Carla Alexandra Parada Rodrigues

Enterobacteriaceae resistant to extended-spectrum β-lactam antibiotics in different settings in Portugal: towards an epidemiological characterization of a Public Health priority

Mestrado em Microbiologia Clínica

Universidade Fernando Pessoa
www.ufp.pt

Faculdade de Ciências de Saúde

Porto, Dezembro de 2012

Enterobacteriaceae resistant to extended-spectrum β-lactam antibiotics in different settings in Portugal: towards an epidemiological characterization of a Public Health priority

Carla Alexandra Parada Rodrigues

Ass:_____

Tutors:

Doutora Ângela Patricia da Silva Novais Amorim

(REQUIMTE - Faculdade de Farmácia da Universidade do Porto)

Prof. Doutora Elisabete Maria Pereira Machado

(Faculdade de Ciências da Saúde da Universidade Fernando Pessoa)

Dissertation presented to

Universidade Fernando Pessoa

to obtain the M.Sc. Degree in Clinical Microbiology

Resumo

O aumento de β-lactamases de espetro alargado (ESBLs) e carbapenemases quer a nível hospitalar quer a nível da comunidade tem vindo a comprometer a utilização dos antibióticos β-lactâmicos no tratamento de infeções causadas por *Enterobacteriaceae*. Em Portugal, uma elevada ocorrência de ESBLs tem sido reportada enquanto que algumas carbapenemases foram apenas recentemente identificadas. Contudo, os dados epidemiológicos recentes sobre a ocorrência e diversidade de *Enterobacteriaceae* produtoras de ESBLs e/ou carbapenemases em Portugal são escassos. O principal objetivo deste estudo é caraterizar a diferentes níveis a epidemiologia molecular de isolados recentes de *Enterobacteriaceae* (2006-2010) resistentes a cefalosporinas de espetro alargado e/ou carbapenemos, provenientes de diferentes nichos ecológicos (hospitais, suiniculturas).

Trezentos e dois isolados de *Enterobacteriaceae* obtidos entre 2006 e 2010 de diferentes origens (5 hospitais, 2 suiniculturas), foram analisados. Estes isolados incluíam: i) 264 isolados produtores de ESBLs de 3 hospitais; ii) 16 isolados com redução da suscetibilidade aos carbapenemos de 2 hospitais e iii) 22 isolados produtores de ESBLs de animais, rações e ambiente de 2 suiniculturas Portuguesas. A identificação bacteriana e o estudos de suscetibilidade a antibióticos foram efectuados por métodos clássicos. Os genes que codificam para ESBL ou carbapenemases e o seu ambiente genético foram identificados por PCR (*bla*$_{\text{CTX-M}}$, *bla*$_{\text{SHV}}$, *bla*$_{\text{TEM}}$, *bla*$_{\text{KPC,}}$ *bla*$_{\text{MBL,}}$ *bla*$_{\text{OXA}}$) e sequenciação. A relação clonal entre isolados foi estabelecida por XbaI-PFGE e MLST. A análise de plasmídeos incluiu a identificação de grupos de incompatibilidade por PCR, sequenciação e hibridação, RFLP e pMLST. As alterações em porina foram investigados por PCR e SDS-PAGE.

A resistência a cefalosporinas de espetro alargado foi frequentemente associada a *Escherichia coli* e *Klebsiella pneumoniae*, mas também a *Enterobacter cloacae*, *Klebsiella oxytoca*, *Proteus mirabilis* e *Serratia marcescens*. Foi detetada uma grande diversidade de ESBLs, sendo que as enzimas do tipo CTX-M (CTX-M-1, -2, -9, -14, -15, -32, -79) e SHV (SHV-2, -5, -12, -28, -55, -106, -145) foram as mais predominantes em *E. coli* e *K. pneumoniae* respetivamente, enquanto que as enzimas do tipo TEM foram

detetadas com menor frequência (TEM-10, -24, -52, -116, -199) em diferentes espécies de enterobactérias. Apesar de ter sido observada uma elevada diversidade clonal (264 isolados/86 pulsotipos), foram identificados clones epidémicos de *E. coli* (ST131) e *K. pneumoniae* (ST15, ST147, ST336) associados à disseminação de determinadas ESBLs (CTX-M-15, diferentes variantes de SHV) durante longos períodos de tempo em diferentes hospitais. Neste estudo, foi identificado um reservatório de genes $bla_{\text{CTX-M-1/-32}}$ e $bla_{\text{TEM-52}}$ em suiniculturas Portuguesas associado à disseminação de plasmídeos (IncI1/ST3 e IncN, respetivamente) e clones (*E. coli* complexo clonal 10) epidémicos frequentemente identificados em humanos e outros animais em diversos países. Foram identificados isolados com suscetibilidade reduzida aos carbapenemos em: i) um surto nosocomial que envolveu diferentes espécies de *Enterobactericeae* com alterações em porinas e produção de ESBLs ou AmpCs plasmídicas; e ii) um isolado clínico de *K. pneumoniae* (ST15) produtor de uma nova variante de VIM-1, designada VIM-34.

Neste trabalho é descrita uma epidemiologia complexa entre as *Enterobacteriaceae* produtoras de ESBLs em Portugal, envolvendo uma diversidade de ESBLs, clones (alguns contendo genes bla_{ESBL} filogeneticamente relacionados) e plasmídeos. A identificação de plasmídeos e clones idênticos produtores de ESBL entre isolados de origem humana e animal sugere um papel relevante da cadeia alimentar na disseminação de bactérias produtoras de ESBLs. A deteção de isolados com suscetibilidade diminuída aos carbapenemos (devido à produção de carbapenemases ou ESBL/AmpC e alterações em porina) pertencentes a clones epidémicos em hospitais Portugueses foi importante para implementar medidas de controle de infeção adequadas e oportunas e reforçar os sistemas de vigilância.

Abstract

The expansion of extended-spectrum β-lactamases (ESBLs) and carbapenemases in both nosocomial and community settings has seriously compromised the use of β-lactam antibiotics in the treatment of infections caused by *Enterobacteriaceae*. In Portugal, a high occurrence of ESBLs has been reported and carbapenemase-producing isolates have recently emerged, although recent epidemiological data on the occurrence, diversity, and epidemiological features of ESBL- or carbapenemase-producing *Enterobacteriaceae* are scarce. The global goal of this study is the multi-level molecular epidemiological characterization of recent *Enterobacteriaceae* isolates (2006-2010) resistant to extended-spectrum cephalosporins and/or carbapenems obtained from different ecological niches (hospitalized patients, pig farms).

Three hundred and two *Enterobacteriaceae* isolates obtained from different sources (5 hospitals, 2 piggeries) between 2006 and 2010 were studied. They included i) 264 ESBL-producing isolates from 3 hospitals; ii) 16 isolates with reduced susceptibility to carbapenems from 2 hospitals; and iii) 22 ESBL-producing isolates from animals, feed and environmental samples of two Portuguese piggeries. Bacterial identification and antibiotic susceptibility testing were performed by standard methods. ESBL or carbapenemase genes and their genetic environment were identified by PCR (*bla*$_{\text{CTX-M}}$, *bla*$_{\text{SHV}}$, *bla*$_{\text{TEM}}$, *bla*$_{\text{KPC,}}$ *bla*$_{\text{MBL,}}$ *bla*$_{\text{OXA}}$) and sequencing. Clonal relatedness was established by *Xba*I-PFGE and MLST. Plasmid analysis included identification of incompatibility groups by PCR, sequencing and hybridization, RFLP and pMLST. Porin changes were investigated by PCR and SDS-PAGE.

Resistance to extended-spectrum cephalosporins was mostly observed in *Escherichia coli* and *Klebsiella pneumoniae*, and also *Enterobacter cloacae, Klebsiella oxytoca, Proteus mirabilis* and *Serratia marcescens*. A high diversity of ESBLs was detected, mostly from CTX-M (CTX-M-1, -2, -9, -14, -15, -32, -79) and SHV-types (SHV-2, -5, -12, -28, -55, -106, -145), particularly among *E. coli* and *K. pneumoniae* isolates, respectively, while the TEM-type enzymes were sporadically reported (TEM-10, -24, -52, -116, -199) in different *Enterobacteriaceae* species. Despite the high clonal diversity observed (264 isolates/86 PFGE-types), a few epidemic *E. coli* (ST131) and *K.*

pneumoniae (ST15, ST147, ST336) clones were associated with the dissemination of particular ESBL-types (CTX-M-15, different SHV-variants) during large periods of time in different hospitals. CTX-M-1/-32 or TEM-52 types were detected among Portuguese piggeries associated with the spread of epidemic plasmids (IncI1/ST3 and IncN, respectively) and clones (*E. coli* clonal complex 10), frequently identified in humans and other animals in several countries. Reduced susceptibility to carbapenems was detected: i) in a nosocomial outbreak involving different ESBL or plasmid-mediated AmpC-producing *Enterobacteriaceae* isolates exhibiting porin alterations; and ii) in a ST15 *K. pneumoniae* clinical isolate producing a novel VIM-1 variant, designated VIM-34.

A complex epidemiology of ESBL-producing *Enterobactericeae* in Portugal is described, with the involvement of a diversity of ESBL-types, epidemic clones (in some cases harbouring closely related bla_{ESBL} genes) and plasmids. The identification of identical ESBL-encoding plasmids and clones between isolates from human and animal origins stresses a link through the food chain. The detection of strains with decreased susceptibility to carbapenems (either by carbapenemase production or ESBL/AmpC production plus porin changes) belonging to widespread clones in Portuguese hospitals was important to implement appropriate and timely infection control measures, and to reinforce continuous surveillance systems.

Enterobacteriaceae resistant to extended-spectrum β-lactam antibiotics in different settings in Portugal: towards an epidemiological characterization of a Public Health priority

This work was financially supported by research grants from:

- Fundação para a Ciência e Tecnologia, Ministério da Ciência, Tecnologia e Ensino Superior of Portugal (grants numbers PEst-C/EQB/ LA0006/2011 and POCI/AMB/61814/2004-FSE/FEDER
- Fundação Ensino e Cultura Fernando Pessoa
- Marie Curie Intra European Fellowship within the 7th European Community Framework Programme (grant number PIEF-GA-2009-255512 to Ângela Novais)
- REQUIMTE (Laboratório Associado)

Aos meus pais,

Por tudo o que são para mim.

“O único lugar onde o sucesso vem antes do trabalho é no dicionário.”

Albert Einstein

Agradecimentos

Em primeiro lugar, gostaria de agradecer a alguém muito especial e que esteve comigo desde o início desta aventura pelo mundo das bactérias, a Doutora Ângela Novais. Por muito que escreva as palavras nunca serão suficientes para expressar a minha admiração, carinho, gratidão e amizade. Muito obrigada pelo empenho, confiança, apoio incondicional e expetativas depositadas em mim. Obrigado pela excelente orientação, disponibilidade total em ensinar-me e por partilhar comigo a sua imensa experiência profissional.

À Prof. Doutora Elisabete Machado pelas palavras de carinho, incentivo, força e motivação proferidas sempre nas alturas certas. Obrigado pela amizade, pelo apoio, compreensão, pela transmissão de conhecimentos e por ter acreditado em mim e no meu trabalho.

Gostaria de agradecer a alguém por quem nutro uma enorme admiração e que me proporcionou a integração numa equipa de trabalho maravilhosa, a Prof. Doutora Luísa Peixe. Muito obrigado por me ter permitido realizar o meu trabalho de mestrado na sua equipa, por me fornecer os meios para a realização do mesmo e por partilhar comigo a sua experiência, mas também pelo carinho, amizade e por acreditar em mim.

Gostaria também de agradecer ao Prof. Doutor João Carlos Sousa, pela oportunidade de realizar este trabalho no âmbito do Mestrado em Microbiologia Clínica na Universidade Fernando Pessoa.

Ao REQUIMTE e à Faculdade de Farmácia da Universidade do Porto, e mais especificamente ao departamento de Microbiologia, por me proporcionarem as condições necessárias à realização deste trabalho.

À Prof. Doutora Carla Novais e à Prof. Doutora Patrícia Antunes pelos conselhos prestados e pela simpatia.

Às amigas e companheiras de bancada, Joana Campos, Joana Mourão, Teresa Ribeiro, Raquel Branquinho, Eduarda Silveira, Filipa Grosso, Clara Sousa, Liliana Silva, Mariana Barros e ao João Pires, o meu muito obrigado por tudo… Obrigado pelas gargalhadas intermináveis, pelo companheirismo, pelo apoio, pelas noitadas infindáveis (sobretudo nesta última fase), por me aturarem (o que nem sempre é fácil!!)…mas mais do que tudo, pela amizade!! Obrigado por fazerem com que todos os dias de trabalho valham a pena.

À Cristina Costa e ao Nuno Oliveira pela disponibilidade, amizade e boa disposição com que sempre pude contar.

Às amigas de uma vida, Maria João, Mafalda, Xana, Bruna e Krys, obrigado pelo apoio incondicional, por perdoarem a minha ausência e por torcerem por mim em todos os momentos…obrigado por estarem sempre lá.

Ao Luís, companheiro de anos, obrigado pelo amor, apoio e compreensão. Sei que nem sempre foi fácil lidar com a distância e ausência, mas mesmo assim estiveste sempre lá. Obrigada.

Aos meus pais e ao meu irmão…sem dúvida as pessoas mais importantes da minha vida… Obrigado por acreditarem em mim, por me obrigarem a lutar pelos meus sonhos e por contribuírem para a pessoa que sou hoje…Sem vocês tudo teria sido mais difícil, nem teria alcançado as minhas metas.

Um muito obrigado a todos os que de forma direta ou indireta contribuíram para a realização deste trabalho.

"Unir-se é um bom começo, manter a união é um progresso, e trabalhar em conjunto é a vitória."

Henry Ford

Table of Contents

1. **Introduction** 3

1.1. **Antibiotic resistance among *Enterobacteriaceae*** 3

1.1.1. Ecology and clinical relevance of *Enterobacteriaceae* 3

1.1.2. Resistance to extended-spectrum β-lactam antibiotics 4

i. Production of β-lactamases 5

ii. Modifications in membrane permeability 15

iii. Increased activity of efflux pumps 16

1.1.3. Resistance to non-β-lactam antibiotics 17

1.2. **Dissemination of β-lactamase (*bla*) genes among *Enterobacteriaceae*** 18

1.2.1. Clonal spread 19

1.2.2. Horizontal gene transfer 22

1.3. **Reservoirs of β-lactam (*bla*) genes** 26

1.4. **References** 28

2. **Aims of the study** 45

3. **Results** 47

3.1. **Epidemiology of extended-spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae* in the clinical setting** 48

3.1.1. Current spread of CTX-M genes in Portuguese hospitals is associated with widespread *Escherichia coli* clones from different phylogenetic groups 49

3.1.2. Amplification of ST15, ST147 and ST336 *Klebsiella pneumoniae* clones producing different extended-spectrum β-lactamases in Portuguese hospitals 65

3.2. **Epidemiology of extended-spectrum β-lactamase (ESBL)-producing Enterobacteriaceae in food-producing animals and farms**..... 79

3.2.1. Emergence of TEM-52 and CTX-M-32 in healthy pigs associated with ST10 complex Escherichia coli isolates and common IncI1/ST3 and IncN plasmids ... 80

3.3. Emergence of carbapenemase-producing *Enterobacteriaceae* in Portugal..... 94

3.3.1. First report of VIM-34, a new VIM-1 variant identified in a ST15 *Klebsiella pneumoniae* isolate in Portugal 94

3.3.2. Spread of carbapenem resistance mediated by porin alterations in *Enterobacteriaceae* from Portuguese clinical settings 102

4. **Conclusions**..... 110

5. **Appendix**..... 114

5.1 Communications in international meetings…….. 117

5.2 Communications in national meetings…...…….. 117

5.3 Sequences submitted to GenBank…….....……..118

5.4 Participation in scientific projects…….....……..119

List of Figures

Figure 1. Mechanisms of resistance to β-lactam antibiotics in Gram-negative bacteria 5

Figure 2. Global distribution of CTX-M-enzymes 10

Figure 3. Distribution of KPC producers across Europe 12

Figure 4. Wordwide (A) and European (B) geographic distribution of VIM- and IMP-producing *Enterobacteriaceae* 13

Figure 5. Spread of OXA-48 and OXA-48-like carbapenemases 14

Figure 6. Cell wall and cytoplasmic membrane of Gram-negative bacteria 15

Figure 7. Schematic diagram of representative drug exporting systems involved in resistance in Gram-negative bacteria 17

Figure 8. Mechanisms of dissemination of antibiotic resistance genes. 19

Figure 9. Population snapshot of widespread *E. coli* clones within the entire MLST database obtained by E-burst V3 (http://eburst.mlst.net/) 21

Figure 10. The modular and hierarchical composition of mobile genetic elements 23

Figure 11. Microorganisms and environment: transmission pathways 27

List of Tables

Table 1. β-lactamase classification schemes 7

Table 2. Major plasmid families and associated resistance genes in antibiotic resistant *Enterobacteriaceae* isolated worldwide from human and animal sources..... 24

1. Introduction

1. Introduction

1.1. Antibiotic resistance among *Enterobacteriaceae*

1.1.1. Ecology and clinical relevance of *Enterobacteriaceae*

Enterobacteriaceae constitute a large family of Gram-negative bacteria comprising more than 40 genera and over 130 species with ubiquitous distribution, being found amongst the intestinal commensal flora of humans and animals, and also in different ecological niches such as plants, soil or water (Murray *et al.*, 2003). However, some *Enterobacteriaceae* species are well recognized gastrointestinal pathogens (causing mild to severe syndromes) or opportunistic pathogens, being one of the most important causes of nosocomial or community acquired infections (Murray *et al.*, 2003; Paterson, 2006). *Escherichia* sp., *Salmonella* sp., *Shigella* sp. and *Yersinia* sp. include species commonly associated with gastrointestinal tract infections (Murray *et al.*, 2003), whereas species *Escherichia coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Enterobacter* spp., *Proteus mirabilis* and *Serratia marcescens* are mainly involved in extraintestinal infections, especially urinary tract infections (UTIs), pneumonia or septicemia (Murray *et al.*, 2003; Gaynes and Edwards, 2005; Paterson and Bonomo, 2005).

E. coli and *K. pneumoniae* are the most frequently isolated *Enterobacteriaceae* species from human clinical samples (Murray *et al.*, 2003). In, particular, *E coli* is the most common opportunistic pathogen associated with extraintestinal infections, mainly UTIs, in the nosocomial or the community settings (Riley, 2004). The analysis of the enzymatic profiles obtained by *multilocus enzyme electrophoresis* (MLEE) allowed the classification of *E. coli* population into four main phylogenetic groups and corresponding subgroups: A (A_0, A_1), B1, B2 ($B2_2$, $B2_3$) and D (D_1, D_2) (Ochman and Selander, 1984; Herzer *et al.*, 1990; Clermont *et al.*, 2000). Multiple studies focusing on the characterization of *E. coli* population structure have traditionally suggested a relationship between phylogeny and pathogenicity in this species, with virulent extraintestinal strains belonging mainly to B2 and D phylogroups, and commensal strains to phylogenetic groups A and B1 (Clermont *et al.*, 2000; Duriez *et al.*, 2001;

Johnson *et al.*, 2001; Pitout, 2012). However, recent epidemiological data highlights the increasing identification of A and B1 *E. coli* involved in extraintestinal infections, suggesting a more promiscuous evolutionary history for *E. coli* (Moreno *et al.*, 2006; Oteo *et al.*, 2009; Valverde *et al.*, 2009; Cooke *et al.*, 2010; Rodríguez-Baño *et al.*, 2012).

K. pneumoniae or *Enterobacter* spp. are opportunistic nosocomial pathogens, which have been associated with outbreaks mainly in intensive care units (ICUs) and newborn units (NUs), representing a high clinical risk especially in immunocompromised patients (Murray *et al.*, 2003; Riley, 2004). Brisse *et al*. (2004) developed a scheme to classify *K. pneumoniae* isolates in three distinct phylogenetic groups (KpI, KpII and KpIII), with KpI being the most frequently detected in clinical samples (Brisse *et al.*, 2004). Other *Enterobactericeae* species, such as *P. mirabilis*, *S. marcescens*, or *Citrobacter* spp., have less frequently been reported in nosocomial infections (Murray *et al.*, 2003).

1.1.2. Resistance to extended-spectrum β-lactam antibiotics

β-lactam antibiotics (mainly extended-spectrum cephalosporins and carbapenems) are considered first-line therapeutic options for the treatment of nosocomial and community infections caused by Enterobacteriaceae isolates, given their therapeutic efficacy, low cost and the low toxicity to humans and animals. Resistance to β-lactams has been reported extensively over the last decades, becoming a serious public health problem that requires full attention and appropriate management (Coque *et al*., 2008a; Nordmann *et al*., 2011a). β-lactams were introduced in the clinical practice in 1940, and since then have become the most widely used class of antibiotics. They are characterized by the presence of a β-lactam ring, essential for their activity, acting by inhibition of bacterial transpeptidases (known as PBPs- Penicillin Binding Proteins), which are involved in the synthesis of peptidoglycan, the main component of the bacterial cell wall (Sousa, 2006). Based on their chemical structure, β-lactams can be divided in four different groups: penicillins, cephalosporins, monobactams and carbapenems. Some β-lactams have a narrow antimicrobial spectrum (penicillins, first- and second-generation cephalosporins) being active mainly in Gram-positive bacteria,

while others have a extended-spectrum (third- and fourth-generation cephalosporins and carbapenems), acting on both Gram-negative and Gram-positive bacteria (Sousa, 2006).

The most common resistance mechanism to β-lactam antibiotics among Gram-negative bacteria is the production of enzymes known as β-lactamases, able to hydrolyse the β-lactam ring. Other resistance mechanisms include the alteration of the antibiotic target (more common among Gram-positive bacteria), changes in membrane permeability (mediated by loss/alteration of porins) and increased activity of efflux pump systems (Figure 1) (Sousa, 2006; Pfeifer *et al.*, 2010).

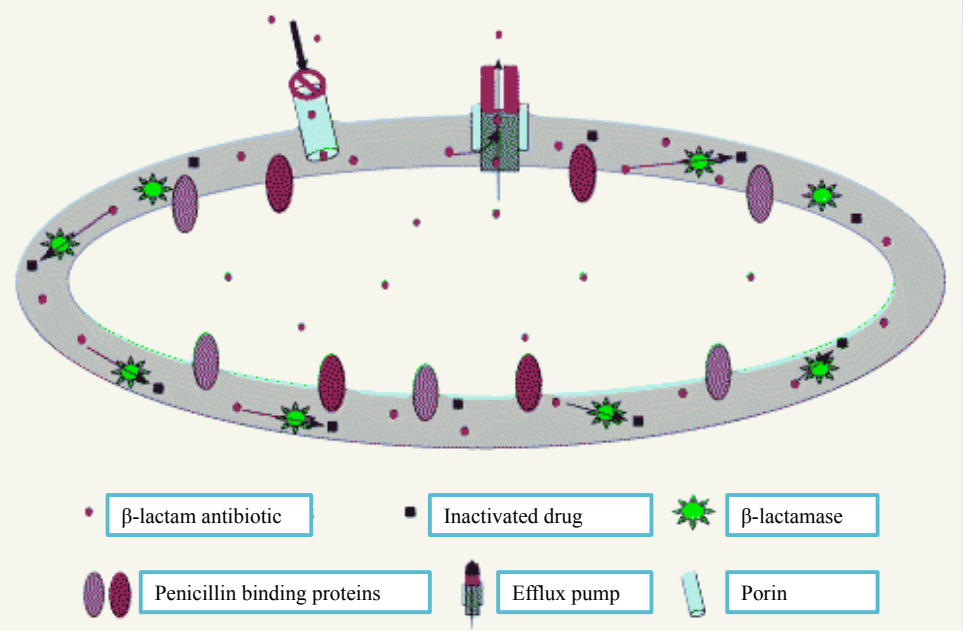


Figure 1. Mechanisms of resistance to β-lactam antibiotics in Gram-negative bacteria (adapted from Thomson and Bonomo, 2005)

i. Production of β-lactamases

β-lactamase production constitutes the most important mechanism of resistance to β-lactam antibiotics among *Enterobacteriaceae* (Pitout, 2010). As previously referred, these enzymes inactivate the β-lactams by hydrolysis of the β-lactam ring. A variety of schemes have been proposed for the classification of the high diversity of β-lactamase

enzymes that have been identified, being the most widely used based on the chemical constitution of their active site (serine or zinc) or their hydrolytic profiles over distinct β-lactams (penicillins, cephalosporins, monobactams, carbapenems) and their inactivation by classical β-lactamase inhibitors (clavulanic acid, sulfabactam and tazobactam) (Ambler, 1980; Bush and Jacoby, 2010). They are: i) the Ambler molecular classification scheme and ii) the Bush-Jacoby-Medeiros functional classification scheme (Table 1) (Ambler, 1980; Bush *et al.*, 1995; Bush and Jacoby, 2010).

i) Ambler classification scheme: divides β-lactamases into four major classes (A, B, C and D) based on amino acid sequence homology (phenotypic characteristics are not considered). In this classification, β-lactamases of class A, C and D are serine-β-lactamases (serine residue in the active site), whereas class B enzymes are metallo-β-lactamases (zinc atom in the active site) (Ambler, 1980; Paterson and Bonomo, 2005).

ii) Bush-Jacoby-Medeiros classification scheme: divides β-lactamases into four main groups, based on functional similarities (substrate and inhibitor profile) (Bush *et al.*, 1995; Bush and Jacoby, 2010):

- Group 1/Ambler class C: includes cephalosporinases that are encoded in the chromosome and/or in plasmids, and that are not inhibited by the classical β-lactamase inhibitors;
- Group 2/Ambler classes A and D: represent the largest and heterogeneous group of β-lactamases and includes penicillinases, cephalosporinases, oxacillinases and carbapenemases, which are inhibited by the classical β-lactamase inhibitors. Different subgroups are further considered, one of which includes the extended-spectrum β-lactamases (ESBLs) (2be subgroup);
- Group 3/ Ambler class B: includes metallo-β-lactamases, which are enzymes with a high hydrolytic activity over carbapenems and that are inhibited by chelating agents, such as EDTA (ethylenediamine tetraacetic acid). They are not inhibited by classical β-lactamase inhibitors and do not hydrolyse monobactams;
- Group 4: includes enzymes that are not inhibited by classical β-lactamase inhibitors and that cannot be classified in the other groups.

