portugal of CTX-M-15-, OXA-1-, and TEM-1-producing *Enterobacteriaceae* strains containing the *aac(6′)-ib-cr* gene, which encodes an aminoglycoside- and fluoroquinolone-modifying enzyme. *Antimicrob. Agents Chemother.* **50**, 3220–3221.

Maltezou, H.C., Giakkoupi, P., Maragos, A., Bolikas, M., Raftopoulos, V., Papahatzaki, H., Vrouhos, G., Liakou, V., and Vatopoulos, A.C. 2009. Outbreak of infections due to KPC-2-producing *Klebsiella pneumoniae* in a hospital in Crete (Greece). *J. Infect.* **58**, 213–219.

Manageiro, V., Ferreira, E., Jones-Dias, D., Louro, D., Pinto, M., Diogo, J., and Caniça, M. 2012. Emergence and risk factors of β-lactamase-mediated resistance to oxyimino-β-lactams in *Enterobacteriaceae* isolates. *Diagn. Microbiol. Infect. Dis.* **72**, 272–277.

Mata, C., Miró, E., Alvarado, A., Garcillán-Barcia, M.P., Toleman, M., Walsh, T.R., de la Cruz, F., and Navarro, F. 2012. Plasmid typing and genetic context of AmpC β-lactamases in *Entero-*

bacteriaceae lacking inducible chromosomal *ampc* genes: Findings from a spanish hospital 1999-2007. *J. Antimicrob. Chemother.* **67**, 115–122.

Mata, C., Miró, E., Toleman, M., Rivera, M.A., Walsh, T.R., and Navarro, F. 2011. Association of bla_{DHA}-1 and *qnrB* genes carried by broad-host-range plasmids among isolates of *Enterobacteriaceae* at a spanish hospital. *Clin. Microbiol. Infect.* **17**, 1514–1517.

Mendonça, N., Ferreira, E., Louro, D., ARSIP Participants, and Caniça, M. 2009. Molecular epidemiology and antimicrobial susceptibility of extended- and broad-spectrum β-lactamase-producing *Klebsiella pneumoniae* isolated in Portugal. *Int. J. Antimicrob. Agents* **34**, 29–37.

Mendonça, N., Leitão, J., Manageiro, V., Ferreira, E., and Caniça, M. 2007. Spread of extended-spectrum β-lactamase CTX-M-producing *Escherichia coli* clinical isolates in community and nosocomial environments in Portugal. *Antimicrob. Agents Chemother.* **51**, 1946–1955.

Mendonça, N., Louro, D., Castro, A.P., Diogo, J., and Caniça, M. 2006. CTX-M-15, OXA-30 and TEM-1-producing *Escherichia coli* in two portuguese regions. *J. Antimicrob. Chemother.* **57**, 1014–1016.

Nicolas-Chanoine, M.H., Blanco, J., Leflon-Guibout, V., Demarty, R., Alonso, M.P., Caniça, M.M., Park, Y.J., Lavigne, J., Pitout, J., and Johnson, J.R. 2008. Intercontinental emergence of *Escherichia coli* clone O25:H4-ST131 producing CTX-M-15. *J. Antimicrob. Chemother.* **61**, 273–281.

Patel, G. and Bonomo, R.A. 2011. Status report on carbapenemases: Challenges and prospects. *Expert. Rev. Anti. Infect. Ther.* **9**, 555–570.

Paterson, D.L. and Bonomo, R.A. 2005. Extended-spectrum β-lactamases: A clinical update. *Clin. Microbiol. Rev.* **18**, 657–686.

Philippon, A., Arlet, G., and Jacoby, G.A. 2002. Plasmid-determined AmpC-type β-lactamases. *Antimicrob. Agents Chemother.* **46**, 1–11.

Queenan, A.M. and Bush, K. 2007. Carbapenemases: The versatile β-lactamases. *Clin. Microbiol. Rev.* **20**, 440–458.

Robicsek, A., Strahilevitz, J., Jacoby, G.A., Macielag, M., Abbanat, D., Park, C.H., Bush, K., and Hooper, D.C. 2006. Fluoroquinolone-modifying enzyme: A new adaptation of a common aminoglycoside acetyltransferase. *Nat. Med.* **12**, 83–88.

Silva-Sánchez, J., Cruz-Trujillo, E., Barrios, H., Reyna-Flores, F., Sánchez-Pérez, A.; Bacterial Resistance Consortium, and Garza-Ramos, U. 2013. Characterization of plasmid-mediated quinolone resistance (PMQR) genes in extended-spectrum β-lactamase-producing *Enterobacteriaceae* pediatric clinical isolates in Mexico. *PLoS ONE* **8**, e77968.

Toleman, M.A. and Walsh, T.R. 2011. Combinatorial events of insertion sequences and ICE in Gram-negative bacteria. *FEMS Microbiol. Rev.* **35**, 912–935.

Verdet, C., Benzerara, Y., Gautier, V., Adam, O., Ould-Hocine, Z., and Arlet, G. 2006. Emergence of DHA-1-producing *Klebsiella* spp. in the Parisian region: Genetic organization of the *ampC* and *ampR* genes originating from *Morganella morganii*. *Antimicrob. Agents Chemother.* **50**, 607–617.

Yano, H., Uemura, M., Endo, S., Kanamori, H., Inomata, S., Kakuta, R., Ichimura, S., Ogawa, M., Shimojima, M., Ishibashi, N., and *et al.* 2013. Molecular characteristics of extended-spectrum β-lactamases in clinical isolates from *Escherichia coli* at a Japanese tertiary hospital. *PLoS ONE* **8**, e64359.