Table 1. β-lactamase classification schemes (adapted from Bush and Jacoby, 2010)

Bush-Jacoby group (2009)	Bush-Jacoby-Medeiros group (1995)	Molecular class (subclass)	Distinctive substrate(s)	Inhibited by		Defining characteristic(s)	Representative enzyme(s)
				CA or TZB[a]	EDTA		
1	1	C	Cephalosporins	No	No	Greater hydrolysis of cephalosporins than benzylpenicillin; hydrolyzes cephamycins	*E. coli* AmpC, P99, ACT-1, CMY-2, FOX-1, MIR-1
1e	NI[b]	C	Cephalosporins	No	No	Increased hydrolysis of ceftazidime and often other oxyimino-β-lactams	GC1, CMY-37
2a	2a	A	Penicillins	Yes	No	Greater hydrolysis of benzylpenicillin than cephalosporins	PC1
2b	2b	A	Penicillins, early cephalosporins	Yes	No	Similar hydrolysis of benzylpenicillin and cephalosporins	TEM-1, TEM-2, SHV-1
2be	2be	A	Extended-spectrum cephalosporins, monobactams	Yes	No	Increased hydrolysis of oxyimino-β-lactams (cefotaxime, ceftazidime, ceftriaxone, cefepime, aztreonam)	TEM-3, SHV-2, CTX-M-15, PER-1, VEB-1
2br	2br	A	Penicillins	No	No	Resistance to clavulanic acid, sulbactam, and tazobactam	TEM-30, SHV-10
2ber	NI	A	Extended-spectrum cephalosporins, monobactams	No	No	Increased hydrolysis of oxyimino-β-lactams combined with resistance to clavulanic acid, sulbactam, and tazobactam	TEM-50
2c	2c	A	Carbenicillin	Yes	No	Increased hydrolysis of carbenicillin	PSE-1, CARB-3
2ce	NI	A	Carbenicillin, cefepime	Yes	No	Increased hydrolysis of carbenicillin, cefepime, and cefpirome	RTG-4
2d	2d	D	Cloxacillin	Variable	No	Increased hydrolysis of cloxacillin or oxacillin	OXA-1, OXA-10
2de	NI	D	Extended-spectrum cephalosporins	Variable	No	Hydrolyzes cloxacillin or oxacillin and oxyimino-β-lactams	OXA-11, OXA-15
2df	NI	D	Carbapenems	Variable	No	Hydrolyzes cloxacillin or oxacillin and carbapenems	OXA-23, OXA-48
2e	2e	A	Extended-spectrum cephalosporins	Yes	No	Hydrolyzes cephalosporins. Inhibited by clavulanic acid but not aztreonam	CepA
2f	2f	A	Carbapenems	Variable	No	Increased hydrolysis of carbapenems, oxyimino-β-lactams, cephamycins	KPC-2, IMI-1, SME-1
3a	3	B (B1)	Carbapenems	No	Yes	Broad-spectrum hydrolysis including carbapenems but not monobactams	IMP-1, VIM-1, CcrA, IND-1
		B (B3)					L1, CAU-1, GOB-1, FEZ-1
3b	3	B (B2)	Carbapenems	No	Yes	Preferential hydrolysis of carbapenems	CphA, Sfh-1
NI	4	Unknown					

[a] CA, clavulanic acid, TZB, tazobactam; [b] NI, not included.

The first plasmid-mediated β-lactamases described in *Enterobacteriaceae* (TEM-1, TEM-2 and SHV-1) appeared soon after the introduction of β-lactam antibiotics in the clinical practice. They have the ability to hydrolyse narrow-spectrum β-lactams (ampicillin, amoxicillin and other penicillins, as well as first- and second-generation cephaslosporins) and are widely distributed in *Enterobacteriaceae* (Paterson, 2005). In response to the increased prevalence of these β-lactamases in certain *Enterobacteriaceae* species (*K. pneumoniae* and *E. coli*) and their spread into new hosts

(e.g. *Neisseria gonorrhoeae*, *Haemophilus influenzae* or *Pseudomonas aeruginosa*), third-generation cephalosporins were introduced in the clinical practice. However, the rapid emergence of extended-spectrum β-lactamases (ESBLs) conferring resistance to these compounds soon, compromised their activity (Paterson and Bonomo, 2005; Paterson, 2006; Bush and Jacoby, 2010).

A) Extended-spectrum β-lactamases (ESBLs)

Extended-spectrum β-lactamases (ESBLs) have the ability to hydrolyse penicillins, first-, second-, third- and four-generation cephalosporins, and monobactams, but not cephamycins and carbapenems, and are inhibited by β-lactamase classical inhibitors such as clavulanic acid, tazobactam or sulfabactam (Paterson and Bonomo, 2005; Paterson, 2006; Bush and Jacoby, 2010). They have been increasingly reported worldwide since their first description in 1983 in Germany, a few years after the introduction of extended-spectrum cephalosporins in the clinical setting (Knothe *et al.*, 1983; Pitout, 2010). The genes coding for ESBLs (bla_{ESBL})$_s$ genes are plasmid-mediated and are the most commonly determinants mechanism of resistance described among *Enterobacteriaceae* species (Paterson and Bonomo, 2005; Pitout, 2010).

ESBLs can be classified into different families, according to their amino acid sequence (http://www.lahey.org/Studies/). ESBLs belonging to the TEM, SHV or CTX-M types are the most frequently reported among *Enterobacteriaceae* and are worldwide distributed, whereas OXA, PER, GES, IBC and VEB seem to be confined to specific geographic areas (Paterson and Bonomo, 2005; Coque *et al.*, 2008a). A high number of variants in each group have already been identified, although only a few of them are highly represented.

A1. TEM and SHV ESBLs

During the 1990s, TEM and SHV ESBLs were the most frequently identified in nosocomial *K. pneumoniae* and *Enterobacter* spp. isolates (Coque *et al.*, 2008a; Hawkey and Jones, 2009; Livermore, 2012). The name SHV refers to sulfhydryl variable, a designation attributed due to the biochemical proprieties of this family, while the TEM name derives from the name of the patient (Temoniera) where the first *E. coli* isolate producing TEM (TEM-1) was isolated in the early 1960s (Paterson and Bonomo, 2005).

TEM and SHV ESBLs are derivatives of TEM-1/-2 and SHV-1 β-lactamases, respectively, with ability to hydrolyse extended-spectrum β-lactams such as third-generation cephalosporins and monobactams (Bradford, 2001; Paterson and Bonomo, 2005). To date, more than 200 TEM and 150 SHV variants have been identified (http://www.lahey.org/Studies/), reflecting the rapid emergence and evolution of these enzymes under the selective pressure of antibiotic usage (Paterson, 2006).

Whereas most TEM and SHV enzymes have been only sporadically described and/or confined to specific geographic areas, others have a global distribution throughout different settings (Paterson, 2006; Livermore, 2012). For example, TEM-24 has been mainly associated with nosocomial outbreaks in different Europeans countries (Spain, Portugal, Belgium and France), while SHV-12 and TEM-52 are widely disseminated in the different settings. SHV-2, SHV-5 and TEM-10 are also widespread but mainly in the hospital setting (Paterson and Bonomo, 2005; Coque *et al.*, 2008a; Hawkey and Jones, 2009; EFSA, 2011; Livermore, 2012).

A2. CTX-M enzymes

During the last decade CTX-M enzymes have become the most prevalent ESBL family in both the nosocomial and community settings, being mainly identified in *E. coli* isolates (Paterson and Bonomo, 2005; Cantón and Coque, 2006; Coque *et al.*,

2008a; Hawkey and Jones, 2009; Cantón *et al.*, 2012). They evolved from genes that have been captured by mobile genetic elements (such as IS*Ecp1* or IS*CR1*) from the chromosome of different species of *Kluyvera* spp. (Cantón *et al.*, 2012; Pitout, 2012). These enzymes were designated as cefotaximases (CTX-M) due to the preferential hydrolysis over cefotaxime than ceftazidime observed in the first enzymes identified. However, a few variants have afterwards been described with ability to hydrolyse both cephalosporins (Novais *et al.*, 2010b; Cantón *et al.*, 2012; Pitout, 2012).

Nowadays, CTX-M β-lactamases include more than 130 different enzymes (http://www.lahey.org/Studies/) that are clustered into six groups according to their amino acid identities, namely the CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, CTX-M-25 and CTX-M-45 groups (Cantón *et al.*, 2012; Pitout, 2012). CTX-M-14 and CTX-M-15 are worldwide disseminated in both community and hospital settings (Figure 2.). Other CTX-M enzymes are confined to particular geographic regions, such as CTX-M-2 (Canada, South America, Israel and Japan), CTX-M-3 (Eastern Europe, South Africa and China), and CTX-M-9 (Spain, United Kingdom and Japan). CTX-M-1 and CTX-M-32 are also frequently identified among nosocomial isolates, mostly in Mediterranean countries, but also among animals and environmental bacteria (Figure 2) (Cantón and Coque, 2006; Coque *et al.*, 2008a; Hawkey and Jones, 2009; EFSA, 2011; Cantón *et al.*, 2012; Pitout, 2012).

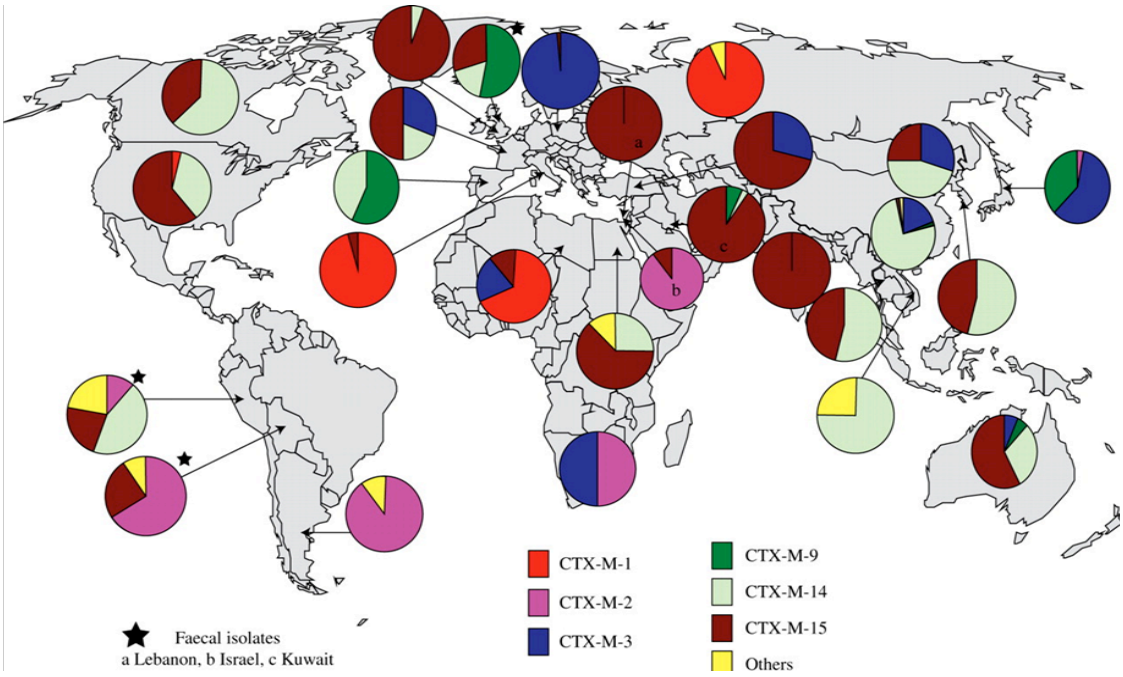


Figure 2. Global distribution of CTX-M-enzymes (Hawkey and Jones, 2009)

B) Carbapenemases

Carbapenemases have the ability to hydrolyse almost all β-lactams (including carbapenems) and most of them are not inhibited by the classical β-lactamase inhibitors (Queenan and Bush, 2007; Cantón *et al.*, 2012). Carbapenemase enzymes are distributed in all four Ambler classes, although Ambler classes A, B and D include the most epidemiologically relevant (Queenan and Bush, 2007; Grundmann *et al.*, 2010). They represent the most versatile family of β-lactamases, with a variable hydrolysis spectrum. Whereas class A and B enzymes usually confer higher resistance levels to carbapenems, variants have been described with low-level resistance profiles, which hinders their identification by susceptibility tests in the laboratory (Nordmann *et al.*, 2011a; Nordmann *et al.*, 2012b).

These enzymes have emerged as a consequence of the increasing use of carbapenems in infections caused by ESBL-producing *Enterobateriaceae* (Livermore, 2012; Nordmann *et al.*, 2012a). *bla* genes coding for carbapenemases (bla_{CARB}) have been frequently reported in *P. aeruginosa* and *Acinetobacter baumanni*, and only in the last decade emerged among *Enterobacteriaceae* species. The first carbapenemase enzyme identified in an *Enterobacteriaceae* isolate was described in Japan in 1991 (a *S. marcescens* producing IMP-1), and since then carbapenemases have been increasingly reported in different *Enterobacteriaceae* species, representing a serious public health problem (Osano *et al.*, 1994; Queenan and Bush, 2007; Nordmann *et al.*, 2011a). The most frequent belong to class A (*Klebsiella pneumoniae* carbapenemases, KPCs), class B (Metallo-β-lactamases, MBLs) and class D (Oxacillinases, OXAs) β-lactamases.

Klebsiella pneumoniae carbapenemases (KPCs) class A carbapenemases are the most common and disseminated carbapenemases among *Enterobacteriaceae* species, conferring resistance to penicillins, to first-, second- and third-generation cephalosporins, carbapenems and monobactams, and being inhibited by clavulanic acid (Queenan and Bush, 2007; Nordmann *et al.*, 2011a). The first KPC enzyme (KPC-1) was identified in a *K. pneumoniae* in 1996 in the United States of America (USA) (Yigit *et al.*, 2001). Since then, they have been considered endemic in the USA, Israel, Greece and Italy, and outbreaks have also been reported in China, Brazil and several European countries (Figure 3) (Grundmann *et al.*, 2010; Nordmann *et al*., 2011a; Cantón *et al.*,

2012; Livermore, 2012). KPC enzymes have also been identified in other *Enterobacteriaceae* species such as *E. coli*, *Enterobacter* spp. and *K. oxytoca* (Naas *et al*., 2008; Rasheed *et al*., 2008).

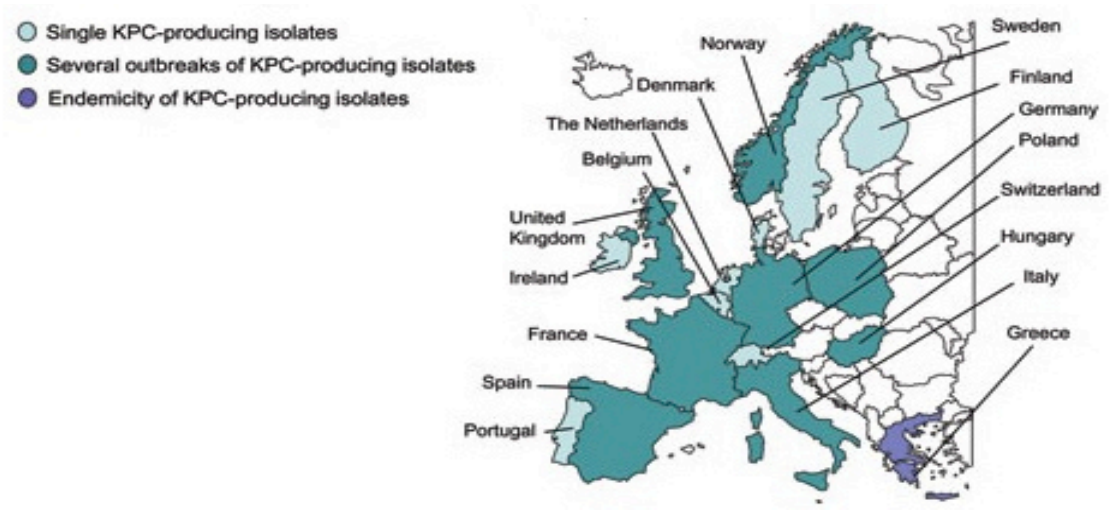


Figure 3. Distribution of KPC producers across Europe (adapted from Nordmann *et al.*, 2011a)

Metallo-β-lactamases (MBLs) are class B β-lactamases able to hydrolyse all β-lactams except aztreonam, which are inhibited by chelator agents such as EDTA, but not by clavulanic acid (Queenan and Bush, 2007; Cornaglia *et al.*, 2011). MBLs are encoded by genes located at the chromosome of several Gram-positive and Gram-negative species (e.g. *Bacillus* spp., *Pseudomonas* spp*., Stenotrophomonas maltophilia*, *Aeromonas* spp.), or by plasmid-encoded genes acquired by horizontal gene transfer (mainly in *K. pneumoniae* and *E. coli*) (Cornaglia *et al*., 2011). The first MBL enzyme, IMP-1 (for "active on imipenem"), was detected in a *S. marcescens* isolate in Japan in 1991 (Osano et al., 1994). Nowadays, the most common plasmid-mediated MBLs detected among *Enterobacteriaceae* species are VIM (Verona integron-encoded metallo-β-lactamase), IMP and, more recently, NDM (New Delhi metallo-β-lactamase-1) types (Cornaglia *et al*., 2011; Nordmann *et al*., 2011a; Cantón *et al*., 2012). MBL-producing *Enterobacteriaceae* have been described worldwide, with VIM-1 and VIM-2 being the most disseminated variants, mainly in *K. pneumoniae* isolates (Nordmann *et al*., 2011a; Cantón *et al*., 2012; Nordmann *et al*., 2012a). VIM-producing *Enterobacteriaceae* are endemic in Greece and associated with multiple outbreaks

across the world have been reported (Spain, Italy, Denmark, Hungary, Brazil, Argentina), whereas IMP-types are more prevalent in Asian countries (Figura 4) (Hawkey and Jones, 2009; Cornaglia *et al.*, 2011; Nordmann *et al*., 2011a; Cantón *et al*., 2012).

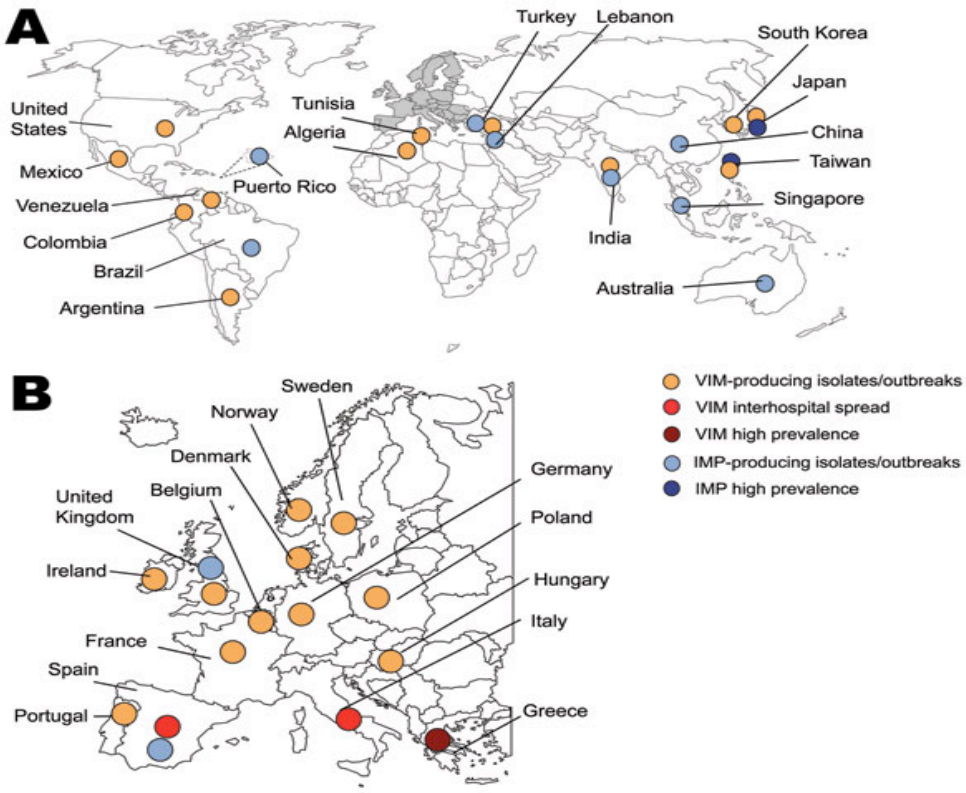


Figure 4. Wordwide (A) and European (B) geographic distribution of VIM- and IMP-producing Enterobacteriaceae (Nordmann *et al*., 2011a)

NDM-1 was identified for the first time in a *K. pneumoniae* isolate from an Indian patient previously hospitalized in New Delhi Sweden in 2008 (Yong *et al.*, 2009), and is now the focus of worldwide attention. It seems to have been imported into Europe, Asia, North America and Australia by those who have travelled or were hospitalized in the Indian subcontinent (Kumarasamy *et al.*, 2010), being identified in different *Enterobacteriaceae* species (mainly *K. pneumoniae* and *E. coli*, but also *Enterobacter* spp., *Citrobacter freundii*, *Morganella morganii*, and *Providencia* spp.). (Kumarasamy *et al.*, 2010; Nordmann *et al.*, 2011b; Walsh *et al.*, 2011).

Oxacillinases (known as OXAs) Ambler class D carbapenemases are a heterogeneous group of β-lactamases with activity over amino- and ureido-penicillins, oxacillin, cloxacillin, and carbapenems, which are inhibited by sodium chloride (NaCl),

and a few of them are able to hydrolyse carbapenems (Poirel *et al.*, 2010). They are more frequently reported among *A. baumanni* and *P. aeruginosa* (OXA-23, OXA-24, OXA-51, OXA-58), but members of this group (most frequently OXA-48) are increasingly being reported among *Enterobacteriaceae* species. (Paterson and Bonomo, 2005; Poirel *et al*., 2010). OXA-48 was first identified in a *K. pneumoniae* isolate in Turkey in 2001 (Poirel *et al*., 2004), and is nowadays disseminated in the Middle East and North Africa, with several outbreaks occurring in Europe (United Kingdom, Belgium, France, Spain and The Netherlands) (Figure 5) (Nordmann *et al.*, 2011a; Cantón *et al.*, 2012; Poirel *et al.*, 2012c). Some OXA-48-like variants (e.g. OXA-163, OXA-181) have also been identified, differing from OXA-48 by a few amino acid substitutions or deletions (Poirel *et al.*, 2012c).

Figure 5. Spread of OXA-48 and OXA-48-like carbapenemases (Livermore, 2012)

C) AmpC β-lactamases

AmpC β-lactamases (Ambler class C) hydrolyse efficiently penicillins and oxyimino-cephalosporins (including cephamycins) and are not inhibited by clavulanic acid. They include chromosomal inducible enzymes in some *Enterobactericeae* species (e.g. *Citrobacter* spp., *Enterobacter* spp., *Morganella* spp., *Serratia* spp.), chromosomal

non-inducible enzymes (e.g. *E. coli, Shigella* spp.), and the plasmid-mediated AmpC β-lactamases which are being increasingly reported in *Enterobacteriaceae*, mainly among *K. pneumoniae* and *E. coli* isolates (Jacoby, 2009). DHA-1 and CMY-2 are the most prevalent plasmid-mediated AmpC variants reported, being implicated in several outbreaks in different countries (Hawkey and Jones, 2009; Jacoby, 2009).

ii. Modifications in membrane permeability

The outer membrane of Gram-negative bacteria excludes large or hydrophobic antibiotics (such as glycopeptides, daptomycin and rifampicin), and slows the entry of those (hydrophilic antibiotics) that cross it through the porins (Figure 6) (Sousa, 2006; Livermore, 2012).

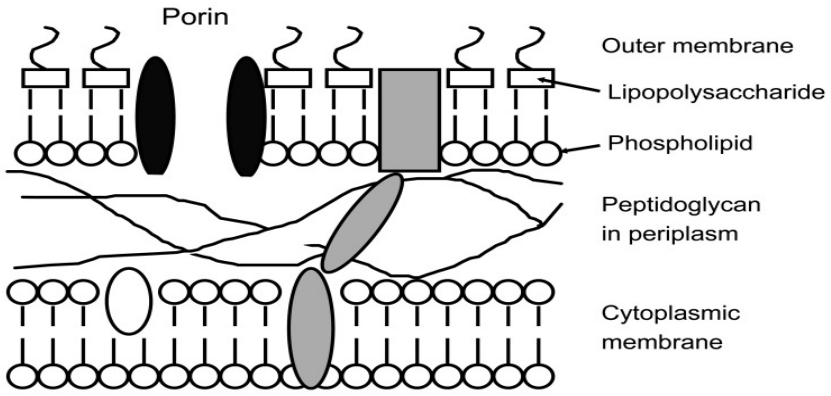


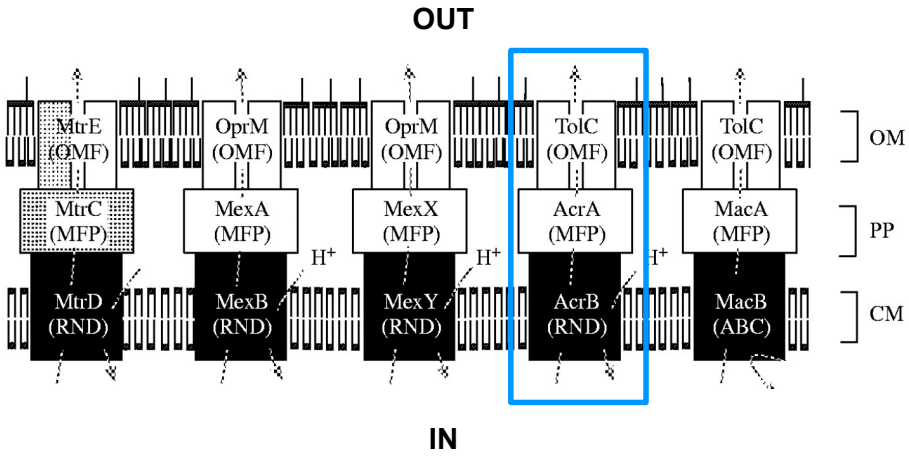
Figure 6. Cell wall and cytoplasmic membrane of Gram-negative bacteria (Livermore, 2012)

Porins are water-filled channels belonging to the family of outer membrane proteins (OMPs) allowing the diffusion of small hydrophilic solutes across the outer membrane. *Enterobacteriaceae* porins are generally divided into two classes: i) specific porins (e.g. LamB), which facilitate the diffusion of specific subtracts (involved in maltose and maltodextrin transport) and ii) non-specific porins (e.g. major non-specific porins as OmpC and OmpF in *E. coli* and *Enterobacter* spp., and their homologous OmpK35 and OmpK36 in *K. pneumoniae*, respectively), which allow the general diffusion of small polar molecules (<600 Da) such as β-lactam antibiotics (Doménech-

Sanchéz *et al*., 1999; Sousa, 2006; Martínez-Martínez, 2008). The expression of genes encoding such major non-specific porins might be affected by mutations causing protein structural changes (e.g. mutations in the transmembrane-β-strand loop 3, responsible for the conductance of the channel), alterations in the promoters and/or regulators, premature termination of translation or gene disruption (Martínez-Martínez, 2008; Doumith *et al.*; 2009). These changes result in decreased susceptibility to β-lactams, with is more prominent when other resistance mechanisms are also present, such as the production of β-lactamases (mainly ESBLs and AmpCs) (Martínez-Martínez, 2008; Doumith *et al*., 2009; García-Fernández *et al*., 2010). Several outbreaks of ESBL or AmpC producers from different *Enterobacteriaceae* species with membrane permeability changes have been described in the literature (Kaczmarek *et al.*, 2006; Doumith *et al.,* 2009; García-Fernández *et al*., 2010).

iii. Increased activity of efflux pumps

The involvement of efflux systems in antibiotic resistance in *Enterobacteriaceae* has been clearly demonstrated for certain classes of antibiotics, including chloramphenicol, tetracyclines and quinolones, but rarely for β-lactams (Poole, 2005; Pages *et al.*, 2009). Bacterial efflux systems are generally divided into five major classes, the major facilitator (MF) superfamily, the ATP-binding cassette (ABC) family, the resistance-nodulation-division (RND) family, the small multidrug resistance (SMR) family and the multidrug and toxic compound extrusion (MATE) family (Poole, 2005). RND family are the most commonly found among Gram-negative bacteria, and typically operate as part of a tripartite system that includes a perisplasmic membrane fusion (MFP) and an outer membrane factor (OMF). AcrAB-TolC is part of the RND family and represents the major pump system in *Enterobacteriaceae* (Figure 7) (Li and Nikaido, 2004; Poole, 2005). It has already been implicated in resistance to cephalosporins (*K. pneumoniae)* and imipenem (*Enterobacter aerogenes)* (Bornet *et al.*, 2003; Pages *et al.*, 2009).



OM, outer membrane; PP, periplasmic space; CM, cytoplasmic membrane

Figure 7. Schematic diagram of representative drug exporting systems involved in resistance in Gram-negative bacteria. The different families of pumps that span the cell envelope are show. The AcrAB-TolC efflux pump is highlighted as the main efflux mechanism in *Enterobacteriaceae* (adapted from Poole, 2005)

1.1.3. Resistance to non-β-lactam antibiotics

ESBL- and/or carbapenemase-producing *Enterobacteriaceae* often exhibit multidrug resistant (MDR) phenotypes, i.e. they are resistant to several other classes of non-β-lactam antibiotics including fluoroquinolones and aminoglycosides, which are also first-line antibiotics used in clinical practice (Paterson, 2006; Coque *et al.*, 2008a; Livermore, 2012). This multidrug resistance pattern is explained by the co-localization of bla_{ESBL} and/or bla_{CARB} with other antibiotic resistance genes within the same horizontal gene transfer element(s) (plasmids, integrons/*gene cassettes* and/or transposons) (Paterson, 2006; Coque *et al.*, 2008a).

Fluoroquinolone resistance is being increasingly reported among *Enterobacteriaceae* (mainly in *E. coli* and *K. pneumoniae*) in the last years (Hawkey and Jones, 2009; Poirel *et al.*, 2012b). It has been associated with mutations at DNA gyrase (GyrA) and/or topoisomerase IV (ParC) chromosomal genes and/or with the emergence and spread of plasmid-mediated quinolone resistance (PMQRs) genes (Cattoir and Nordmann, 2009; Poirel *et al.*, 2012b). Mechanisms of plasmid-mediated

quinolone resistance are: i) protection of target enzymes by Qnr proteins (e.g. QnrA, QnrB, QnrC, QnrD and QnrS), ii) production of acetylases that affect the activity of some fluoroquinolones and aminoglycosides [AAC(6')-Ib-cr], and iii) efflux systems that pump fluoroquinolones out of the bacterial cell (QepA and OqxAB) (Cattoir and Nordmann, 2009; Poirel *et al.*, 2012b). Some PMQR mechanisms have been associated with the production of particular ESBL-types, such as AAC(6')-Ib-cr and CTX-M-15, QnrA and CTX-M-9 group enzymes, or QnrS1 and VIM-1 (Cattoir and Nordmann, 2009), suggesting that the corresponding genes are located on common genetic platforms (e.g. plasmids), sometimes circulating among different *Enterobacteriaceae* species (Coque *et al.*, 2008a; Rogers *et al.*, 2011).

Aminoglycoside resistance can be caused by: i) production of aminoglycoside-modifying enzymes, usually encoded by *gene cassettes* located in integrons; ii) decrease in intracellular antibiotic accumulation; iii) substitution of ribosomal proteins or mutations on rRNA; iv) and more recently, the production of 16S rRNA methylases, which results in high-level resistance to all aminoglycosides (Davies and Wright, 1997; Galimand *et al.*, 2003). Genes encoding acetyltransferases (*aac*) and adenyltransferases (*aad*) are widely distributed in integron platforms, whereas those encoding 16S rRNA methylases (*armA*, *rmtA, rmtB*, *rmtC* and *npmA*) have emerged recently, are predominant in Asian countries and have been associated with the spread of plasmids harbouring genes encoding SHV-12, CTX-M-14, or NDM-1 (Kang *et al.*, 2009; Berçot *et al.*, 2011).

1.2. Dissemination of β-lactamase (*bla*) genes among *Enterobacteriaceae*

The epidemiology of antibiotic resistance represents the result of an interplay of resistance genes, genetic structures and bacterial clonality (Cantón *et al*., 2003). Epidemiological data demonstrated that the expansion of bla_{ESBL} or bla_{CARB} genes has been influenced by both clonal spread of particular *E. coli* and *K. pneumoniae* lineages widely disseminated in different geographic regions and/or by the horizontal transmission of genetic elements (plasmids, transposons, integrons/*gene cassettes*) between the same or different *Enterobacteriaceae* species (Coque *et al*., 2008a;

Woodford *et al*., 2011) (Figure 8). Although still controversial, the identification of the same clones or mobile genetic elements in isolates from human and animal origin suggests a link along the food chain.

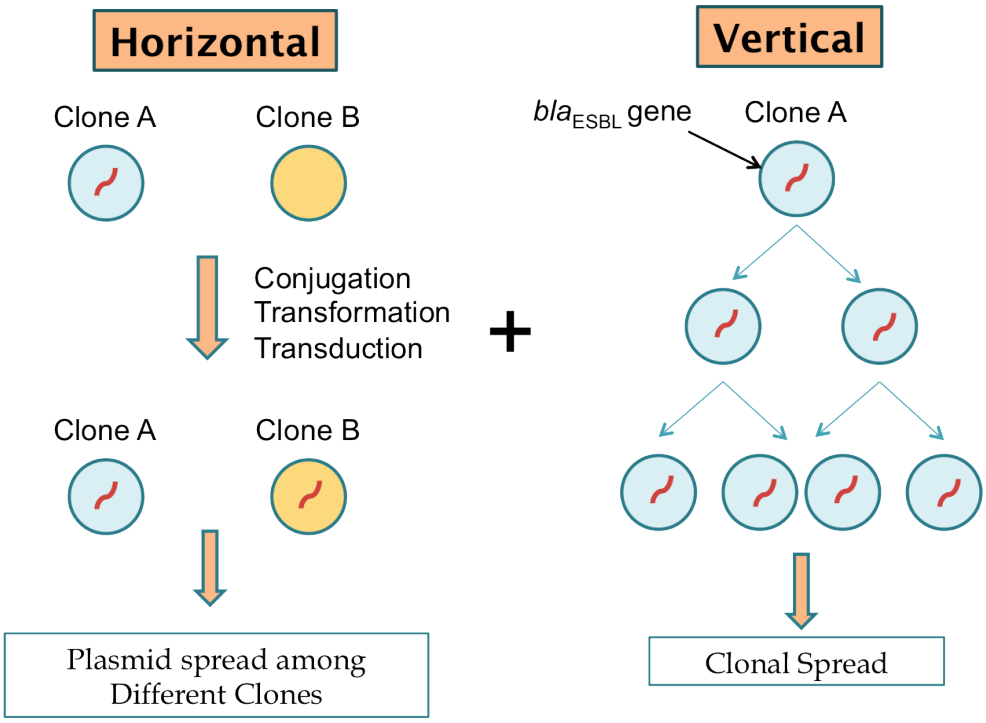


Figure 8. Mechanisms of dissemination of antibiotic resistance genes

1.2.1. Clonal spread

A 'clone' is defined as an isolate or a group of isolates descending from a common precursor exhibiting identical or closely similar phenotypic or genotypic traits, which are characterized by a strain-typing method as belonging to the same group (Riley, 2004). The most common strain-typing methods currently used for bacteria genotyping are *pulsed-field gel electrophoresis* (PFGE) and *multi-locus sequence typing* (MLST). PFGE is based on the analysis of restriction fragments after digestion of genomic DNA with a macrorrestriction enzyme (e.g. *Xba*I in *E. coli* and *K. pneumoniae*). This method provides a high degree of discrimination, which is particularly useful for outbreak situations (Tenover *et al.*, 1995). MLST is based on allelic variations in a number of *housekeeping genes* to define sequence types (ST) and

is valuable to perform evolutionary studies and interlaboratory comparisons (Urwin and Maiden, 2003).

One of the most representative examples linked to the clonal expansion of ESBL-producing *Enterobacteriaceae* is the global dissemination of the highly virulent and multidrug resistant B2-*E. coli* ST131 clone, which has been responsible for the worldwide spread of CTX-M-15 (Coque *et al.*, 2008a; Rogers *et al.*, 2011; Woodford *et al.*, 2011). This clone has also been detected encoding other ESBLs (CTX-M-1, -2, -3, -9, -14; SHV-12) or carbapenemases (NDM-1), highlighting its potential for diversification (Oteo *et al.*, 2009; Rooney *et al.*, 2009; Suzuki *et al.*, 2009; Cerquetti *et al.*, 2010; Peirano *et al.*, 2010; Courpon-Claudinon *et al.*, 2011; Park *et al.*, 2012)**.** Other *E. coli* clonal groups belonging to phylogenetic group D (ST69, ST393, ST405) are also widely spread among different hosts, often causing urinary tract infections and producing ESBLs (mainly CTX-M enzymes), cephamycinases (plasmid-mediated AmpCs), carbapenemases (NDM) and/or methylases (AmrA, RmtB) (Coque *et al.*, 2008b; Johnson *et al.*, 2009; Jakobsen *et al.*, 2010; Lee *et al.*, 2010; Blanco *et al.*, 2011; Fam *et al.*, 2011; Ruiz *et al.*, 2011; Tian *et al.*, 2011). In addition, A and B1 *E. coli* (frequently belonging to widespread clonal complexes such as CC10 or CC23) are increasingly being identified among ESBL-producing isolates in the nosocomial setting (Figure 9) (Oteo *et al.*, 2009; Fam *et al.*, 2011; Rodríguez-Baño *et al.*, 2012).

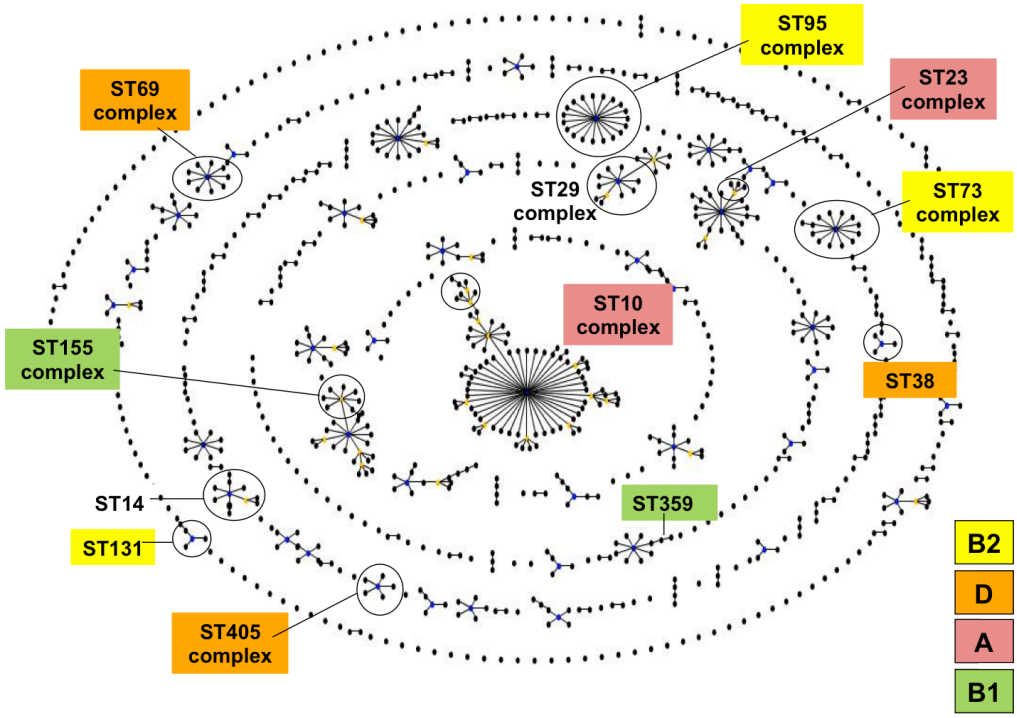


Figure 9. Population snapshot of widespread *E. coli* clones within the entire MLST database obtained by E-burst V3 (http://eburst.mlst.net/).

Particular *K. pneumoniae* clones have also been linked to the worldwide amplification of certain ESBLs or carbapenemases. The international ST15 *K. pneumoniae* clone has been associated with the spread of CTX-M-15 in different European and Asian countries (Damjanova *et al.*, 2008; Lee *et al.*, 2011; Nielsen *et al.*, 2011; Shin *et al.*, 2011; Novais *et al.*, 2012), whereas the ST258 clone has been involved in the worldwide spread of KPC enzymes (Samuelsen *et al.*, 2009; Toth *et al.*, 2010; Andrade *et al.*, 2011; Gomez *et al.*, 2011; Ho *et al.*, 2011; Morris *et al.*, 2012). ST11 *K. pneumoniae* isolates are also extensively distributed in different continents and linked to the production of diverse ESBLs (mainly CTX-M) or carbapenemases (KPC, OXA-48, NDM, VIM) (Kristof *et al.*, 2010; Lee *et al.*, 2011; Qi *et al.*, 2011; Shin *et al.*, 2011; Giske *et al.*, 2012; Pereira *et al.*, 2012; Voulgari *et al.*, 2012).

Outbreaks of ESBL-producing *Enterobacteriaceae* other than *E. coli* or *K. pneumoniae* isolates have also been described, but in most cases they seem to be of

local significance. An epidemic *E. aerogenes* strain encoding TEM-24 has been detected in different European countries (Novais *et al.*, 2010a), although it was able to acquire other ESBLs (SHV-12, SHV-5, TEM-20) (Biendo *et al.*, 2008). Other reports included outbreaks of *Enterobacter cloacae* (CTX-M-9), *P. mirabilis* (CTX-M-2), *S. marcescens* (CTX-M-3) and *K. oxytoca* (TEM-7) in different countries (Decré *et al.*, 2004; Machado *et al.*, 2007; Paauw *et al.*, 2007; Ivanova *et al.*, 2008; Novais *et al.*, 2010a; Nakano *et al.*, 2012).

1.2.2. Horizontal gene transfer

Mobile genetic elements can be generally divided in two types: elements that can move from one bacteria to another, as conjugative plasmids and transposons (the latter uncommon among *Enterobactericeae*), and elements that can move from one genetic location to another in the same cell (transposons, *gene cassettes* and insertion sequences) (Bennett, 2008; Partridge, 2011). These mobile genetic elements are crucial in the adaptation of bacterial cells to environmental conditions, but they are also very important in the dissemination and persistence of antibiotic resistance genes among *Enterobacteriaceae* species (Figure 10) (Bennett, 2004).

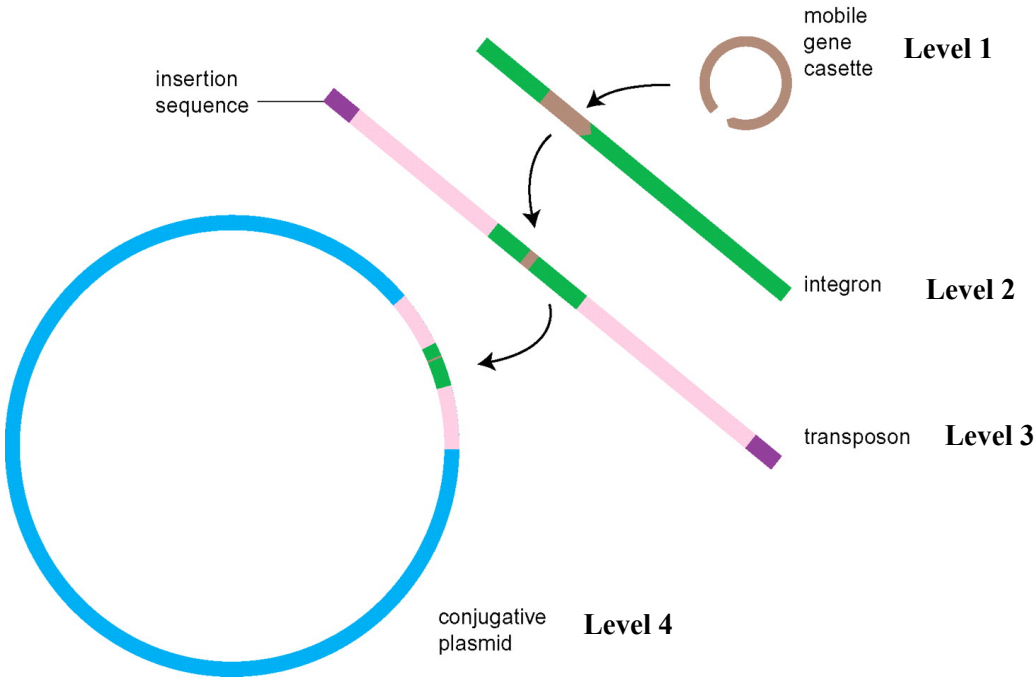


Figure 10. The modular and hierarchical composition of mobile genetic elements (adapted from Norman *et al.*, 2009)

- **Plasmids**

Plasmids are extra-chromosomal genetic units able to replicate autonomously in a given host bacterial cell, which can be transmitted to other cells by conjugation, transformation or transduction (Hayes, 2001). They are composed of genes coding for essential functions (replication, maintenance and transfer) and genes encoding accessory functions (e.g. antibiotic/metal resistance or virulence), which mediate bacterial adaptation (Hayes, 2001). Plasmids have been strongly impacting the dissemination of antimicrobial resistance genes among *Enterobacteriaceae* (Carattoli, 2009). They are classified according to different criteria such as the number of copies, the host range, the ability to transfer between cells and the incompatibility group, the latter being the most commonly used (Hayes, 2001). According to the incompatibility group (Inc) classification scheme (including 21 groups), two plasmids belong to the same Inc when they cannot stably coexist in the same cell line (Novick, 1987; Snyder and W., 2007; Carattoli, 2009). The identification of incompatibility groups has recently

been simplified by the development of a PCR-based replicon-typing scheme, targeting sequences encoding replication (Carattoli *et al.*, 2005).

The most frequent conjugative plasmids associated with the spread of resistance genes among *Enterobacteriaceae* are the narrow host range IncFII and IncI1 plasmids and the broad host range IncP, IncN and IncA/C plasmids (Taylor *et al.*, 2004; Carattoli *et al.*, 2006). Particular plasmid types belonging to these groups have been responsible for the spread of specific ESBLs or carbapenemases among different *Enterobacteriaceae* species and/or niches. One of the most representative examples is the pandemic spread of an IncFII plasmid carrying *bla*$_{\text{CTX-M-15}}$ and also *bla*$_{\text{TEM-1,}}$ *bla*$_{\text{OXA-1,}}$ and *aac(6')-Ib-cr* resistance genes in different *E. coli* backgrounds (mainly ST131 and ST405) in hospitalized humans and animals (Coque *et al.*, 2008b; Madec *et al.*, 2012). Other plasmid types have been responsible for the spread of CTX-M-1 (IncN, IncI1), CTX-M-32 (IncN), CTX-M-9 (IncP, IncHI2), CTX-M-14 (IncK), TEM-52 (IncI1), TEM-24 (IncA/C), VIM-1 (IncN, IncI1, IncHI2), OXA-48 (IncL/M), NDM-1 (IncN, IncFII, IncHI2) and KPC (IncN) in isolates of human and/or non-human origins (animals, food products and the environment) (Table 2) (Valverde *et al.*, 2004; Novais *et al.*, 2006; Novais *et al.*, 2007; Bortolaia *et al.*, 2010; Miriagou *et al.*, 2010; Novais *et al.*, 2010a; Tato *et al.*, 2010; Bielak *et al.*, 2011; Leverstein-van Hall *et al.*, 2011; Chen *et al.*, 2012; Coelho *et al.*, 2012; Dhanji *et al.*, 2012; Dolejska *et al.*, 2012; Mataseje *et al.*, 2012; Poirel *et al.*, 2012c).

Table 2. Major plasmid families and associated resistance genes in antibiotic resistant *Enterobacteriaceae* isolated worldwide from human and animal sources (adapted from Carattoli, 2009).

Replicon	Antibiotic resistance genes	Species
F	*aac (6')-Ib-cr*, *bla*$_{\text{CMY-2}}$, *bla*$_{\text{CTX-M-1-2-3-9-14-15-24-27}}$, *bla*$_{\text{DHA-1}}$, *bla*$_{\text{SHV-2-5-12}}$, *bla*$_{\text{TEM-1}}$, *bla*$_{\text{NDM-1}}$, *armA, rmtB, qepA, qepA2, qnrA1, qnrB2, qnrB4, qnrB6, qnrB19, qnrS1*	*E. aerogenes, E. cloacae, E. coli, K. pneumoniae, S. enterica, S.marcencens, S. sonnei*
A/C	*bla*$_{\text{CMY-2-4}}$, *bla*$_{\text{CTX-M-2-3-14-15-56}}$, *bla*$_{\text{SHV-2-5-12}}$, bla$_{\text{TEM-3-21-24}}$, *bla*$_{\text{IMP-4-8-13}}$, *bla*$_{\text{VIM-4}}$, *bla*$_{\text{VEB-1}}$, *armA, rmtB, qnrA1*	*C. freundii, C. koseri, E. cloacae, E. coli, K. oxytoca, K. pneumoniae, P. mirabilis, P. stuartii, S. enterica, S. marcescens*
L/M	*aac (6')-Ib-cr*, *bla*$_{\text{CTX-M-1-3-15-42}}$, *bla*$_{\text{TEM-3-10}}$, *bla*$_{\text{SHV-5}}$ *bla*$_{\text{IMP-4-8}}$, *bla*$_{\text{OXA-48}}$, *armA, qnrA1, qnrB1, qnrB2, qnrB4, qnrS1*	*C. amalonaticus, C. freundii, E. aerogenes, E. cloacae, E. coli, K. oxytoca, K. pneumoniae, M. morganii, P. mirabilis, S. enterica, S. flexneri, S. marcescens*
I1	*bla*$_{\text{CMY-2-7-21}}$, *bla*$_{\text{CTX-M-1-2-3-9-14-15-24}}$, *bla*$_{\text{SHV-12,}}$ *bla*$_{\text{VIM-1}}$ *bla*$_{\text{TEM-1-3-52}}$, *bla*$_{\text{VIM-1}}$, *armA, rmtB, mphA, qnrA1*	*E. coli, K. pneumoniae, S. enterica, S. sonnei*
HI2	*bla*$_{\text{CMY-2-3-9-14}}$, *bla*$_{\text{SHV-12}}$, *bla*$_{\text{IMP-4}}$, *bla*$_{\text{IMP-4}}$, *bla*$_{\text{VIM-1}}$, *bla*$_{\text{NDM-1}}$, *armA, qnrA1, qnrS1*	*C. youngae, E. cloacae, E. coli, K. pneumoniae, S. enterica*
N	*bla*$_{\text{KPC-2}}$, *bla*$_{\text{CTX-M-1-3-15-32-40}}$, *bla*$_{\text{VIM-1}}$,*bla*$_{\text{NDM-1,}}$ *qnrA3, qnrB2, qnrB19, qnrS1, armA*	*E. coli, K. ascorbata, K. pneumoniae, S. enterica*

- **Integrons/*gene cassettes* and Transposons**

Integrons/*gene cassettes* and transposons [insertion sequences (IS), composite transposons] are the main types of mobile genetic elements involved in the capture and mobilization of antibiotic resistance genes among *Enterobacteriaceae* (Partridge, 2011).

Integrons are site-specific recombination systems able to capture and express *gene cassettes*, small mobile elements comprising one gene (often encoding antibiotic resistance) and a recombination site (*att*C). Several *gene cassettes* can be integrated originating multiple *gene cassette* arrays (Bennett, 2004; Partridge *et al.*, 2009; Partridge, 2011). Integrons are classified into five different classes differing in the integrase *(intI)* sequence, being *class 1 integrons* the most frequently reported among *Enterobacteriaceae* (Mazel, 2006). Class 1 integrons are derivatives of Tn*402* transposons but they are defective for self-transposition and their dissemination occurs through association with transposons (mostly Tn*3*/Tn*21* family) and/or plasmids (Partridge *et al.*, 2009). A few *bla* genes, such as $bla_{\text{CTX-M-2/-9}}$, bla_{IMP} and bla_{VIM} have been associated with class 1 integrons, in some cases as part of *gene cassettes* (Cantón *et al.*, 2003; Bennett, 2004; Bush, 2010).

Insertion sequences (IS) correspond to the simplest transposable element consisting on a gene encoding a transposase, usually bounded by short, identical or imperfect inverted repeats (IR), able to move from one location to another in the genome (Partridge, 2011). They are widely distributed in bacterial genomes providing a high plasticity (Bennett, 2004). A few ISs (IS*Ecp1*, IS*26*, IS*CR1* or IS*903*) have been identified in the boundaries of some bla_{ESBL} and seem to have contributed to their mobilization and/or expression (e.g. IS*Ecp1* flanking $bla_{\text{CTX-M-15}}$, IS*26* flanking bla_{SHV} and $bla_{\text{CTX-M}}$, or IS*CR1* flanking $bla_{\text{CTX-M-2}}$ or $bla_{\text{CTX-M-9}}$) (Lartigue *et al.*, 2004; Partridge, 2011; Cantón *et al.*, 2012).

Transposons have been subdivided in four classes depending on the mechanism of transposition, being class I (also called composite transposons) or class II transposons the most frequently found among *Enterobacteriaceae* (Partridge, 2011). Class I transposons consist in two copies of the same IS (or two closely related ISs) flanking a given (antibiotic resistance) gene. Tn*10* (*tetR-tetA*, encoding tetracyclin resistance) and

Tn*1999* (*bla*$_{OXA-48}$) are a few examples of widespread class I transposons (Sherburne *et al.*, 2000; Aubert *et al.*, 2006). Class II transposons contain genes encoding a transposase (*tnpA*) and a resolvase (*tnpR*), and one variable DNA fragment flanked by two inverted repeats (IR). Depending on the orientation of *tnpA* and *tnpR*, two subtypes can be defined: Tn*21*-like mercurial transposons (which confer mercury resistance due to the presence of *mer* operon), which have been associated with the spread of *bla*$_{CTX-M-2}$, *bla*$_{CTX-M-9}$ or *bla*$_{VIM-1}$ genes (Novais *et al.*, 2006; Soler Bistue *et al.*, 2006; Tato *et al.*, 2010); and ii) Tn*3-like* transposons, which are also extensively distributed among *Enterobacteriaceae*, being responsible for the dissemination of *bla*$_{TEM}$ (e.g. *bla*$_{TEM-1}$, *bla*$_{TEM-2}$, *bla*$_{TEM-3}$, *bla*$_{TEM-24}$) or *bla*$_{KPC}$ genes (Tn*4401*) (Mabilat *et al.*, 1992; Partridge and Hall, 2005; Novais *et al.*, 2010a; Cuzon *et al.*, 2011).

1.3. Reservoirs of β-lactamase (*bla*) genes

The *bla*$_{ESBL}$ genes seem to be common among healthy volunteers, food-producing animals (mainly poultry and swine, but also cattle), companion (dogs) and wild animals (e.g. Iberian lynx), food products and environmental samples (e.g. consumption or sewage waters) (Carattoli, 2008; EFSA, 2011; Ewers *et al.*, 2012; Nicolas-Chanoine *et al.*, 2012). *bla*$_{CTX-M-1}$, *bla*$_{SHV-12}$ and *bla*$_{TEM-52}$ are the most prevalent among non-human hosts (Carattoli, 2008; EFSA, 2011). *bla*$_{CTX-M-1}$ is widely disseminated among *E. coli* isolates recovered from food-producing (poultry, swine, cattle) and companion animals (dogs), mostly in European countries, whereas *bla*$_{TEM-52}$ and *bla*$_{SHV-12}$ are widespread among *E. coli* and *Salmonella* spp. recovered from poultry (Carattoli, 2008; Coque *et al.*, 2008a; EFSA, 2011). *bla*$_{CTX-M-32}$, *bla*$_{CTX-M-14}$ and *bla*$_{CTX-M-9}$ are also frequently reported among animals (mainly poultry) in the Mediterranean (Spain, Greece) countries (Carattoli, 2008; Coque *et al.*, 2008a; EFSA, 2011). *bla*$_{CARB}$ genes have less frequently been recovered from non-clinical origins (VIM-1 in pigs and healthy persons; VIM-2, KPC-2 and NDM-1 in sewage, river and consumption waters) (Walsh *et al.*, 2011; Fischer *et al.*, 2012; Gijón *et al.*, 2012; Poirel *et al.*, 2012a). The wide distribution of these antibiotic resistance genes in different compartments highlights the existence of reservoirs in different ecological niches, and eventually a dynamic genetic exchange between them (Ewers *et al.*, 2012) (Figure 11).

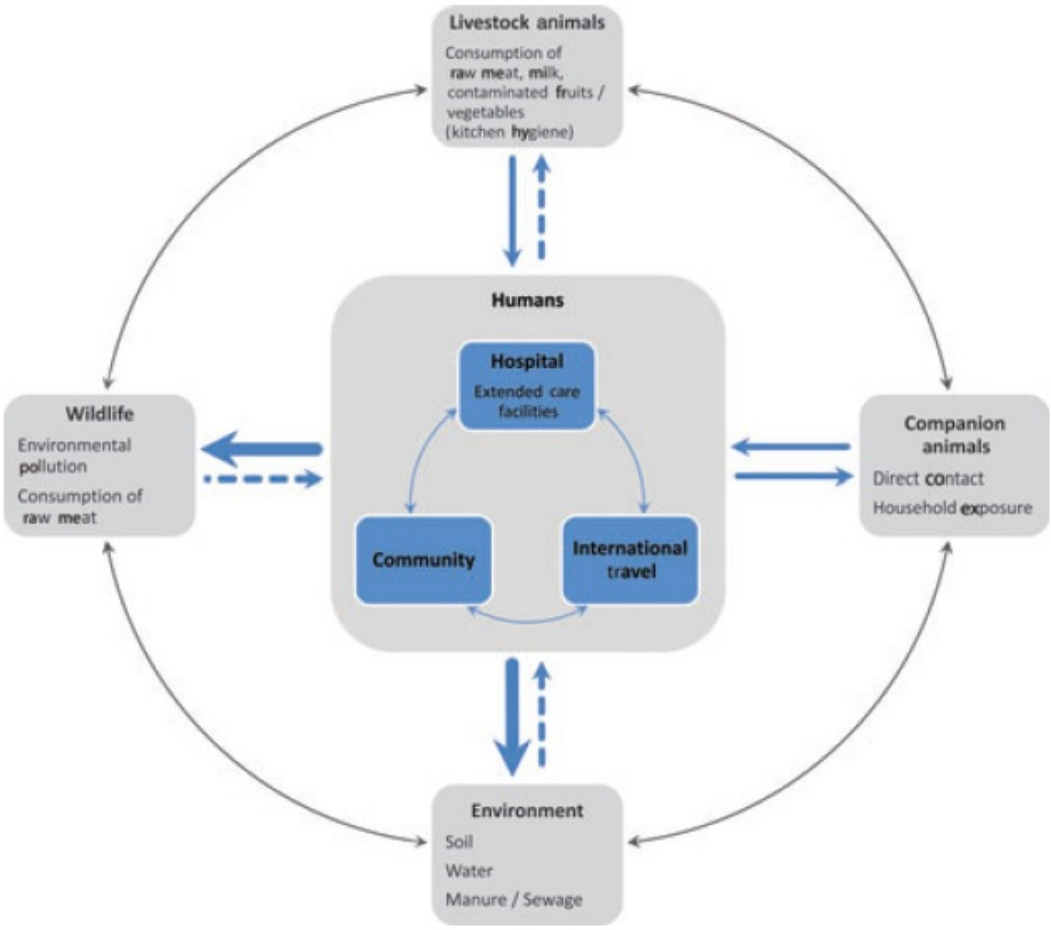


Figure 11. Microorganisms and environment: transmission pathways (adapted from Ewers *et al.*, 2012)

Moreover, some recent studies highlighted the presence of clones and/or mobile genetic elements homologous to those frequently implicated in human infections among healthy humans and non-human hosts (Leflon-Guibout *et al.*, 2008; Vincent *et al.*, 2010; Leverstein-van Hall *et al.*, 2011; Platell *et al.*, 2011; Bergeron *et al.*, 2012), suggesting a food-animal source of antibiotic resistance and their potential direct or indirect transmission through the food chain (Leflon-Guibout *et al.*, 2008; Belanger *et al.*, 2011; Manges and Johnson, 2012), although a more complex scenario cannot be discarded (Ewers *et al.*, 2012).

1.4. References

Ambler, R. P. 1980. The structure of beta-lactamases. *Philos Trans R Soc Lond B Biol Sci,* 289**,** 321-31.

Andrade, L. N.*, et al.* 2011. Dissemination of *bla*$_{KPC-2}$ by the spread of *Klebsiella pneumoniae* clonal complex 258 clones (ST258, ST11, ST437) and plasmids (IncFII, IncN, IncL/M) among *Enterobacteriaceae* species in Brazil. *Antimicrob Agents Chemother,* 55**,** 3579-83.

Aubert, D.*, et al.* 2006. Functional characterization of IS1999, an IS4 family element involved in mobilization and expression of beta-lactam resistance genes. *J Bacteriol,* 188**,** 6506-14.

Belanger, L.*, et al.* 2011. *Escherichia coli* from animal reservoirs as a potential source of human extraintestinal pathogenic E. coli. *FEMS Immunol Med Microbiol,* 62**,** 1-10.

Bennett, P. M. 2004. Genome plasticity: insertion sequence elements, transposons and integrons, and DNA rearrangement. *Methods Mol Biol,* 266**,** 71-113.

Bennett, P. M. 2008. Plasmid encoded antibiotic resistance: acquisition and transfer of antibiotic resistance genes in bacteria. *Br J Pharmacol,* 153 Suppl 1**,** S347-57.

Berçot, B.*, et al.* 2011. Updated multiplex polymerase chain reaction for detection of 16S rRNA methylases: high prevalence among NDM-1 producers. *Diagn Microbiol Infect Dis,* 71**,** 442-5.

Bergeron, C. R.*, et al.* 2012. Chicken as reservoir for extraintestinal pathogenic *Escherichia coli* in humans, Canada. *Emerg Infect Dis,* 18**,** 415-21.

Bielak, E.*, et al.* 2011. Investigation of diversity of plasmids carrying the *bla*TEM-52 gene. *J Antimicrob Chemother,* 66**,** 2465-74.

Biendo, M.*, et al.* 2008. Successive emergence of extended-spectrum beta-lactamase-producing and carbapenemase-producing *Enterobacter aerogenes* isolates in a university hospital. *J Clin Microbiol,* 46**,** 1037-44.

Blanco, J., *et al.* 2011. National survey of *Escherichia coli* causing extraintestinal infections reveals the spread of drug-resistant clonal groups O25b:H4-B2-ST131, O15:H1-D-ST393 and CGA-D-ST69 with high virulence gene content in Spain. *J Antimicrob Chemother,* 66**,** 2011-21.

Bornet, C., *et al.* 2003. Imipenem and expression of multidrug efflux pump in *Enterobacter aerogenes*. *Biochem Biophys Res Commun,* 301**,** 985-90.

Bortolaia, V., *et al.* 2010. High diversity of extended-spectrum beta-lactamases in *Escherichia coli* isolates from Italian broiler flocks. *Antimicrob Agents Chemother,* 54**,** 1623-6.

Bradford, P. A. 2001. Extended-spectrum beta-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. *Clin Microbiol Rev,* 14**,** 933-51, table of contents.

Brisse, S., *et al.* 2004. Development of a rapid identification method for *Klebsiella pneumoniae* phylogenetic groups and analysis of 420 clinical isolates. *Clin Microbiol Infect,* 10**,** 942-5.

Bush, K. 2010. Alarming beta-lactamase-mediated resistance in multidrug-resistant *Enterobacteriaceae*. *Curr Opin Microbiol,* 13**,** 558-64.

Bush, K. & Jacoby, G. A. 2010. Updated functional classification of beta-lactamases. *Antimicrob Agents Chemother,* 54**,** 969-76.

Bush, K., *et al.* 1995. A functional classification scheme for beta-lactamases and its correlation with molecular structure. *Antimicrob Agents Chemother,* 39**,** 1211-33.

Cantón, R., *et al.* 2012. Rapid evolution and spread of carbapenemases among *Enterobacteriaceae* in Europe. *Clinical Microbiology and Infection,* 18**,** 413-431.

Cantón, R. & Coque, T. M. 2006. The CTX-M beta-lactamase pandemic. *Curr Opin Microbiol,* 9**,** 466-75.

Cantón, R., *et al.* 2003. Multi-resistant Gram-negative bacilli: from epidemics to endemics. *Curr Opin Infect Dis,* 16**,** 315-25.

Cantón, R., *et al.* 2012. CTX-M Enzymes: Origin and Diffusion. *Front Microbiol,* 3**,** 110.

Carattoli, A. 2008. Animal reservoirs for extended spectrum beta-lactamase producers. *Clin Microbiol Infect,* 14 Suppl 1**,** 117-23.

Carattoli, A. 2009. Resistance plasmid families in *Enterobacteriaceae*. *Antimicrob Agents Chemother,* 53**,** 2227-38.

Carattoli, A., *et al.* 2005. Identification of plasmids by PCR-based replicon typing. *J Microbiol Methods,* 63**,** 219-28.

Carattoli, A., *et al.* 2006. Replicon typing of plasmids encoding resistance to newer beta-lactams. *Emerg Infect Dis,* 12**,** 1145-8.

Cattoir, V. & Nordmann, P. 2009. Plasmid-mediated quinolone resistance in gram-negative bacterial species: an update. *Curr Med Chem,* 16**,** 1028-46.

Cerquetti, M., *et al.* 2010. Ciprofloxacin-resistant, CTX-M-15-producing *Escherichia coli* ST131 clone in extraintestinal infections in Italy. *Clin Microbiol Infect,* 16**,** 1555-8.

Chen, Y.T., *et al.* 2012. Sequence of Closely Related Plasmids Encoding NDM-1 in Two Unrelated *Klebsiella pneumoniae* Isolates in Singapore. *PLoS One,* 7**,** e48737.

Clermont, O., *et al.* 2000. Rapid and simple determination of the *Escherichia coli* phylogenetic group. *Appl Environ Microbiol,* 66**,** 4555-8.

Coelho, A., *et al.* 2012. Role of IncHI2 plasmids harbouring *bla*VIM-1, *bla*CTX-M-9, *aac(6')-Ib* and *qnrA* genes in the spread of multiresistant *Enterobacter cloacae* and *Klebsiella pneumoniae* strains in different units at Hospital Vall d'Hebron, Barcelona, Spain. *Int J Antimicrob Agents,* 39**,** 514-7.

Cooke, N. M., *et al.* 2010. Major differences exist in frequencies of virulence factors and multidrug resistance between community and nosocomial *Escherichia coli* bloodstream isolates. *J Clin Microbiol,* 48**,** 1099-104.

Coque, T. M., *et al.* 2008a. Increasing prevalence of ESBL-producing *Enterobacteriaceae* in Europe. *Euro Surveill,* 13.

Coque, T. M., *et al.* 2008b. Dissemination of clonally related *Escherichia coli* strains expressing extended-spectrum beta-lactamase CTX-M-15. *Emerg Infect Dis,* 14**,** 195-200.

Cornaglia, G., *et al.* 2011. Metallo-beta-lactamases: a last frontier for beta-lactams? *Lancet Infect Dis,* 11**,** 381-93.

Courpon-Claudinon, A., *et al.* 2011. Bacteraemia caused by third-generation cephalosporin-resistant *Escherichia coli* in France: prevalence, molecular epidemiology and clinical features. *Clin Microbiol Infect,* 17**,** 557-65.

Cuzon, G., *et al.* 2011. Functional characterization of Tn4401, a Tn3-based transposon involved in bla_{KPC} gene mobilization. *Antimicrob Agents Chemother,* 55**,** 5370-3.

Damjanova, I., *et al.* 2008. Expansion and countrywide dissemination of ST11, ST15 and ST147 ciprofloxacin-resistant CTX-M-15-type beta-lactamase-producing *Klebsiella pneumoniae* epidemic clones in Hungary in 2005--the new 'MRSAs'? *J Antimicrob Chemother,* 62**,** 978-85.

Davies, J. & Wright, G. D. 1997. Bacterial resistance to aminoglycoside antibiotics. *Trends Microbiol,* 5**,** 234-40.

Decré, D., *et al.* 2004. Outbreak of multi-resistant *Klebsiella oxytoca* involving strains with extended-spectrum beta-lactamases and strains with extended-spectrum activity of the chromosomal beta-lactamase. *J Antimicrob Chemother,* 54**,** 881-8.

Dhanji, H., *et al.* 2012. Dissemination of pCT-like IncK plasmids harboring CTX-M-14 extended-spectrum beta-lactamase among clinical *Escherichia coli* isolates in the United Kingdom. *Antimicrob Agents Chemother,* 56**,** 3376-7.

Dolejska, M., *et al.* 2012. Complete sequencing of an IncHI1 plasmid encoding the carbapenemase NDM-1, the ArmA 16S RNA methylase and a resistance-nodulation-cell division/multidrug efflux pump. *J Antimicrob Chemother*.

Doménech-Sánchez, A., *et al.* 1999. Identification and characterization of a new porin gene of *Klebsiella pneumoniae*: its role in beta-lactam antibiotic resistance. *J Bacteriol,* 181**,** 2726-32.

Doumith, M., *et al.* 2009. Molecular mechanisms disrupting porin expression in ertapenem-resistant *Klebsiella* and *Enterobacter* spp. clinical isolates from the UK. *J Antimicrob Chemother,* 63**,** 659-67.

Duriez, P., *et al.* 2001. Commensal *Escherichia coli* isolates are phylogenetically distributed among geographically distinct human populations. *Microbiology,* 147**,** 1671-6.

EFSA 2011. European Food Safety Authority and European Centre Disease Prevention Control. The European Union Summary Report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in the European Union in 2009.

Ewers, C., *et al.* 2012. Extended-spectrum beta-lactamase-producing and AmpC-producing *Escherichia coli* from livestock and companion animals, and their putative impact on public health: a global perspective. *Clin Microbiol Infect,* 18**,** 646-55.

Fam, N., *et al.* 2011. CTX-M-15-producing *Escherichia coli* clinical isolates in Cairo (Egypt), including isolates of clonal complex ST10 and clones ST131, ST73, and ST405 in both community and hospital settings. *Microb Drug Resist,* 17**,** 67-73.

Fischer, J., *et al.* 2012. *Escherichia coli* producing VIM-1 carbapenemase isolated on a pig farm. *J Antimicrob Chemother,* 67**,** 1793-5.

Galimand, M., *et al.* 2003. Plasmid-mediated high-level resistance to aminoglycosides in *Enterobacteriaceae* due to 16S rRNA methylation. *Antimicrob Agents Chemother,* 47**,** 2565-71.

García-Fernández, A., *et al.* 2010. An ertapenem-resistant extended-spectrum-beta-lactamase-producing *Klebsiella pneumoniae* clone carries a novel OmpK36 porin variant. *Antimicrob Agents Chemother,* 54**,** 4178-84.

Gaynes, R. & Edwards, J. R. 2005. Overview of nosocomial infections caused by gram-negative bacilli. *Clin Infect Dis,* 41**,** 848-54.

Gijón, D., *et al.* 2012. Fecal carriage of carbapenemase-producing *Enterobacteriaceae*: a hidden reservoir in hospitalized and nonhospitalized patients. *J Clin Microbiol,* 50**,** 1558-63.

Giske, C. G., *et al.* 2012. Diverse sequence types of *Klebsiella pneumoniae* contribute to the dissemination of bla_{NDM-1} in India, Sweden, and the United Kingdom. *Antimicrob Agents Chemother,* 56**,** 2735-8.

Gomez, S. A., *et al.* 2011. Clonal dissemination of *Klebsiella pneumoniae* ST258 harbouring KPC-2 in Argentina. *Clin Microbiol Infect,* 17**,** 1520-4.

Grundmann, H., *et al.* 2010. Carbapenem-non-susceptible *Enterobacteriaceae* in Europe: conclusions from a meeting of national experts. *Euro Surveill,* 15.

Hawkey, P. M. & Jones, A. M. 2009. The changing epidemiology of resistance. *J Antimicrob Chemother,* 64 Suppl 1**,** i3-10.

Hayes, F. 2001. The Horizontal Gene Pool — Bacterial Plasmids and Gene Spread. Christopher M. Thomas (ed.). Harwood Academic Publishers, Amsterdam. 2000., hardback. *Heredity,* 86**,** 251-252.

Herzer, P. J., *et al.* 1990. Phylogenetic distribution of branched RNA-linked multicopy single-stranded DNA among natural isolates of *Escherichia coli. J Bacteriol,* 172**,** 6175-81.

Ho, P. L., *et al.* 2011. Emergence of *Klebsiella pneumoniae* ST258 with KPC-2 in Hong Kong. *Int J Antimicrob Agents,* 37**,** 386-7.

Ivanova, D., *et al.* 2008. Extended-spectrum beta-lactamase-producing *Serratia marcescens* outbreak in a Bulgarian hospital. *J Hosp Infect,* 70**,** 60-5.

Jacoby, G. A. 2009. AmpC beta-lactamases. *Clin Microbiol Rev,* 22**,** 161-82, Table of Contents.

Jakobsen, L., *et al.* 2010. Detection of clonal group A *Escherichia coli* isolates from broiler chickens, broiler chicken meat, community-dwelling humans, and urinary tract infection (UTI) patients and their virulence in a mouse UTI model. *Appl Environ Microbiol,* 76**,** 8281-4.

Johnson, J. R., *et al.* 2009. Epidemic clonal groups of *Escherichia coli* as a cause of antimicrobial-resistant urinary tract infections in Canada, 2002 to 2004. *Antimicrob Agents Chemother,* 53**,** 2733-9.

Johnson, J. R., *et al.* 2001. Clonal Relationships and Extended Virulence Genotypes among *Escherichia coli* Isolates from Women with a First or Recurrent Episode of Cystitis. *Journal of Infectious Diseases,* 183**,** 1508-1517.

Kaczmarek, F. M., *et al.* 2006. High-Level Carbapenem Resistance in a *Klebsiella pneumoniae* Clinical Isolate Is Due to the Combination of *bla*ACT-1 β-Lactamase Production, Porin OmpK35/36 Insertional Inactivation, and Down-Regulation of the Phosphate Transport Porin PhoE. *Antimicrob Agents Chemother,* 50**,** 3396-3406.

Kang, H. Y., *et al.* 2009. Characterization of conjugative plasmids carrying antibiotic resistance genes encoding 16S rRNA methylase, extended-spectrum beta-lactamase, and/or plasmid-mediated AmpC beta-lactamase. *J Microbiol,* 47**,** 68-75.

Knothe, H., *et al.* 1983. Transferable resistance to cefotaxime, cefoxitin, cefamandole and cefuroxime in clinical isolates of *Klebsiella pneumoniae* and *Serratia marcescens*. *Infection,* 11**,** 315-7.

Kristof, K., *et al.* 2010. Identification of a *bla*VIM-4 gene in the internationally successful *Klebsiella pneumoniae* ST11 clone and in a *Klebsiella oxytoca* strain in Hungary. *J Antimicrob Chemother,* 65**,** 1303-5.

Kumarasamy, K. K., *et al.* 2010. Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study. *Lancet Infect Dis,* 10**,** 597-602.

Lartigue, M. F., *et al.* 2004. Diversity of genetic environment of *bla*(CTX-M) genes. *FEMS Microbiol Lett,* 234**,** 201-7.

Lee, M. Y., *et al.* 2010. Dissemination of ST131 and ST393 community-onset, ciprofloxacin-resistant *Escherichia coli* clones causing urinary tract infections in Korea. *J Infect,* 60**,** 146-53.

Lee, M. Y., *et al.* 2011. High prevalence of CTX-M-15-producing *Klebsiella pneumoniae* isolates in Asian countries: diverse clones and clonal dissemination. *Int J Antimicrob Agents,* 38**,** 160-3.

Leflon-Guibout, V., *et al.* 2008. Absence of CTX-M enzymes but high prevalence of clones, including clone ST131, among fecal *Escherichia coli* isolates from healthy subjects living in the area of Paris, France. *J Clin Microbiol,* 46**,** 3900-5.

Leverstein-Van Hall, M. A., *et al.* 2011. Dutch patients, retail chicken meat and poultry share the same ESBL genes, plasmids and strains. *Clin Microbiol Infect,* 17**,** 873-80.

Li, X. Z. & Nikaido, H. 2004. Efflux-mediated drug resistance in bacteria. *Drugs,* 64**,** 159-204.

Livermore, D. M. 2012. Current epidemiology and growing resistance of gram-negative pathogens. *Korean J Intern Med,* 27**,** 128-42.

Mabilat, C., *et al.* 1992. A new example of physical linkage between Tn1 and Tn21: the antibiotic multiple-resistance region of plasmid pCFF04 encoding extended-spectrum beta-lactamase TEM-3. *Mol Gen Genet,* 235**,** 113-21.

Machado, E., *et al.* 2007. High diversity of extended-spectrum beta-lactamases among clinical isolates of *Enterobacteriaceae* from Portugal. *J Antimicrob Chemother,* 60**,** 1370-4.

Madec, J. Y., *et al.* 2012. Non-ST131 *Escherichia coli* from cattle harbouring human-like *bla*(CTX-M-15)-carrying plasmids. *J Antimicrob Chemother,* 67**,** 578-81.

Manges, A. R. & Johnson, J. R. 2012. Food-borne origins of *Escherichia coli* causing extraintestinal infections. *Clin Infect Dis,* 55**,** 712-9.

Martínez-Martínez, L. 2008. Extended-spectrum beta-lactamases and the permeability barrier. *Clin Microbiol Infect,* 14 Suppl 1**,** 82-9.

Mataseje, L. F., *et al.* 2012. Carbapenem-resistant Gram-negative bacilli in Canada 2009-10: results from the Canadian Nosocomial Infection Surveillance Program (CNISP). *J Antimicrob Chemother,* 67**,** 1359-67.

Mazel, D. 2006. Integrons: agents of bacterial evolution. *Nat Rev Microbiol,* 4**,** 608-20.

Miriagou, V., *et al.* 2010. Sequence of pNL194, a 79.3-kilobase IncN plasmid carrying the *bla*VIM-1 metallo-beta-lactamase gene in *Klebsiella pneumoniae. Antimicrob Agents Chemother,* 54**,** 4497-502.

Moreno, E., *et al.* 2006. Characterization of *Escherichia coli* isolates derived from phylogenetic groups A and B1 causing extraintestinal infection. *Enferm Infecc Microbiol Clin,* 24**,** 483-9.

Morris, D., *et al.* 2012. Inter-hospital outbreak of *Klebsiella pneumoniae* producing KPC-2 carbapenemase in Ireland. *J Antimicrob Chemother,* 67**,** 2367-72.

Murray, P., *et al.* 2003. *Manual of Clinical Microbiology*, American Society of Microbiology Press.

Naas, T., *et al.* 2008. Genetic structures at the origin of acquisition of the beta-lactamase *bla* KPC gene. *Antimicrob Agents Chemother,* 52**,** 1257-63.

Nakano, R., *et al.* 2012. Regional outbreak of CTX-M-2 beta-lactamase-producing *Proteus mirabilis* in Japan. *J Med Microbiol,* 61**,** 1727-35.

Nicolas-Chanoine, M. H., *et al.* 2012. Patient's origin and lifestyle associated with CTX-M-producing *Escherichia coli*: a case-control-control study. *PLoS One,* 7**,** e30498.

Nielsen, J. B., *et al.* 2011. Identification of CTX-M15-, SHV-28-producing *Klebsiella pneumoniae* ST15 as an epidemic clone in the Copenhagen area using a semi-automated Rep-PCR typing assay. *Eur J Clin Microbiol Infect Dis,* 30**,** 773-8.

Nordmann, P., *et al.* 2012a. Carbapenem resistance in *Enterobacteriaceae*: here is the storm! *Trends Mol Med,* 18**,** 263-72.

Nordmann, P., *et al.* 2012b. Identification and screening of carbapenemase-producing *Enterobacteriaceae*. *Clin Microbiol Infect,* 18**,** 432-8.

Nordmann, P., *et al.* 2011a. Global spread of Carbapenemase-producing *Enterobacteriaceae. Emerg Infect Dis,* 17**,** 1791-8.

Nordmann, P., *et al.* 2011b. The emerging NDM carbapenemases. *Trends Microbiol,* 19**,** 588-95.

Norman, Â., *et al.* 2009. Conjugative plasmids: vessels of the communal gene pool. *Philos Trans R Soc Lond B Biol Sci,* 364**,** 2275-89.

Novais, Â., *et al.* 2010a. International spread and persistence of TEM-24 is caused by the confluence of highly penetrating *enterobacteriaceae* clones and an IncA/C2 plasmid containing Tn1696::Tn1 and IS5075-Tn21. *Antimicrob Agents Chemother,* 54**,** 825-34.

Novais, Â., *et al.* 2007. Emergence and dissemination of *Enterobacteriaceae* isolates producing CTX-M-1-like enzymes in Spain are associated with IncFII (CTX-M-15) and broad-host-range (CTX-M-1, -3, and -32) plasmids. *Antimicrob Agents Chemother,* 51**,** 796-9.

Novais, Â., *et al.* 2006. Dissemination and persistence of *bla*CTX-M-9 are linked to class 1 integrons containing CR1 associated with defective transposon derivatives from Tn402 located in early antibiotic resistance plasmids of IncHI2, IncP1-alpha, and IncFI groups. *Antimicrob Agents Chemother,* 50**,** 2741-50.

Novais, Â., *et al.* 2010b. Evolutionary trajectories of beta-lactamase CTX-M-1 cluster enzymes: predicting antibiotic resistance. *PLoS Pathog,* 6**,** e1000735.

Novais, Â., *et al.* 2012. Spread of an OmpK36-modified ST15 *Klebsiella pneumoniae* variant during an outbreak involving multiple carbapenem-resistant *Enterobacteriaceae* species and clones. *Eur J Clin Microbiol Infect Dis,* 31**,** 3057-63.

Novick, R. P. 1987. Plasmid incompatibility. *Microbiol Rev,* 51**,** 381-95.

Ochman, H. & Selander, R. K. 1984. Standard reference strains of *Escherichia coli* from natural populations. *J Bacteriol,* 157**,** 690-3.

Osano, E., *et al.* 1994. Molecular characterization of an enterobacterial metallo beta-lactamase found in a clinical isolate of *Serratia marcescens* that shows imipenem resistance. *Antimicrob Agents Chemother,* 38**,** 71-8.

Oteo, J., *et al.* 2009. Extended-spectrum beta-lactamase-producing *Escherichia coli* in Spain belong to a large variety of multilocus sequence typing types, including ST10 complex/A, ST23 complex/A and ST131/B2. *Int J Antimicrob Agents,* 34**,** 173-6.

Paauw, A., *et al.* 2007. Failure to control an outbreak of qnrA1-positive multidrug-resistant *Enterobacter cloacae* infection despite adequate implementation of recommended infection control measures. *J Clin Microbiol,* 45**,** 1420-5.

Pages, J. M., *et al.* 2009. Efflux pump, the masked side of beta-lactam resistance in *Klebsiella pneumoniae* clinical isolates. *PLoS One,* 4**,** e4817.

Park, S. H., *et al.* 2012. Molecular epidemiology of extended-spectrum beta-lactamase-producing *Escherichia coli* in the community and hospital in Korea: emergence of ST131 producing CTX-M-15. *BMC Infect Dis,* 12**,** 149.

Partridge, S. R. 2011. Analysis of antibiotic resistance regions in Gram-negative bacteria. *FEMS Microbiol Rev,* 35**,** 820-55.

Partridge, S. R. & Hall, R. M. 2005. Evolution of transposons containing *bla*TEM genes. *Antimicrob Agents Chemother,* 49**,** 1267-8.

Partridge, S. R., *et al.* 2009. Gene cassettes and cassette arrays in mobile resistance integrons. *FEMS Microbiol Rev,* 33**,** 757-84.

Paterson, D. L. 2006. Resistance in gram-negative bacteria: *Enterobacteriaceae*. *Am J Med,* 119**,** S20-8; discussion S62-70.

Paterson, D. L. & Bonomo, R. A. 2005. Extended-spectrum beta-lactamases: a clinical update. *Clin Microbiol Rev,* 18**,** 657-86.

Peirano, G., *et al.* 2010. High prevalence of ST131 isolates producing CTX-M-15 and CTX-M-14 among extended-spectrum-beta-lactamase-producing *Escherichia coli* isolates from Canada. *Antimicrob Agents Chemother,* 54**,** 1327-30.

Pereira, P. S., *et al.* 2012. Update of the molecular epidemiology of KPC-2-producing *Klebsiella pneumoniae* in Brazil: spread of clonal complex 11 (ST11, ST437 and ST340). *J Antimicrob Chemother*.

Pfeifer, Y., *et al.* 2010. Resistance to cephalosporins and carbapenems in Gram-negative bacterial pathogens. *Int J Med Microbiol,* 300**,** 371-9.

Pitout, J. D. 2010. Infections with extended-spectrum beta-lactamase-producing *Enterobacteriaceae*: changing epidemiology and drug treatment choices. *Drugs,* 70**,** 313-33.

Pitout, J. D. 2012. Extraintestinal Pathogenic *Escherichia coli:* A Combination of Virulence with Antibiotic Resistance. *Front Microbiol,* 3**,** 9.

Platell, J. L*., et al.* 2011. Multidrug-resistant extraintestinal pathogenic *Escherichia coli* of sequence type ST131 in animals and foods. *Vet Microbiol,* 153**,** 99-108.

Poirel, L.*, et al.* 2012a. Environmental KPC-producing *Escherichia coli* isolates in Portugal. *Antimicrob Agents Chemother,* 56**,** 1662-3.

Poirel, L.*, et al.* 2012b. Plasmid-Mediated Quinolone Resistance; Interactions between Human, Animal, and Environmental Ecologies. *Front Microbiol,* 3**,** 24.

Poirel, L*., et al.* 2004. Emergence of oxacillinase-mediated resistance to imipenem in *Klebsiella pneumoniae*. *Antimicrob Agents Chemother,* 48**,** 15-22.

Poirel, L*., et al.* 2010. Diversity, epidemiology, and genetics of class D beta-lactamases. *Antimicrob Agents Chemother,* 54**,** 24-38.

Poirel, L*., et al.* 2012c. OXA-48-like carbapenemases: the phantom menace. *J Antimicrob Chemother,* 67**,** 1597-606.

Poole, K. 2005. Efflux-mediated antimicrobial resistance. *J Antimicrob Chemother,* 56**,** 20-51.

Qi, Y.*, et al.* 2011. ST11, the dominant clone of KPC-producing *Klebsiella pneumoniae* in China. *J Antimicrob Chemother,* 66**,** 307-12.

Queenan, A. M. & Bush, K. 2007. Carbapenemases: the versatile beta-lactamases. *Clin Microbiol Rev,* 20**,** 440-58, table of contents.

Rasheed, J. K.*, et al.* 2008. Detection of the *Klebsiella pneumoniae* carbapenemase type 2 Carbapenem-hydrolyzing enzyme in clinical isolates of *Citrobacter freundii* and *K. oxytoca* carrying a common plasmid. *J Clin Microbiol,* 46**,** 2066-9.

Riley, L. 2004. *Molecular Epidemiology of Infectious Diseases: Principles and Practices,* Washington, D.C., American Society Microbiology Press.

Rodríguez-Baño, J.*, et al.* 2012. Virulence profiles of bacteremic extended-spectrum beta-lactamase-producing *Escherichia coli*: association with epidemiological and clinical features. *PLoS One,* 7**,** e44238.

Rogers, B. A.*, et al.* 2011. *Escherichia coli* O25b-ST131: a pandemic, multiresistant, community-associated strain. *J Antimicrob Chemother,* 66**,** 1-14.

Rooney, P. J.*, et al.* 2009. Nursing homes as a reservoir of extended-spectrum beta-lactamase (ESBL)-producing ciprofloxacin-resistant *Escherichia coli*. *J Antimicrob Chemother,* 64**,** 635-41.

Ruiz, S. J.*, et al.* 2011. First characterization of CTX-M-15-producing *Escherichia coli* ST131 and ST405 clones causing community-onset infections in South America. *J Clin Microbiol,* 49**,** 1993-6.

Samuelsen, O.*, et al.* 2009. Emergence of clonally related *Klebsiella pneumoniae* isolates of sequence type 258 producing plasmid-mediated KPC carbapenemase in Norway and Sweden. *J Antimicrob Chemother,* 63**,** 654-8.

Sherburne, C. K.*, et al.* 2000. The complete DNA sequence and analysis of R27, a large IncHI plasmid from *Salmonella typhi* that is temperature sensitive for transfer. *Nucleic Acids Res,* 28**,** 2177-86.

Shin, J., ***et al.*** **2011.** Comparison of CTX-M-14- and CTX-M-15-producing *Escherichia coli* and *Klebsiella pneumoniae* isolates from patients with bacteremia. *J Infect,* 63**,** 39-47.

Snyder, L. & W., C. 2007. *Molecular Genetics of Bacteria*, ASM Press.

Soler Bistue, A. J.*, et al.* 2006. *Vibrio cholerae* InV117, a class 1 integron harboring aac(6')-Ib and *bla*CTX-M-2, is linked to transposition genes. *Antimicrob Agents Chemother,* 50**,** 1903-7.

Sousa, J. C. 2006. *Manual de Antibióticos Antibacterianos,* Porto, Portugal.

Suzuki, S., *et al.* 2009. Change in the prevalence of extended-spectrum-beta-lactamase-producing *Escherichia coli* in Japan by clonal spread. *J Antimicrob Chemother,* 63**,** 72-9.

Tato, M.*, et al.* 2010. Dispersal of carbapenemase *bla*VIM-1 gene associated with different Tn402 variants, mercury transposons, and conjugative plasmids in *Enterobacteriaceae* and *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother,* 54**,** 320-7.

Taylor, D. E*., et al.* 2004. Antibiotic Resistance Plasmids. *In:* ASM (ed.) *Plasmid Biology.* Washington D. C.

Tenover, F. C*., et al.* 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J Clin Microbiol,* 33**,** 2233-9.

Thomson, J. M. & Bonomo, R. A. 2005. The threat of antibiotic resistance in Gram-negative pathogenic bacteria: beta-lactams in peril! *Curr Opin Microbiol,* 8**,** 518-24.

Tian, G. B*., et al.* 2011. Sequence type ST405 *Escherichia coli* isolate producing QepA1, CTX-M-15, and RmtB from Detroit, Michigan. *Antimicrob Agents Chemother,* 55**,** 3966-7.

Toth, A.*, et al.* 2010. Emergence of a colistin-resistant KPC-2-producing *Klebsiella pneumoniae* ST258 clone in Hungary. *Eur J Clin Microbiol Infect Dis,* 29**,** 765-9.

Urwin, R. & Maiden, M. C. 2003. Multi-locus sequence typing: a tool for global epidemiology. *Trends Microbiol,* 11**,** 479-87.

Valverde, A*., et al.* 2009. Spread of bla(CTX-M-14) is driven mainly by IncK plasmids disseminated among *Escherichia coli* phylogroups A, B1, and D in Spain. *Antimicrob Agents Chemother,* 53**,** 5204-12.

Valverde, A*., et al.* 2004. Dramatic increase in prevalence of fecal carriage of extended-spectrum beta-lactamase-producing *Enterobacteriaceae* during nonoutbreak situations in Spain. *J Clin Microbiol,* 42**,** 4769-75.

Vincent, C., *et al.* 2010. Food reservoir for *Escherichia coli* causing urinary tract infections. *Emerg Infect Dis,* 16**,** 88-95.

Voulgari, E., *et al.* 2012. Outbreak of OXA-48 carbapenemase-producing *Klebsiella pneumoniae* in Greece involving an ST11 clone. *J Antimicrob Chemother*.

Walsh, T. R., *et al.* 2011. Dissemination of NDM-1 positive bacteria in the New Delhi environment and its implications for human health: an environmental point prevalence study. *Lancet Infect Dis,* 11**,** 355-62.

Woodford, N., *et al.* 2011. Multiresistant Gram-negative bacteria: the role of high-risk clones in the dissemination of antibiotic resistance. *FEMS Microbiol Rev,* 35**,** 736-55.

Yigit, H., *et al.* 2001. Novel carbapenem-hydrolyzing beta-lactamase, KPC-1, from a carbapenem-resistant strain of *Klebsiella pneumoniae. Antimicrob Agents Chemother,* 45**,** 1151-61.

Yong, D., *et al.* 2009. Characterization of a new metallo-beta-lactamase gene, *bla*(NDM-1), and a novel erythromycin esterase gene carried on a unique genetic structure in *Klebsiella pneumoniae* sequence type 14 from India. *Antimicrob Agents Chemother,* 53**,** 5046-54.

2. Aims of the study

2. Aims of the study

Extended-spectrum cephalosporins and carbapenems constitute β-lactam antibiotics considered first-line therapeutic options to treat infections caused by *Enterobacteriaceae*. However, the exponential and worldwide expansion of extended-spectrum-β-lactamases (ESBL) and carbapenemases among *Enterobacteriaceae* isolates and/or the emergence of ESBL-producing isolates with permeability changes have been compromising the use of these antibiotics.

In Portugal, available epidemiological studies report a high occurrence and diversity of ESBLs in different *Enterobacteriaceae* species from human (hospitalized and non-hospitalized patients), animal and environmental settings. However, most of these studies included only *E. coli* or *K. pneumoniae* isolates from particular geographic locations, settings or time periods, having poorly addressed the role of particular clones and/or plasmids in the dissemination and persistence of (bla_{ESBL}) and carbapenemase (bla_{CARB}) genes in different niches. Moreover, the recent identification of carbapenemase-producing *Enterobacteriaceae* in our country is worrisome, alerting for the need of continuous surveillance and characterization.

The **global goal** of this work is the multi-level molecular epidemiological characterization of recent (2006-2010) *Enterobacteriaceae* isolates resistant to extended-spectrum cephalosporins and/or carbapenems from different ecological niches (hospitalized patients, pig farms).

The **specific objectives** are:

i) To analyse the diversity of bla_{ESBL} genes in recent *Enterobacteriaceae* isolates (2006-2010) from different Portuguese hospitals and pig farms;

ii) To evaluate the contribution of clones and/or horizontal gene transfer elements (plasmids, integrons/*gene cassettes*) in the spread and persistence of bla_{ESBL} genes and bla_{CARB} genes;

iii) To monitor the emergence of *Enterobacteriaceae* isolates with decreased susceptibility to carbapenems and to characterize them at the molecular level.

3. Results

3.1.

Epidemiology of extended-spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae* in the clinical setting

3.1.1.

Current spread of CTX-M genes in Portuguese hospitals is associated with widespread *Escherichia coli* clones from different phylogenetic groups

Current spread of CTX-M genes in Portuguese hospitals is associated with widespread *Escherichia coli* clones from different phylogenetic groups

Carla Rodrigues[1, 2], *Elisabete Machado*[1, 2], *João Pires*[1], *Luísa Peixe*[1] and *Ângela Novais*[1]

[1]**REQUIMTE. Faculdade de Farmácia, Universidade do Porto, Porto, Portugal**;

[2]**CEBIMED, Faculdade de Ciências da Saúde, Universidade Fernando Pessoa, Porto, Portugal.**

Running title: ESBL-producing *Escherichia coli* in Portuguese hospitals

Keywords: ESBL, CTX-M-15, SHV-12, ST131, ST117, ST10, ST155

Manuscript Final Draft

ABSTRACT

Objectives: The diversity of extended-spectrum β-lactamase (ESBL)-producing *E. coli* has significantly been reduced by the expansion in different geographic areas of particular high-risk clones. Previous reports document a high occurrence of ESBLs in Portugal, but population structure of ESBL-producing *E. coli* remains largely unknown. Our aim is to characterize recent ESBL-types and *E. coli* clones recovered from different Portuguese hospitals during two time periods (2006-07 and 2010).

Methods: One hundred and seventy-three ESBL-producing *E. coli* isolates recovered from 3 Portuguese hospitals [A (North), B and C (Centre); 2006-07 and 2010] were analysed. Bacterial identification and antibiotic susceptibility testing were performed by standard methods. ESBL characterization included DDST, PCR ($bla_{\text{CTX-M}}$, bla_{SHV} and bla_{TEM}) and sequencing. Clonal relatedness was established by *Xba*I-PFGE and MLST. O25b-ST131 clone and *E. coli* phylogroups were identified by PCR.

Results: CTX-M-15 was the most prevalent ESBL-type (68%) detected in all hospitals and time periods, although other CTX-M variants (24%; CTX-M-1, -2, -9, -14, -32, -79) were also identified, mainly in 2010. SHV (6%; only SHV-12) and TEM (2%; only TEM-52) were less frequent. ESBL-producing *E. coli* clones belonged to phylogenetic groups B2 (67%), A (12%), B1 (12%) or D (9%). The B2-ST131 *E. coli* clone (n=114/66%; 7 PFGE-types) was detected since 2006 in different hospitals harbouring mainly $bla_{\text{CTX-M-15}}$ (97%), but also $bla_{\text{CTX-M-1}}$ (1%), $bla_{\text{CTX-M-32}}$ (1%), $bla_{\text{CTX-M-14}}$ (1%), or $bla_{\text{SHV-12}}$ (1%). A high clonal diversity was observed among other phylogroups, although a few widespread clones or clonal complexes such as D-ST117, D-ST648, A-CC10, A-CC23 and B1-CC155 were detected carrying diverse bla_{ESBL} genes (CTX-M-1, -9, -14, -15, -32, -79; SHV-12; TEM-52).

Conclusion: We describe current spread of a high diversity of ESBLs (mostly CTX-M-types) among *E. coli* in Portuguese hospitals, associated with widespread clones from different phylogenetic groups. The genetic diversity (PFGE- and ESBL-types) observed for ST131 and other *E. coli* clones suggests intraclonal evolution by both genomic and plasmid diversification.

INTRODUCTION

In the last decade, *Escherichia coli* has emerged as a major extended-spectrum-β-lactamase (ESBL)-producing pathogen in the hospital setting. This expansion has greatly been influenced by the spread of particular high-risk *E. coli* clonal complexes (CC) belonging to different phylogenetic groups in distinct geographic regions.[1,2] The worldwide disseminated B2-ST131 *E. coli* clone is one of the most representative examples,[1,2] but other D-*E. coli* clones (ST69, ST393, ST405) exhibiting multidrug resistance profiles (including ESBL production) are also commonly identified in hospitalized patients.[3,4] In addition, A and B1 *E. coli* (frequently belonging to widespread clonal complexes such as CC10 or CC23) are increasingly being identified among ESBL-producing isolates in the nosocomial setting.[5-7]

In Portugal, previous surveys covering periods between 2002 and 2007 report a high occurrence of TEM-type enzymes (TEM-24, -52, -116) among ESBL-producing *E. coli*, and recent emergence of CTX-M enzymes (CTX-M-1, -9 -14, -15).[8-11] However, the population structure of ESBL-producing *E. coli* in Portuguese clinical institutions is largely unknown, with only a few studies giving insights into a small sample population.[12,13] Our aim is to characterize recent ESBL-types and *E. coli* clones recovered from different Portuguese hospitals during two time periods (2006-07 and 2010).

MATERIALS AND METHODS

One hundred and seventy-three ESBL-producing *E. coli* recovered at three hospitals located in the North (Hospital A, a central hospital) and Centre (Hospitals B and C, local hospitals) regions of Portugal were studied. The ESBL-producing *E. coli*

isolates of the 2006-2007 period (n=103/173, 60%) were obtained from Hospital A (n=49) or Hospitals B and C (n=54). The ESBL-producing *E. coli* isolates of the 2010 period (n=70/173, 40%) were recovered from Hospital A. Only one isolate per patient and hospitalization week was studied.

Bacterial identification and preliminary antimicrobial susceptibility testing were performed with the automated PHOENIX (BD Diagnostic Systems, Sparks, MD) or VITEK (bioMérieux, Marcy l'Étoile, France) systems. Antimicrobial susceptibility to non-β-lactam antibiotics (aminoglycosides, quinolones, tetracycline, sulphonamides, trimethoprim, chloramphenicol and nitrofurantoin) was further determined by the standard disk diffusion method.[14] ESBL production was inferred by the double disk synergy test (DDST) and further confirmed by PCR (bla_{TEM}, bla_{SHV} and bla_{CTX-M}) and sequencing.[12] Clonal relatedness was investigated by pulsed-field gel electrophoresis (PFGE) of *Xba*I-digested genomic DNA (10s-40s pulses for 21h, 14°C, 6 V/cm^2) and multi-locus sequence typing (MLST) (http://mlst.ucc.ie/mlst/dbs/Ecoli).[12] The sequence types (ST) were identified when 3 alleles (*fumC*, *gyrB* and/or *mdh*) were compatible with those from high-risk *E. coli* clonal complexes. Identification of *E. coli* phylogenetic groups and O25b-ST131 lineage were performed by PCR.[15,16]

RESULTS AND DISCUSSION

ESBL-producing *E. coli* isolates were mainly recovered from urine samples (85%). CTX-M-15 was the most prevalent ESBL-type (n=117/68%) detected in all hospitals and time periods analysed (Table 1). Other CTX-M (n=17/10% CTX-M-14, n=14/8% CTX-M-1, n=6/3% CTX-M-32, n=2/1% CTX-M-2, n=1/0.6% CTX-M-9, and n=1/0.6% CTX-M-79), SHV-12 (n=11/6%) and TEM-52 (n=4/2%) enzymes were also detected (Table 1). Although the proportion of CTX-M enzymes was similar between

both time periods analyzed (93% in 2006-07 *versus* 89% in 2010), a higher diversity of CTX-M-types was detected more recently (2010) (Table 1). Our results demonstrate current spread and dominance of diverse CTX-M enzymes (mainly CTX-M-15) and a low prevalence of other ESBL variants (SHV-12 and TEM-52), suggesting a shift in ESBL-types among *E. coli* isolates from Portuguese hospitals**,** as described all over the world.[1,17,18] ESBL-producing *E. coli* belonged to a high diversity of clones from phylogenetic groups B2 (n=116/67%), A (n=20/12%), B1 (n=21/12%) or D (n=16/9%), harbouring a diversity of bla_{ESBL} genes. Whereas B2-*E. coli* were extensively identified in both periods (81% in 2006-07 *versus* 47% in 2010), other phylogroups (A, B1, D) were more frequently observed in 2010 (52%) than in 2006-07 (19%).

The B2-*E. coli* isolates mainly belonged to ST131 (n=114/116, 7 PFGE-types), which represented 66% (n=114/173) of all *E. coli* isolates identified in both time periods, and most of them harboured $bla_{\text{CTX-M-15}}$ (n=110/97%; sporadically associated with $bla_{\text{TEM-10}}$ or $bla_{\text{TEM-116}}$), but also $bla_{\text{CTX-M-1}}$ (n=1/1%), $bla_{\text{CTX-M-32}}$ (n=1/1%), $bla_{\text{CTX-M-14}}$ (n=1/1%), or $bla_{\text{SHV-12}}$ (n=1/1%). Non-ST131 B2-*E. coli* isolates were diverse (*fumC11*, *fumC103*) and harboured $bla_{\text{CTX-M-1}}$ or $bla_{\text{CTX-M-2}}$ (Table 1). The high diversity of PFGE-types, bla_{ESBL} genes and/or antibiotic resistance profiles associated with ST131 isolates in this and other studies,[7,19-21] suggests a high recombinogenic potential for ST131, evolving either by genomic diversification or by acquisition of diverse ESBL-encoding genetic platforms, supporting previous observations.[22,23]

E. coli isolates of phylogroup D (n=16/9%) increased from 4% in 2006-07 to 17% in 2010 and were genetically diverse (14 PFGE-types) (Table 1). While most of them were linked to diverse sequence types (including the ST648), five isolates corresponded to ST117 (n=4) or a new ST (n=1, a double locus variant of ST117) producing different ESBLs (CTX-M-1, CTX-M-1 plus TEM-116, CTX-M-14 or SHV-

12), which were identified in different hospitals since 2006 (Table 1). Both ST648 and ST117 clones are here firstly reported in Portuguese hospitals. They have been described in clinical isolates and also among non-human hosts (poultry, food products) in different countries associated with diverse ESBLs and/or carbapenemases.[7,24-31] Interestingly, the ST69, ST393 and ST405 *E. coli* clones, which are widely spread in other geographic locations, were not detected.[3,4,32,33]

E. coli isolates from phylogenetic groups A (n=20/12%) or B1 (n=21/12%) were more frequently observed in the 2010 (19%-A, 17%-B1) than in the 2006-07 (7%-A, 9%-B1) period (Table 1). Despite the high clonal diversity detected (17 and 18 PFGE-types, respectively), clones belonging to the widespread clonal complexes (CC) A-CC10 (2 ST10, 2 ST44, 2 ST617, 1 ST167), A-CC23 (1 ST88, 1 ST410) or B1-CC155 (2 ST155, 1 ST58) and producing a diversity of ESBLs (CTX-M-1, -14, -15, -32; SHV-12; TEM-52) were detected in different hospitals since 2006 (Table 1). A and B1 *E. coli* are increasingly being detected among ESBL producers from hospitalized patients in different countries and continents.[6,7,19,34-36] Some of these clones have recently been described in ESBL-producing isolates from hemodialyzed patients,[13] suggesting a wider distribution in our country.

In this study, we describe current spread of a high diversity of ESBLs (mainly CTX-M types) in different Portuguese hospitals, which is associated with different widespread *E. coli* clones from B2 (ST131), D (ST117), A (CC10, CC23) or B1 (CC155) phylogenetic groups. The genomic diversity (diverse PFGE-types) detected among ST131 and other *E. coli* clones and their association with different bla_{ESBL} genes suggests intraclonal evolution by both genomic and plasmid diversification.

ACKNOWLEDGEMENTS

We are grateful to Helena Ramos (Hospital Geral de Santo António, Porto, Portugal), José Luís Grañeda (Centro Hospitalar Cova da Beira, EPE), and Serviço de Patologia Clínica, Centro Hospitalar Tondela-Viseu, EPE, for the clinical isolates included in this study.

FUNDING

The present work has been supported by Fundação para a Ciência e a Tecnologia (grant no. PEst-C/EQB/LA0006/2011), Marie Curie Intra European Fellowship and Fundação Ensino e Cultura Fernando Pessoa. Ângela Novais is supported by a Marie Curie Intra European Fellowship within the 7th European Community Framework Programme (PIEF-GA-2009-255512).

TRANSPARENCY DECLARATIONS

No conflicts of interest to declare.

REFERENCES

1. Coque TM, Baquero F, Cantón R. Increasing prevalence of ESBL-producing *Enterobacteriaceae* in Europe. *Euro surveillance* 2008; **13**.

2. Woodford N, Turton JF, Livermore DM. Multiresistant Gram-negative bacteria: the role of high-risk clones in the dissemination of antibiotic resistance. *FEMS Microbiology Reviews* 2011; **35**: 736-55.

3. Blanco J, Mora A, Mamani R *et al*. National survey of *Escherichia coli* causing extraintestinal infections reveals the spread of drug-resistant clonal groups O25b:H4-

B2-ST131, O15:H1-D-ST393 and CGA-D-ST69 with high virulence gene content in Spain. *The Journal of Antimicrobial Chemotherapy* 2011; **66**: 2011-21.

4. Johnson JR, Menard M, Johnston B *et al*. Epidemic clonal groups of *Escherichia coli* as a cause of antimicrobial-resistant urinary tract infections in Canada, 2002 to 2004. *Antimicrobial Agents and Chemotherapy* 2009; **53**: 2733-9.

5. Rodriguez-Bano J, Mingorance J, Fernandez-Romero N *et al*. Virulence profiles of bacteremic extended-spectrum beta-lactamase-producing *Escherichia coli*: association with epidemiological and clinical features. *PloS one* 2012; **7**: e44238.

6. Fam N, Leflon-Guibout V, Fouad S *et al*. CTX-M-15-producing *Escherichia coli* clinical isolates in Cairo (Egypt), including isolates of clonal complex ST10 and clones ST131, ST73, and ST405 in both community and hospital settings. *Microb Drug Resist* 2011; **17**: 67-73.

7. Oteo J, Diestra K, Juan C *et al*. Extended-spectrum beta-lactamase-producing *Escherichia coli* in Spain belong to a large variety of multilocus sequence typing types, including ST10 complex/A, ST23 complex/A and ST131/B2. *International Journal of Antimicrobial Agents* 2009; **34**: 173-6.

8. Machado E, Coque TM, Cantón R *et al.* High diversity of extended-spectrum beta-lactamases among clinical isolates of *Enterobacteriaceae* from Portugal. *The Journal of Antimicrobial Chemotherapy* 2007; **60**: 1370-4.

9. Machado E, Coque TM, Canton R *et al*. 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. *Antimicrobial Agents and Chemotherapy* 2006; **50**: 3220-1.

10. Mendonça N, Leitão J, Manageiro V *et al*. Spread of extended-spectrum beta-lactamase CTX-M-producing *Escherichia coli* clinical isolates in community and nosocomial environments in Portugal. *Antimicrobial Agents and Chemotherapy* 2007; **51**: 1946-55.

11. Fernandes R, Gestoso A, Freitas JM *et al*. High resistance to fourth-generation cephalosporins among clinical isolates of *Enterobacteriaceae* producing extended-spectrum beta-lactamases isolated in Portugal. *International Journal of Antimicrobial Agents* 2009; **33**: 184-5.

12. Novais A, Rodrigues C, Branquinho R *et al*. Spread of an OmpK36-modified ST15 *Klebsiella pneumoniae* variant during an outbreak involving multiple carbapenem-resistant *Enterobacteriaceae* species and clones. *European Journal of Clinical Microbiology & Infectious Diseases* 2012; **31**: 3057-63.

13. Correia S, Pacheco R, Radhouani H *et al*. High prevalence of ESBL-producing *Escherichia coli* isolates among hemodialysis patients in Portugal: appearance of ST410 with the bla(CTX-M-14) gene. *Diagnostic Microbiology and Infectious Disease* 2012; **74**: 423-5.

14. Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing: *Nineteenth Informational Supplement M100*-S20 CLSI, Wayne, PA, USA, 2011.

15. Clermont O, Bonacorsi S, Bingen E. Rapid and simple determination of the *Escherichia coli* phylogenetic group. *Applied and Environmental Microbiology* 2000; **66**: 4555-8.

16. Clermont O, Dhanji H, Upton M *et al*. Rapid detection of the O25b-ST131 clone of *Escherichia coli* encompassing the CTX-M-15-producing strains. *The Journal of Antimicrobial hemotherapy* 2009; **64**: 274-7.

17. Hawkey PM, Jones AM. The changing epidemiology of resistance. *The Journal of Antimicrobial Chemotherapy* 2009; **64 Suppl 1**: i3-10.

18. Cantón R, Coque TM. The CTX-M beta-lactamase pandemic. *Current Opinion in Microbiology* 2006; **9**: 466-75.

19. Park SH, Byun JH, Choi SM *et al*. Molecular epidemiology of extended-spectrum beta-lactamase-producing *Escherichia coli* in the community and hospital in Korea: emergence of ST131 producing CTX-M-15. *BMC Infectious Diseases* 2012; **12**: 149.

20. Peirano G, Richardson D, Nigrin J *et al*. High prevalence of ST131 isolates producing CTX-M-15 and CTX-M-14 among extended-spectrum-beta-lactamase-producing *Escherichia coli* isolates from Canada. *Antimicrobial Agents and Chemotherapy* 2010; **54**: 1327-30.

21. Rogers BA, Sidjabat HE, Paterson DL. *Escherichia coli* O25b-ST131: a pandemic, multiresistant, community-associated strain. *The Journal of Antimicrobial Chemotherapy* 2011; **66**: 1-14.

22. Novais A, Pires J, Ferreira H *et al*. Characterization of globally spread *Escherichia* i ST131 isolates (1991 to 2010). *Antimicrobial Agents and Chemotherapy* 2012; **56**: 3973-6.

23. Novais A, Viana D, Baquero F *et al*. Contribution of IncFII and broad-host IncA/C and IncN plasmids to the local expansion and diversification of phylogroup B2 *Escherichia coli* ST131 clones carrying blaCTX-M-15 and *qnrS1* genes. *Antimicrobial Agents and Chemotherapy* 2012; **56**: 2763-6.

24. Leverstein-van Hall MA, Dierikx CM, Cohen Stuart J *et al*. Dutch patients, retail chicken meat and poultry share the same ESBL genes, plasmids and strains. *Clinical Microbiology and Infection* 2011; **17**: 873-80.

25. Cortés P, Blanc V, Mora A *et al*. Isolation and characterization of potentially pathogenic antimicrobial-resistant *Escherichia coli* strains from chicken and pig farms in Spain. *Applied and Environmental Microbiology* 2010; **76**: 2799-805.

26. van der Bij AK, Peirano G, Goessens WH *et al*. Clinical and molecular characteristics of extended-spectrum-beta-lactamase-producing *Escherichia coli* causing bacteremia in the Rotterdam Area, Netherlands. *Antimicrobial Agents and Chemotherapy* 2011; **55**: 3576-8.

27. Mora A, Blanco M, Lopez C *et al*. Emergence of clonal groups O1:HNM-D-ST59, O15:H1-D-ST393, O20:H34/HNM-D-ST354, O25b:H4-B2-ST131 and ONT:H21,42-B1-ST101 among CTX-M-14-producing *Escherichia coli* clinical isolates in Galicia, northwest Spain. *International journal of antimicrobial agents* 2011; **37**: 16-21.

28. Vincent C, Boerlin P, Daignault D *et al*. Food reservoir for *Escherichia coli* causing urinary tract infections. *Emerging Infectious Diseases* 2010; **16**: 88-95.

29. Mora A, Lopez C, Herrera A *et al*. Emerging avian pathogenic *Escherichia coli* strains belonging to clonal groups O111:H4-D-ST2085 and O111:H4-D-ST117 with high virulence-gene content and zoonotic potential. *Veterinary Microbiology* 2012; **156**: 347-52.

30. Hornsey M, Phee L, Wareham DW. A novel variant, NDM-5, of the New Delhi metallo-beta-lactamase in a multidrug-resistant *Escherichia coli* ST648 isolate recovered from a patient in the United Kingdom. *Antimicrobial Agents and Chemotherapy* 2011; **55**: 5952-4.

31. Mushtaq S, Irfan S, Sarma JB *et al*. Phylogenetic diversity of *Escherichia coli* strains producing NDM-type carbapenemases. *The Journal of Antimicrobial Chemotherapy* 2011; **66**: 2002-5.

32. Coque TM, Novais A, Carattoli A *et al*. Dissemination of clonally related *Escherichia coli* strains expressing extended-spectrum beta-lactamase CTX-M-15. *Emerging Infectious Diseases* 2008; **14**: 195-200.

33. Matsumura Y, Yamamoto M, Nagao M *et al*. Emergence and spread of B2-ST131-O25b, B2-ST131-O16 and D-ST405 clonal groups among extended-spectrum-beta-lactamase-producing *Escherichia coli* in Japan. *The Journal of Antimicrobial Chemotherapy* 2012; **67**: 2612-20.

34. Valverde A, Cantón R, Garcillan-Barcia MP *et al*. Spread of bla(CTX-M-14) is driven mainly by IncK plasmids disseminated among *Escherichia coli* phylogroups A, B1, and D in Spain. *Antimicrobial Agents and Chemotherapy* 2009; **53**: 5204-12.

35. Peirano G, van der Bij AK, Gregson DB *et al*. Molecular epidemiology over an 11-year period (2000 to 2010) of extended-spectrum beta-lactamase-producing *Escherichia coli* causing bacteremia in a centralized Canadian region. *Journal of Clinical Microbiology* 2012; **50**: 294-9.

36. Aibinu I, Odugbemi T, Koenig W *et al*. Sequence type ST131 and ST10 complex (ST617) predominant among CTX-M-15-producing *Escherichia coli* isolates from Nigeria. *Clinical Microbiology and Infection* 2012; **18**: E49-51.

Table 1. Epidemiological features of ESBL-producing *E. coli* from Portuguese hospitals (2006-07 and 2010)

PhG[a] (no./%)	Sequence Type (ST)/ Clonal Complex (CC) (no. of isolates/ no. of PFGE-types)	ESBLs (no. of isolates)	Isolation period	Hospital[b]	Antibiotic resistance to non-β-lactam antibiotics[c,d]
B2 (116/67%)	ST131/- (114/8)	CTX-M-15 (106)	2006-07; 2010	A, B, C	(AMK), (CIP), (CLO), (GEN), (KAN), (NAL), (NET), (SUL), (STR), (TET), (TMP), (TOB)
		CTX-M-15 + TEM-116 (3)	2006-07	C	(CIP), GEN, KAN, (NAL), (NET), (SUL), (STR), TET, TOB
		CTX-M-15 + TEM-10 (1)	2006-07	C	AMK, CIP, GEN, KAN, NAL, NET, SUL, STR, TET, TMP, TOB
		CTX-M-32 (1)	2006-07	A	NAL, CIP, SUL, STR, TET, TMP
		CTX-M-1 (1)	2010	A	GEN, NAL, SUL, TET, TMP, TOB
		CTX-M-14 (1)	2010	A	NAL, TET
		SHV-12 (1)	2006-07	B	NAL, SUL, STR, TMP, TET
	fumC103, mdh23 (1/1)	CTX-M-1 (1)	2006-07	A	CIP, GEN, KAN, NAL, NET, STR, TET, TOB
	fumC11 (1/1)	CTX-M-2 (1)	2010	A	SUL, STR, TET, TMP
D (16/9%)	ST117/- (5/4)	CTX-M-1 (2)	2010	A	(NAL), KAN, (SUL), STR, TET, TMP
		CTX-M-1 + TEM-116 (1)	2006-07	C	CIP, CLO, KAN, NAL, SUL, STR, TET, TMP
		CTX-M-14 (1)	2006-07	C	CLO, NAL, SUL, STR, TET
	(DLV of ST117)/- (1/1)	SHV-12 (1)	2010	A	CIP, NAL, SUL
	ST648/- (1/1)	CTX-M-15 (1)	2006-07	A	CIP, NAL, SUL, STR, TMP
	fumC31, gyrB5 (3/2)	CTX-M-14 (2)	2010	A	CIP, CLO, NAL, (NIT), SUL, STR, TET, (TMP)
		CTX-M-1 (1)	2010	A	SUL, TET, TMP
	ST1011/- (2/1)	CTX-M-1 (1)	2010	A	CIP, NAL, NIT, SUL, STR, TET, TMP
		CTX-M-32 (1)	2010	A	CIP, NAL, SUL, STR, TMP
	fumC88, gyrB97 (1/1)	CTX-M-79 (1)	2010	A	CIP, GEN, NAL, SUL, STR, TET, TMP, TOB
	fumC88 (1/1)	CTX-M-14 (1)	2010	A	CIP, GEN, KAN, NAL, NET, STR, TOB
	fumC38, gyrB84 (1/1)	CTX-M-1 (1)	2010	A	KAN, NAL, SUL, TET
	fumC219 (1/1)	TEM-52 (1)	2010	A	CIP, NAL, GEN, STR, TET, TMP

Table 1. Epidemiological features of ESBL-producing *E. coli* from Portuguese hospitals (2006-07 and 2010) (cont.)

PhG[a] (no./%)	Sequence Type (ST)/ Clonal Complex (CC) (no. of isolates/ no. of PFGE-types)	ESBLs (no. of isolates)	Isolation period	Hospital[b]	Antibiotic resistance to non-β-lactam antibiotics[c,d]
A (20/12%)	ST10/CC10 (2/2)	CTX-M-15 (1)	2006-07	C	CLO, NAL, SUL, STR, TET, TMP
		CTX-M-32 (1)	2006/07	C	SUL, STR, TET
	ST44/CC10 (2/1)	CTX-M-15 (2)	2010	A	CIP, (GEN), KAN, NAL, NET, (NIT), STR, SUL, (TET), TMP, TOB
	ST617/CC10 (2/1)	CTX-M-32 (2)	2010	A	CIP, (KAN), NAL, (SUL), STR, TET, (TMP), TOB
	ST167/CC10 (1/1)	CTX-M-1 (1)	2010	A	CIP, CLO, NAL, SUL, STR, TET, TMP
	ST410/CC23 (1/1)	CTX-M-15 (1)	2006-07	A	CIP, GEN, KAN, NAL, NET, STR, SUL, TET, TMP, TOB
	ST88 /CC23 (1/1)	TEM-52 (1)	2006-07	A	STR
	fumC11, gyrB4 (4/4)	CTX-M-1 (1)	2010	A	NAL, SUL, TET, TMP
		CTX-M-32 (1)	2010	A	CIP, CLO, GEN, NAL, SUL, STR, TET, TMP, TOB
		CTX-M-14 (1)	2010	A	CIP, KAN, NAL, SUL, STR, TET, TMP
		SHV-12 (1)	2010	A	STR
	ST2228/- (1/1)	CTX-M-15 (1)	2006-07	C	AMK, CIP, GEN, KAN, NAL, NET, STR, TET, TOB
	ST2230/- (1/1)	CTX-M-1 (1)	2006-07	B	GEN, KAN, NAL, NET, SUL, STR, TET, TMP, TOB
	fumC4, gryB33 (2/1)	CTX-M-14 (2)	2010	A	(KAN), SUL, STR, TET
	fumC11, gyrB135 (1/1)	SHV-12 (1)	2010	A	CLO, CIP, KAN, NAL, SUL, STR, TET, TMP
	fumC11 (1/1)	CTX-M-2 (1)	2006-07	A	CIP, NAL, SUL, STR, TET, TMP
	fumC23, gyrB15 (1/1)	CTX-M-15 (1)	2010	A	CIP, KAN, NAL, SUL, STR, TMP
B1 (21/12%)	ST155/CC155 (2/1)	CTX-M-1 (2)	2006-07	A	CIP, (CLO), (KAN), NAL, STR, SUL, TMP
	ST58/CC155 (3/2)	SHV-12 (1)	2006-07	A	AMK, CIP, KAN, NAL, NET, SUL, STR, TMP
		TEM-52 (1)	2006-07	C	NAL, KAN, SUL, STR, TET, TMP
		CTX-M-14 (1)	2010	A	SUL, STR, TMP
	ST348/CC156 (2/1)	CTX-M-14 (1)	2006-07	C	CLO, NAL, SUL STR
		SHV-12 (1)	2006-07	A	CLO, NAL, SUL STR, TET
	fumC6, gyrB12 (2/2)	CTX-M-14 (2)	2010	A	(CIP), (NAL), (SUL), STR, (TET), (TMP)

Table 1. Epidemiological features of ESBL-producing *E. coli* from Portuguese hospitals (2006-07 and 2010) (cont.).

PhG[a] (no./%)	Sequence Type (ST)/ Clonal Complex (CC) (no. of isolates/ no. of PFGE-types)	ESBLs (no. of isolates)	Isolation period	Hospital[b]	Antibiotic resistance to non-β-lactam antibiotics[c,d]
	fumC4, gyrB5 (2/1)	CTX-M-14 (2)	2010	A	CIP, CLO, GEN, KAN, NAL, SUL, STR, (TET), TMP
	fumC6, mdh11 (2/1)	SHV-12 (2)	2010	A	CIP, CLO, (KAN), NAL, SUL, STR, TET, TMP
	fumC23, mdh9 (1/1)	CTX-M-1 (1)	2010	A	CIP, KAN, NAL, SUL, STR, TMP
	fumC6, gyrB5 (1/1)	CTX-M-14 (1)	2006-07	A	CIP, KAN, NAL, SUL, STR, TET, TMP
	fumC4, gyrB12 (1/1)	CTX-M-14 (1)	2006-07	A	CLO, SUL, STR, TET, TMP
	ST2229/CC101 (1/1)	CTX-M-14 (1)	2010	A	CLO, SUL, STR, TET, TMP
	fumC219 (1/1)	CTX-M-9 (1)	2010	A	CIP, NAL, SUL, STR, TET, TMP
	ST1431/- (1/1)	SHV-12 (1)	2006-07	C	CLO, NAL, SUL
	fumC6, mdh24 (1/1)	SHV-12 (1)	2010	A	CIP, CLO, NAL, SUL, STR, TET, TMP
	fumC4, gyrB33 (1/1)	SHV-12 (1)	2010	A	CIP, CLO, NAL, SUL, STR, TET, TMP

[a] PhG, *E. coli* phylogenetic group. [b]Hospital A is located at the North region of Portugal; Hospitals B and C are located at the Centre region of Portugal. [c]AMK, amikacin; CLO, chloramphenicol; CIP, ciprofloxacin; GEN, gentamicin; KAN, kanamycin; NAL, nalidixic acid; NET, netilmicin; NIT, nitrofurantoin; STR, streptomycin; SUL, sulphonamides; TOB, tobramycin; TET, tetracycline; TMP, trimethoprim; [d] Variable presence of resistance phenotype is indicated by parenthesis.

3.1.2.

Amplification of ST15, ST147 and ST336 *Klebsiella pneumoniae* clones producing different extended-spectrum β-lactamases in Portuguese hospitals

Amplification of ST15, ST147 and ST336 *Klebsiella pneumoniae* clones producing different extended-spectrum β-lactamases in Portuguese hospitals

Carla Rodrigues[1,2], Ângela Novais[1], Elisabete Machado[1,2], and Luísa Peixe[1]

[1]REQUIMTE. Faculdade de Farmácia, Universidade do Porto, Porto, Portugal;

[2]CEBIMED, Faculdade de Ciências da Saúde, Universidade Fernando Pessoa, Porto, Portugal.

Running title: Clonal spread of ESBL-producing *K. pneumoniae* in Portuguese hospitals

Keywords: *Enterobacteriaceae,* clonal spread, ESBL, CTX-M-15, SHV-12

Manuscript Final Draft

ABSTRACT

Objectives: Scarce studies trace the trends in ESBL-types and clones of ESBL-producing *Enterobacteriaceae* in Portugal. We investigated the shifts in ESBL-types and the role of clonal spread in the dissemination of ESBLs among non-*Escherichia coli Enterobacteriaceae* from Portuguese hospitals during two recent time periods.

Methods: A total of ninety-one ESBL-producing non-*E. coli Enterobacteriaceae* isolates (73 *Klebsiella pneumoniae,* 13 *Enterobacter cloacae,* 3 *Klebsiella oxytoca*, 1 *Proteus mirabilis* and 1 *Serratia marcescens*) recovered from 3 Portuguese hospitals [North (A) and Centre (B, C) regions; 2006-07 and 2010] were studied. Bacterial identification and antibiotic susceptibility testing were performed by standard methods. ESBL characterization included DDST, PCR and sequencing. Clonal relatedness was established by PFGE and *K. pneumoniae* clones were identified by MLST.

Results: Isolates produced mostly CTX-M-15 (45%) and SHV-12 (29%), and less frequently other CTX-M (1%; CTX-M-32) or SHV (15%; SHV-2, -5, -28, -55, -106, -145) types, or TEM (10%; TEM-10, -24, -116, -199) enzymes. Three *K. pneumoniae* epidemic clones (ST15, ST147, ST336) were identified during large periods of time: i) ST336 (n=32/44%; 1 PFGE-type) producing CTX-M-15 (97%) or SHV-12 (3%); ii) ST15 (n=16/21%; 3 PFGE-types) producing CTX-M-15 (31%) and a diversity of closely related SHV-types (69%; SHV-2, -12, -28, -55, -106); and iii) ST147 (n=8/11%; 1 PFGE-type) encoding SHV-12. Sporadic *K. pneumoniae* clones (n=17/23%; 16 PFGE-types), and isolates of *E. cloacae* (n=13/14%; 4 PFGE-types), *K. oxytoca* (n=3/3%; 3 PFGE-types), *S. marcescens* (n=1/1%,;1 PFGE-type) and *P. mirabilis* (n=1/1%; 1 PFGE-type) producing different ESBLs (TEM-10, -24, -116, -199; SHV-2, -5, -12; CTX-M-15, -32) were also detected, mostly in the 2006-07 period.

Conclusion: The amplification of CTX-M-15 and SHV (mostly SHV-12) among non-*E. coli Enterobacteriaceae* species circulating in Portuguese hospitals was linked to three *K. pneumoniae* epidemic clones (ST15, ST147, ST336), some exhibiting a high intraclonal diversity. Their spread should be monitored due to the risk of further expansion.

INTRODUCTION

Extended-spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae* are amongst the most frequent pathogens involved in nosocomial infections worldwide.[1] Their diversity has significantly been reduced by the selection and amplification of particular clones belonging mostly to *Escherichia coli* (e.g. ST131, ST69 or ST393) and *Klebsiella pneumoniae* (e.g. ST11, ST15, ST147 or ST258) species, which have been responsible for the spread of particular ESBL-types (mainly CTX-M) and/or other antibiotic resistance mechanisms (e.g., carbapenemases, 16S rRNA methylases) in different countries all over the world.[2-6] ESBLs have also been identified in other *Enterobacteriaceae* species, but epidemiological data is scarce and limited to particular outbreak situations.[1,7-10]

Cross-sectional and temporal evolution studies are important to understand epidemiological features and trends associated with the spread of ESBL-producing *Enterobacteriaceae* in a given country and to improve infection control strategies. In Portugal, previous surveys restricted to particular species and/or time periods (1999, 2002-04 and 2006) highlighted a high diversity of ESBLs (mainly SHV- and TEM-types) and clones among *Enterobacteriaceae* isolates of clinical origin, and anticipated the emergence of CTX-M enzymes.[11,12] However, they poorly addressed the role of particular clones and/or plasmids in the dissemination and persistence of ESBL genes in Portuguese hospitals. The aim of this study is to characterize the recent trends in ESBL-types and to investigate the role of clonal spread in the dissemination of ESBLs among *Enterobacteriaceae* other than *E. coli* identified in different Portuguese hospitals in two time periods (2006-07 and 2010).

MATERIALS AND METHODS

Ninety-one ESBL-producing non-*E. coli Enterobacteriaceae* isolates (73 *Klebsiella pneumoniae*, 13 *Enterobacter cloacae*, 3 *Klebsiella oxytoca*, 1 *Proteus mirabilis, 1 Serratia marcescens*) recovered during 2006-07 (n=33) and 2010 (n=58) from 3 hospitals located at the North (Hospital A, general hospital) and Centre (Hospitals B and C, local hospitals) of Portugal were characterized. They represented one isolate per patient and hospitalization week. Isolates were mostly recovered from urine (n=55/60%) or sputum (n=16/18%) (Table 1), and from patients at medicine wards (n=43/47%) or outpatients (n=29/32%). Bacterial identification and preliminary antimicrobial susceptibility testing were performed using the automated PHOENIX (BD Diagnostic Systems, Sparks, MD) or VITEK (bioMérieux, Marcy l'Étoile, France) systems. Susceptibility to non-β-lactam antibiotics (aminoglycosides, quinolones, tetracycline, sulphonamides, trimethoprim, chloramphenicol and nitrofurantoin) was determined using the standard disk diffusion method.[13] All intermediate isolates were considered as resistant. ESBL production was inferred by the standard double disk synergy test (DDST) and confirmed by PCR (*bla*$_{TEM}$, *bla*$_{SHV}$ and *bla*$_{CTX-M}$) and sequencing.[5] Clonal relatedness was investigated by pulsed-field gel electrophoresis (PFGE), using *Xba*I or *Sma*I as macrorrestriction enzymes,[3,12] and *K. pneumoniae* clones were identified by multi-locus sequence typing (MLST) (http://www.pasteur.fr/recherche/genopole/PF8/mlst/website).

RESULTS AND DISCUSSION

A high diversity of ESBLs was identified, being CTX-M (n=42/46%; 41 CTX-M-15, 1 CTX-M-32) or SHV (n=40/44%; 26 SHV-12, 4 SHV-106, 3 SHV-55, 3 SHV-28, 2 SHV-2, 1 SHV-5, 1 SHV-145) enzymes more frequently observed than TEM variants (n=9/10%; 5 TEM-10, 2 TEM-24, 1 TEM-116, 1 TEM-199) (Table 1). The novel SHV-145 (containing mutation L122R), and TEM-199 (containing mutations Q39K, E104K, M155I and G238S) ESBL-types were first described in this work (Genbank accession numbers JX013655 and JX050178, respectively). CTX-M-15 was the most prevalent ESBL (n=41/45%), being identified among *K. pneumoniae* (n=38/93%), *E. cloacae* (n=2/5%) and *K. oxytoca* (n=1/2%) isolates more recently (n=38/93% in 2010 versus n=3/7% in 2006-07). SHV-12 was also frequently identified (n=26/29%) among *K. pneumoniae* (n=16/62%) and *E. cloacae* (n=10/38%) from both time periods (n=18/69% in 2006-07; n=8/31% in 2010). Other SHV variants (n=14/15%) were associated with *K. pneumoniae* isolates mostly recovered in 2010 (n=11/79%). Diverse TEM-type ESBLs were less prevalent (n=9/10%) and identified among different *Enterobacteriaceae* species (*K. pneumoniae*, *K. oxytoca*, *P. mirabilis*, *S. marcescens*) almost exclusively in the first time period (n=8/89% in 2006-07 versus n=1/11% in 2010). A few isolates produced simultaneously SHV-12 and TEM-116 (n=3; *E. cloacae*). Our results confirm a shift in ESBL-types among non-*E. coli* *Enterobacteriaceae* isolates since our previous survey in 2002-04, with the recent increase of CTX-M-15 and diverse SHV-types mainly in *K. pneumoniae* and the reduction of TEM-type ESBLs, as observed in other European countries.[1,12]

Most ESBL-producing *K. pneumoniae* isolates (n=56/73, 77%) corresponded to three epidemic clones belonging to pandemic lineages and responsible for the spread of CTX-M-15 and/or different SHV-types during large periods of time (Table 1):

i) ST336 producing CTX-M-15 (n=31/42%; 1 PFGE-type) was involved in an outbreak affecting mostly kidney transplanted patients located at different wards of Hospital A during 11 months (February-December 2010). An additional SHV-12-producing ST336 isolate was identified. ST336 belongs to the clonal complex (CC) 17, which seems to be widespread in different American, Asian and European countries, and associated with dissemination of CTX-M-15.[6,14,15] However, to the best of our knowledge, we report for the first time an outbreak of CTX-M-15-producing ST336 *K. pneumoniae* clone;

ii) ST15 (n=16/22%) producing different ESBLs, identified in Hospital A in both time periods analysed (2006, n=1; March-November 2010, n=15). ST15 isolates were assigned to 3 PFGE-types (profiles differing in more than 7 bands): Kp2 (n=5) encoding CTX-M-15, Kp3 (n=6) producing SHV-55 or the highly related SHV-106, and Kp4 (n=5) coding for the closely related SHV-2, SHV-12 or SHV-28 enzymes. ST15 has previously been identified in other Portuguese hospitals associated with the spread of CTX-M-15 or the novel VIM-34 carbapenemase[3] (Rodrigues *et al*., unpublished results), suggesting endemicity. This pandemic clone has also been involved in the spread of CTX-M-15 and carbapenemases (VIM-1, NDM-1) in other European (Hungary, Denmark, Spain) and Asian (South Korea, Malaysia, Singapore and Thailand) countries.[4,5,15-17] The identification of isolates belonging to the ST15 *K. pneumoniae* clone sharing the same PFGE profile and carrying bla_{ESBL} genes differing only in one or two amino acids (e.g. ST15/Kp3 with $bla_{\text{SHV-55}}$ or $bla_{\text{SHV-106}}$) might suggest an intra-clonal evolution of ESBLs.

iii) ST147 encoding SHV-12 (n=8/11%; 1 PFGE-type), detected since 2007 in Hospital A. This clone has been involved in the dissemination of CTX-M-15 in

Hungary,[4] and more recently in the worldwide spread of different carbapenemases (OXA-48, NDM-1, VIM-1 and KPC-2).[18-21]

Other sporadic *K. pneumoniae* clones (n=17/73, 23%; 16 PFGE-types) were linked to SHV (SHV-2, -5, -12, -55, -145), TEM (TEM-10, -24, -116) or CTX-M-15 production in different hospitals (Table 1).

Other ESBL-producing species (*E. cloacae*, *K. oxytoca*, *P. mirabilis*, *S. marcescens*) were mostly identified in Hospitals B and C (Table 1). Despite the identification of two epidemic *E. cloacae* clones producing SHV-12 or SHV-12 plus TEM-116 (n=10, 2 PFGE-types), other *E. cloacae* (n=3, 2 PFGE-types), *K. oxytoca* (n=3, 3 PFGE-types), *P. mirabilis* (n=1) and *S. marcescens* (n=1) isolates were linked to diverse ESBLs (CTX-M-15, CTX-M-32, TEM-10, TEM-24, TEM-199). The identification of identical ESBL-types between different *Enterobacteriaceae* species highlights the role of horizontal gene transfer in the spread of these enzymes.[22]

This study constitutes an update on ESBL epidemiology among non-*E. coli* *Enterobacteriaceae* species implicated in infectious diseases in Portuguese hospitals. We show current dominance of CTX-M-15 and diverse SHV-types (mainly SHV-12) associated with the emergence and spread of three *K. pneumoniae* pandemic clones/clonal complexes (ST15, ST147, ST336) during large periods of time. Moreover, the identification of variable PFGE profiles and bla_{ESBL} genes in the same clone suggests anticipates a more complex scenario involving genomic diversification, intraclonal evolution of bla_{ESBL} genes and/or the acquisition of different ESBL-encoding plasmids. Monitorization of the pandemic *K. pneumoniae* clones identified in this study is crucial to avoid further expansion and endemic spread both at the nosocomial and the community settings.

ACKNOWLEDGEMENTS

We are grateful to Helena Ramos (Hospital Geral de Santo António, Porto, Portugal), José Luís Grañeda (Centro Hospitalar Cova da Beira, EPE), and Serviço de Patologia Clínica, Centro Hospitalar Tondela-Viseu, EPE, for the clinical isolates included in this study.

FUNDING

The present work was supported by Fundação para a Ciência e Tecnologia, which belongs to the Ministry of Science, Technology and Innovation of Portugal (through grant no. PEst-C/EQB/LA0006/2011), and Fundação Ensino e Cultura Fernando Pessoa. Ângela Novais is supported by a Marie Curie Intra European Fellowship within the 7th European Community Framework Programme (PIEF-GA-2009-255512).

TRANSPARENCY DECLARATIONS

No conflicts of interest to declare.

REFERENCES

1. Coque TM, Baquero F, Cantón R. Increasing prevalence of ESBL-producing *Enterobacteriaceae* in Europe. *Euro surveillance* 2008; **13**.

2. Woodford N, Turton JF, Livermore DM. Multiresistant Gram-negative bacteria: the role of high-risk clones in the dissemination of antibiotic resistance. *FEMS Microbiology Reviews* 2011; **35**: 736-55.

3. Novais Â, Rodrigues C, Branquinho R *et al*. Spread of an OmpK36-modified ST15 *Klebsiella pneumoniae* variant during an outbreak involving multiple carbapenem-resistant *Enterobacteriaceae* species and clones. *European Journal of Clinical Microbiology & Infectious Diseases* 2012; **31**: 3057-63.

4. Damjanova I, Toth A, Paszti J *et al*. Expansion and countrywide dissemination of ST11, ST15 and ST147 ciprofloxacin-resistant CTX-M-15-type beta-lactamase-producing *Klebsiella pneumoniae* epidemic clones in Hungary in 2005--the new 'MRSAs'? *The Journal of Antimicrobial Chemotherapy* 2008; **62**: 978-85.

5. Nielsen JB, Skov MN, Jorgensen RL *et al.* Identification of CTX-M15-, SHV-28-producing *Klebsiella pneumoniae* ST15 as an epidemic clone in the Copenhagen area using a semi-automated Rep-PCR typing assay. *European Journal of Clinical Microbiology & Infectious Diseases* 2011; **30**: 773-8.

6. Oteo J, Cuevas O, Lopez-Rodriguez I *et al*. Emergence of CTX-M-15-producing *Klebsiella pneumoniae* of multilocus sequence types 1, 11, 14, 17, 20, 35 and 36 as pathogens and colonizers in newborns and adults. *The Journal of Antimicrobial Chemotherapy* 2009; **64**: 524-8.

7. Decre D, Burghoffer B, Gautier V *et al*. Outbreak of multi-resistant *Klebsiella oxytoca* involving strains with extended-spectrum beta-lactamases and strains with extended-spectrum activity of the chromosomal beta-lactamase. *The Journal of Antimicrobial Chemotherapy* 2004; **54**: 881-8.

8. Ivanova D, Markovska R, Hadjieva N *et al.* Extended-spectrum beta-lactamase-producing *Serratia marcescens* outbreak in a Bulgarian hospital. *The Journal of Hospital Infection* 2008; **70**: 60-5.

9. Machado E, Coque TM, Canton R *et al*. High diversity of extended-spectrum beta-lactamases among clinical isolates of *Enterobacteriaceae* from Portugal. *The Journal of Antimicrobial Chemotherapy* 2007; **60**: 1370-4.

10. Novais A, Baquero F, Machado E *et al*. International spread and persistence of TEM-24 is caused by the confluence of highly penetrating *enterobacteriaceae* clones and an IncA/C2 plasmid containing Tn1696::Tn1 and IS5075-Tn21. *Antimicrobial Agents and Chemotherapy* 2010; **54**: 825-34.

11. Mendonça N, Ferreira E, Louro D *et al.* Molecular epidemiology and antimicrobial susceptibility of extended- and broad-spectrum beta-lactamase-producing *Klebsiella pneumoniae* isolated in Portugal. *International Journal of Antimicrobial Agents* 2009; **34**: 29-37.

12. Machado E, Coque TM, Cantón R *et al.* High diversity of extended-spectrum β-lactamases among clinical isolates of *Enterobacteriaceae* from Portugal. *Journal of Antimicrobial Chemotherapy* 2007; **60**: 1370-4.

13. Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing: *Nineteenth Informational Supplement M100*-S20 CLSI, Wayne, PA, USA, 2011.

14. Peirano G, Sang JH, Pitondo-Silva A *et al.* Molecular epidemiology of extended-spectrum-beta-lactamase-producing *Klebsiella pneumoniae* over a 10 year period in Calgary, Canada. *The Journal of antimicrobial chemotherapy* 2012; **67**: 1114-20.

15. Lee MY, Ko KS, Kang C-I *et al.* High prevalence of CTX-M-15-producing *Klebsiella pneumoniae* isolates in Asian countries: diverse clones and clonal dissemination. *International Journal of Antimicrobial Agents* 2011; **38**: 160-3.

16. Sanchez-Romero I, Asensio A, Oteo J *et al*. Nosocomial outbreak of VIM-1-producing *Klebsiella pneumoniae* isolates of multilocus sequence type 15: molecular basis, clinical risk factors, and outcome. *Antimicrobial Agents and Chemotherapy* 2012; **56**: 420-7.

17. Poirel L, Benouda A, Hays C *et al*. Emergence of NDM-1-producing *Klebsiella pneumoniae* in Morocco. *The Journal of Antimicrobial Chemotherapy* 2011; **66**: 2781-3.

18. Lascols C, Peirano G, Hackel M *et al*. Surveillance and molecular epidemiology of *Klebsiella pneumoniae* that produce carbapenemases; the first report of OXA-48-like enzymes in North America. *Antimicrobial Agents and Chemotherapy* 2012.

19. Giske CG, Froding I, Hasan CM *et al.* Diverse sequence types of *Klebsiella pneumoniae* contribute to the dissemination of blaNDM-1 in India, Sweden, and the United Kingdom. *Antimicrobial Agents and Chemotherapy* 2012; **56**: 2735-8.

20. Samuelsen O, Toleman MA, Hasseltvedt V *et al*. Molecular characterization of VIM-producing *Klebsiella pneumoniae* from Scandinavia reveals genetic relatedness with international clonal complexes encoding transferable multidrug resistance. *Clinical Microbiology and Infection* 2011; **17**: 1811-6.

21. Giakkoupi P, Papagiannitsis CC, Miriagou V *et al*. An update of the evolving epidemic of blaKPC-2-carrying *Klebsiella pneumoniae* in Greece (2009-10). *The Journal of Antimicrobial Chemotherapy* 2011; **66**: 1510-3.

22. Carattoli A. Resistance plasmid families in *Enterobacteriaceae. Antimicrobial Agents and Chemotherapy* 2009; **53**: 2227-38.

Table 1. Epidemiological characterization of ESBL-producing non-*E. coli Enterobactericeae* from three Portuguese hospitals (2006-07 and 2010)

Species (no.)	**PFGE** (no.)	**MLST**[a]	**ESBLs** (no.)	**Hospital**[b]	**Isolation period**	**Sample** (no.)	**Antibiotic resistance to non-β-lactam antibiotics** [c, d]
K. pneumoniae (n=73)	Kp1 (32)	**ST336**	CTX-M-15 (31) SHV-12 (1)	A	2010	Urine (28), Blood (3), Exsudate (1)	(AMK), (CIP), (CLO), (GEN), (KAN), (NAL), (NET), (NIT), STR, (SUL), (TMP), (TOB)
	Kp2 (5)	**ST15**	CTX-M-15 (5)	A	2006-07; 2010	Urine (4), Unknown (1)	CIP, (CLO), GEN, KAN, NAL, (NET), (NIT), STR, SUL, (TET), TMP, TOB
	Kp3 (6)	**ST15**	SHV-106 (4) SHV-55 (2)	A	2010	Urine (5), Sputum (1)	(CIP), CLO, (GEN), (KAN), NAL, (NET), (NIT), STR, SUL, (TET), TMP, (TOB)
	Kp4 (5)	**ST15**	SHV-28 (3) SHV-2 (1) SHV-12 (1)	A	2010	Urine (3), Blood (1), Sputum (1)	(AMK), CIP, (CLO), (GEN), (KAN), NAL, (NET), (NIT), STR, SUL, TMP, (TET), (TOB)
	Kp5 (8)	**ST147**	SHV-12	A	2006-07; 2010	Urine (4), Blood (2), Unknown (2)	(AMK), CIP, (CLO), (GEN), (KAN), NAL, (NET), (NIT), STR, (SUL), (TET), (TMP), (TOB)
	Kp6 (2)	**ST*new***	SHV-12	B	2006-07	Urine (1), Sputum (1)	(AMK), CIP, (CLO), GEN, KAN, NAL, (STR), SUL, (TET), TOB
	Kp7 – Kp10 (4)	ND	SHV-12	A, B	2006-07	Sputum (1), Exsudate (1), Unknown (2)	(AMK), (CIP), (CLO), (GEN), (KAN), (NAL), (NET), (STR), SUL, (TMP), (TET), (TOB)
	Kp11 – Kp13 (3)	ND	TEM-10	B	2006-07	Sputum (2), Urine (1)	(AMK), (CLO), GEN, KAN, (NAL), NET, (STR), TET, TOB
	Kp14, Kp15 (2)	ND	CTX-M-15	A	2010	Urine (1), Exsudate (1)	CIP, (CLO), GEN, (KAN), NAL, (NIT), STR, SUL, TET, TMP, (TOB)
	Kp16 (1)	ND	SHV-2	A	2006-07	Unknown	CIP, NAL, STR, SUL, TMP
	Kp17 (1)	ND	SHV-5	C	2006-07	Urine	-
	Kp18 (1)	ND	SHV-55	B	2010	Sputum	CIP, CLO, GEN, NAL, STR, SUL, TOB
	Kp19 (1)	ND	SHV-145 [e]	A	2006-07	Sputum	AMK, CIP, CLO, GEN, KAN, NET, SUL, TET, TOB
	Kp20 (1)	ND	TEM-24	A	2006-07	Unknown	AMK, CIP, CLO, KAN, NAL, NET, STR, SUL, TMP, TOB
	Kp21 (1)	ND	TEM-116	B	2006-07	Urine	CIP, CLO, KAN, NAL, STR, SUL, TMP, TOB

Table 1. Epidemiological characterization of ESBL-producing non-*E. coli Enterobactericeae* from three Portuguese hospitals (2006-07 and 2010) (cont.)

Species (no.)	**PFGE** (no.)	**MLST**[a]	**ESBLs** (no.)	**Hospital**[b]	**Isolation period**	**Sample** (no.)	**Antibiotic resistance to non-β-lactam antibiotics** [c, d]
E. cloacae (n=13)	Ecl1 (7)	ND	SHV-12 (5) SHV-12 + TEM-116 (2)	B	2006-07	Sputum (4), Urine (3)	(AMK), (CIP), (CLO), GEN, KAN, (NAL), (NET), (SUL), TET, TOB
	Ecl2 (3)	ND	SHV-12 (2) SHV-12 + TEM-116 (1)	B	2006-07	Exsudate (2), Urine (1)	AMK, (CIP), CLO, GEN, KAN, NAL, NET, STR, SUL, TET, TOB
	Ecl3 (2)	ND	CTX-M-15	B, C	2006-07	Urine	CIP, KAN, GEN, NAL, NET, TET, TOB
	Ecl4 (1)	ND	CTX-M-32	B	2006-07	Sputum	CIP, CLO, KAN, GEN, NAL, SUL, TET, TOB
K. oxytoca (n=3)	Ko1 (1)	ND	CTX-M-15	A	2010	Sputum	CIP, KAN, GEN, NAL, NIT, SUL, STR, TET, TMP, TOB
	Ko2 (1)	ND	TEM-10	B	2006-07	Urine	KAN, GEN, NAL, NET, SUL, TMP, TOB
	Ko3 (1)	ND	TEM-24	A	2010	Sputum	CIP, CLO, NAL, KAN, SUL, STR, TET, TMP, TOB
P. mirabilis (n=1)	Pm1 (1)	ND	TEM-199[f]	B	2006-07	Blood	AMK, CIP, CLO, KAN, GEN, NAL, NET, SUL, STR, TET, TMP, TOB
S. marcescens (n=1)	Sm1 (1)	ND	TEM-10	B	2006-07	Sputum	KAN, GEN, NET, STR, TET, TOB

[a]ND, not done; [b]Hospital A is located at the North region of Portugal; Hospitals B and C are located at the Centre region of Portugal; [c]AMK, amikacin; CLO, chloramphenicol; CIP, ciprofloxacin; GEN, gentamicin; KAN, kanamycin; NAL, nalidixic acid; NET, netilmicin; NIT, nitrofurantoin; STR, streptomycin; SUL, sulphonamides; TOB, tobramycin; TET, tetracycline; TMP, trimethoprim; [d]Variable presence of resistance phenotype is indicated by parenthesis; [e] New SHV variant, Genbank number JX013655; [f] New TEM variant, GenBank number JX050178.

3.2.

Epidemiology of extended-spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae* in food-producing animals and farms

3.2.1.

Emergence of TEM-52 and CTX-M-32 in healthy pigs associated with ST10 complex *Escherichia coli* isolates and common IncI1/ST3 and IncN plasmids

Emergence of TEM-52 and CTX-M-32 in healthy pigs associated with ST10 complex *Escherichia coli* isolates and common IncI1/ST3 and IncN plasmids

Carla Rodrigues [1,2], , *Elisabete Machado*[1,2*], *Luísa Peixe*[1] and *Ângela Novais* [1]

[1]**REQUIMTE. Laboratório de Microbiologia. Faculdade de Farmácia, Universidade do Porto, Porto, Portugal**;

[2]**CEBIMED. Faculdade de Ciências da Saúde, Universidade Fernando Pessoa, Porto, Portugal;**

Running title: Spread of ESBL-plasmids and *E. coli* clones in piggeries

Keywords: CTX-M, TEM-52, ST10, swine, IncI1, pMLST

Manuscript Final Draf

ABSTRACT

Objectives: The spread of ESBL-producing Enterobacteriaceae from food producing animals/food products has been linked to clones and/or plasmids homologous to those circulating in humans. We aim to evaluate their contribution for the spread of $bla_{\text{TEM-52}}$ and $bla_{\text{CTX-M}}$ genes in swine.

Methods: Twenty-two ESBL (13 TEM-52, 6 CTX-M-32, 3 CTX-M-1)-producing *Escherichia coli* isolates from healthy pigs and swine production environments of two geographically distant Portuguese piggeries were studied. Clonal relatedness was assessed by X*ba*I-PFGE and MLST. Plasmid analysis included S1-PFGE, identification of incompatibility groups, pMLST and RFLP. $bla_{\text{CTX-M}}$ genetic context and the presence of plasmid-mediated fluoroquinolone resistance (PMQR) genes were investigated by PCR and sequencing.

Results: TEM-52 was the most prevalent ESBL (59%, 13/22), followed by CTX-M-32 (27%, 6/22) and CTX-M-1 (14%, 3/22). A high clonal diversity was observed among ESBL-producing *E. coli*, which belonged to phylogroups A (55%), B1 (27%), B2 (9%) and D (9%). However, isolates belonging to ST10 clonal complex were identified among TEM-52 (n=6) and CTX-M-32 (n=3) producers. The $bla_{\text{TEM-52}}$ gene was identified within an IncI1/ST3 plasmid variant identical to those circulating in Portuguese hospital settings (2003-04), while $bla_{\text{CTX-M-1}}$ and most $bla_{\text{CTX-M-32}}$ genes were located on highly related 40kb IncN plasmids.

Conclusions: We report for the first time a piggery reservoir of $bla_{\text{TEM-52}}$ and $bla_{\text{CTX-M-32}}$ genes associated with plasmids and clones widespread in humans and other animal hosts, in different EU countries, and carrying different bla_{ESBL} genes. The high plasticity observed in these genetic platforms might explain local diversification and further amplification events.

INTRODUCTION

The increased occurrence of extended-spectrum beta-lactamase (ESBL)-producing Enterobacteriaceae in humans, food-producing animals or food products has been raising concern about their possible transmission through the food chain.[1-3] In fact, the identification of clones and/or mobile genetic elements (plasmids and/or transposons) in non-human origins homologous to those circulating in humans suggests a common source.[4-7] TEM-52, SHV-12, CTX-M-1 and CTX-M-32 are the most frequent ESBLs reported among Enterobacteriaceae from food animals, although their distribution seems to be uneven in different hosts (poultry, pigs and/or cattle).[1, 2, 7] The spread of these ESBL types has been previously associated with particular epidemic plasmids (mostly from IncI1 or IncN types),[5, 8-10] in most cases identified in a diverse *E. coli* population.[5, 11, 12] Epidemiological data concerning ESBL-producing Enterobacteriaceae from food animals and their production environment is important to identify reservoirs and transmission pathways of bla_{ESBL} genes in order to better control their spread, but it has been scarce and limited to specific countries.[2,3,7]

In previous surveys, we found a low incidence (5.7%) of ESBLs (only SHV-12 in *Citrobacter freundii*) among Enterobacteriaceae from swine in Portugal (1998/2004).[13] Spread of CTX-M-1-producing *E. coli* has also recently (2007) been reported in a Portuguese intensive swine farm.[14] In this study, we investigate the contribution of clones and plasmids for the spread of $bla_{\text{TEM-52}}$ and different $bla_{\text{CTX-M}}$ genes in distinct Portuguese piggeries.

MATERIALS AND METHODS

Twenty-two ESBL (13 TEM-52, 6 CTX-M-32, 3 CTX-M-1)-producing *E. coli* isolates identified in swine (n=10; faeces and skin) and piggery environment (n=12; feed, waste waters) samples were studied. They corresponded to all ESBL producers detected in a large-scale study

including 43 samples recovered from 5 intensive-production piggeries located in the North (n=22, Piggeries E and F), Centre (n=10, Piggery C) and South (n=11, Piggeries A and B) regions of Portugal (April 2006-May 2007). Samples were pre-enriched in buffered peptone water for 18 h at 37ºC, plated (0,2 mL) on MacConkey agar plates supplemented with ceftazidime (1 mg/L) or cefotaxime (1 mg/L), and each different morphotype was selected for screening of ESBL-production by the standard double-disc synergy test.[13] ESBL characterization was performed by PCR and sequencing of bla_{TEM}, bla_{SHV}, and $bla_{\text{CTX-M}}$ products.[13]

Bacterial identification, antibiotic susceptibility tests and conjugation assays were performed as described previously.[13] Clonal relatedness was investigated by *Xba*I-PFGE and multi-locus sequence typing (MLST) (http://mlst.ucc.ie/mlst/dbs/Ecoli), and *E. coli* phylogenetic groups were identified by a multiplex PCR.[15, 16] ESBL-encoding plasmids were characterized by replicon typing (PCR, sequencing and hybridization), restriction fragment length polymorphism (RFLP) with *Eco*RI and *Hinc*II restriction enzymes and pMLST as described.[15, 8] Epidemic plasmids identified among ESBL-producing *E. coli* isolates circulating in representative Portuguese human and poultry isolates were also compared.[13, 17] The presence of sequences linked to $bla_{\text{CTX-M}}$ genetic environments (IS*Ecp1*, IS*26*, *orf477* and/or $bla_{\text{OXA-1}}$) and plasmid-mediated quinolone

resistance (PMQR; *qnr*, *aac(6')-Ib-cr*, *qepA*) genes was investigated by PCR and sequencing.[9, 13, 18]

RESULTS AND DISCUSSION

TEM-52 was the most prevalent ESBL (n=13/22; 59%), followed by CTX-M-32 (n=6/22; 27%) and CTX-M-1 (n=3/22; 14%), indicating a higher diversity than that observed in our previous survey [13]. A few CTX-M-1- and CTX-M-32-producing *E. coli* isolates also harbored $bla_{\text{OXA-1}}$ (n=2/9) and/or $bla_{\text{TEM-1}}$ (n=6/9) genes (Table 1). Isolates were recovered from food (n=8, 36%; 3 TEM-52, 1 CTX-M-1, 4 CTX-M-32), swine feces (n=6, 27%; 5 TEM-52, 1 CTX-M-1), swine hide (n=4, 18%; 2 TEM-52, 2 CTX-M-32) and manure (n=4, 18%; 3 TEM-52, 1 CTX-M-1). Both TEM-52 and CTX-M-1 were detected in Piggery F, whereas CTX-M-32 was only identified in Piggery E (Table 1). These ESBL-types seem to be common among Enterobacteriaceae of both human and non-human origins in different European countries, including Portugal.[5, 7, 11-14, 17, 19-22] However, our study constitutes the first report of TEM-52 and CTX-M-32-producing Enterobacteriaceae from healthy pigs and swine production environments.

ESBL-producing *E. coli* isolates belonged to phylogroups A (n=12/22, 55%), B1 (n=6/22, 27%), B2 (n=2/22, 9%) or D (n=2/22, 9%) (Table 1). A high clonal diversity was identified among TEM-52 (n=13; 8 clones), CTX-M-1 (n=3; 3 clones) and CTX-M-32 (n=6; 4 clones) producers. However, 3 CTX-M-32-producing isolates belonged to ST10 and 6 TEM-52 producers belonged to ST34 (n=1) or ST227 (n=5), corresponding to single and double locus variants of ST10, respectively (Table 1). They were identified in different piggeries and samples (feces, food, liquid manure or hide swab), confirming the widespread distribution of clonal complex 10 (CC10) isolates and the independent acquisition of different ESBLs. In fact, ST10 *E. coli* isolates have been frequently

identified in humans (patients, healthy individuals), animals (poultry, swine) and food products (retail meat), and associated with a variety of ESBLs, including TEM-52.[5, 15, 23-25] However, the high variability of PFGE patterns and/or serotypes prevents from establishing a direct link between isolates from different niches and consequently transmission pathways.[23, 25, 26]

Conjugative transfer was achieved in 96% (21/22) of the isolates and bla_{ESBL} genes were identified in epidemic IncI1, IncN and occasionally IncFII plasmids (Table 1). Remarkably, the $bla_{\text{TEM-52c}}$ gene was located on a 90kb-IncI1$_{\text{pSL476}}$/ST3 plasmid identical to an epidemic IncI::$bla_{\text{TEM-52}}$ plasmid identified in *E. coli* from Portuguese hospital settings (2003-04) (Figure 1), and highly similar to CTX-M-1 or SHV-12-encoding IncI1/ST3 plasmids identified in *E. coli* and *Salmonella* from food-producing animals (poultry, cattle, goat), pets and humans in France, Italy and the Netherlands.[5,8,17,27,28] The plasmid variant detected in this study ($bla_{\text{TEM-52c}}$/IncI1/ST3) among humans and swine differs from that widespread in humans and poultry in other EU countries ($bla_{\text{TEM-52c}}$/IncI1/ST36) [29], suggesting local emergence and spread.

The $bla_{\text{CTX-M-1}}$ and most $bla_{\text{CTX-M-32}}$ (67%, 4/6) genes were located on closely related 40kb-IncN$_{\text{R46}}$ plasmids (Figure 1) and linked to common genetic environments (IS*26*-ΔIS*Ecp1*-80bp-$bla_{\text{CTX-M-1}}$-*orf477* vs IS*Ecp1*-IS*5*-80bp-$bla_{\text{CTX-M-32}}$-*orf477*) within IncN and IncI1 plasmids circulating among diverse *E. coli* and *Salmonella* from multiple origins [9, 30-33], illustrating the high plasticity of these platforms. A common RFLP pattern was also identified in an IncN::$bla_{\text{CTX-M-32}}$ plasmid recovered from an *E. coli* identified in marine waters close to clandestine discharge points of water streams contaminated by faecal coliforms [34], but it was different from those previously identified among IncN::$bla_{\text{CTX-M-1}}$ in representative isolates from Portuguese hospitals and poultry (2003-

05) (Figure 1) [13, 17], suggesting a higher plasmid diversity. One CTX-M-32-producing isolate contained *qnrS1* and other PMQR determinants were not found.

In summary, we describe for the first time a piggery reservoir of $bla_{\text{TEM-52}}$ and $bla_{\text{CTX-M-32}}$ genes associated with epidemic plasmids (IncI1/ST3 and IncN, respectively) and clones (ST10 clonal complex) frequently recovered in humans and other animal hosts in different EU countries. Besides their widespread distribution along the food chain, the high promiscuity of these antibiotic resistance platforms seems to contribute to local diversification and amplification of ESBL-encoding plasmids and strains.

ACKNOWLEDGEMENTS

We thank all the contributing piggeries for allowing the collection of samples included in this study.

FUNDING

The present work was supported by Fundação para a Ciência e Tecnologia, which belongs to the Ministry of Science, Technology and Innovation of Portugal (through grants no. PEst-C/EQB/LA0006/2011 and POCI/AMB/61814/2004-FSE/FEDER), and Fundação Ensino e Cultura Fernando Pessoa. Ângela Novais is supported by a Marie Curie Intra European Fellowship within the 7th European Community Framework Programme (Ref: PIEF-GA-2009-255512).

TRANSPARENCY DECLARATIONS

No conflicts of interest to declare.

REFERENCES

1. Ewers C, Bethe A, Semmler T et al. Extended-spectrum beta-lactamase-producing and AmpC-producing *Escherichia coli* from livestock and companion animals, and their putative impact on public health: a global perspective. *Clin Microbiol Infect* 2012; **18**: 646-55.

2. Carattoli A. Animal reservoirs for extended spectrum beta-lactamase producers. *Clin Microbiol Infect* 2008; **14(Suppl 1)**: 117-23.

3. EFSA. Scientific Opinion of the Panel on Biological Hazards on a request from the European Food Safety Authority on foodborne antimicrobial resistance as a biological hazard. *The EFSA Journal* 2008; **765**: 1-87.

4. Vincent C, Boerlin P, Daignault D et al. Food reservoir for *Escherichia coli* causing urinary tract infections. *Emerg Infect Dis* 2010; **16**: 88-95.

5. Leverstein-van Hall MA, Dierikx CM, Cohen Stuart J et al. Dutch patients, retail chicken meat and poultry share the same ESBL genes, plasmids and strains. *Clin Microbiol Infect* 2011; **17**: 873-80.

6. Manges AR, Johnson JR. Food-borne origins of *Escherichia coli* causing extraintestinal infections. *Clin Infect Dis* 2012; **55**: 712-9.

7. Coque TM, Baquero F, Cantón R. Increasing prevalence of ESBL-producing *Enterobacteriaceae* in Europe. *Euro Surveill* 2008; **13(47)**. pii: 19044.

8. García-Fernández A, Chiaretto G, Bertini A et al. Multilocus sequence typing of IncI1 plasmids carrying extended-spectrum beta-lactamases in *Escherichia coli* and *Salmonella* of human and animal origin. *J Antimicrob Chemother* 2008; **61**: 1229-33.

9. Novais Â, Cantón R, Moreira R et al. Emergence and dissemination of *Enterobacteriaceae* isolates producing CTX-M-1-like enzymes in Spain are associated with IncFII (CTX-M-15) and broad-host-range (CTX-M-1, -3, and -32) plasmids. *Antimicrob Agents Chemother* 2007; **51**: 796-9.

10. Bortolaia V, Guardabassi L, Trevisani M et al. High diversity of extended-spectrum beta-lactamases in *Escherichia coli* isolates from Italian broiler flocks. *Antimicrob Agents Chemother* 2010; **54**: 1623-6.

11. Endimiani A, Rossano A, Kunz D et al. First countrywide survey of third-generation cephalosporin-resistant *Escherichia coli* from broilers, swine, and cattle in Switzerland. *Diagn Microbiol Infect Dis* 2012; **73**: 31-8.

12. Overdevest I, Willemsen I, Rijnsburger M et al. Extended-spectrum beta-lactamase genes of *Escherichia coli* in chicken meat and humans, The Netherlands. *Emerg Infect Dis* 2011; **17**: 1216-22.

13. Machado E, Coque TM, Cantón R et al. Antibiotic resistance integrons and extended-spectrum beta-lactamases among *Enterobacteriaceae* isolates recovered from chickens and swine in Portugal. *J Antimicrob Chemother* 2008; **62**: 296-302.

14. Gonçalves A, Torres C, Silva N et al. Genetic characterization of extended-spectrum beta-lactamases in *Escherichia coli* isolates of pigs from a Portuguese intensive swine farm. *Foodborne Pathog Dis* 2010; **7**: 1569-73.

15. Novais Â, Baquero F, Machado E et al. International spread and persistence of TEM-24 is caused by the confluence of highly penetrating Enterobacteriaceae clones and an IncA/C_2 plasmid containing Tn*1696*::Tn*1* and IS*5075*-Tn*21*. *Antimicrob Agents Chemother* 2010; **54**: 825-34.

16. Clermont O, Bonacorsi S, Bingen E. Rapid and simple determination of the *Escherichia coli* phylogenetic group. *Appl Environ Microbiol* 2000; **66**: 4555-8.

17. Machado E, Coque TM, Cantón R et al. High diversity of extended-spectrum beta-lactamases among clinical isolates of *Enterobacteriaceae* from Portugal. *J Antimicrob Chemother* 2007; **60**: 1370-4.

18. Minarini LA, Poirel L, Cattoir V et al. Plasmid-mediated quinolone resistance determinants among enterobacterial isolates from outpatients in Brazil. *J Antimicrob Chemother* 2008; **62**: 474-8.

19. Jorgensen CJ, Cavaco LM, Hasman H et al. Occurrence of CTX-M-1-producing *Escherichia coli* in pigs treated with ceftiofur. *J Antimicrob Chemother* 2007; **59**: 1040-2.

20. Escudero E, Vinué L, Teshager T et al. Resistance mechanisms and farm-level distribution of fecal *Escherichia coli* isolates resistant to extended-spectrum cephalosporins in pigs in Spain. *Res Vet Sci* 2010; **88**: 83-7.

21. Geser N, Stephan R, Hachler H. Occurrence and characteristics of extended-spectrum beta-lactamase (ESBL) producing *Enterobacteriaceae* in food producing animals, minced meat and raw milk. *BMC Vet Res* 2012; **8**: 21.

22. Machado E, Silva V, Costa S et al. Occurrence of TEM-52- and CTX-M-producing *Escherichia coli* among Portuguese piggeries. In: Societies FEMS- *Forth Congress of European Microbiologists*. Geneva, Switzerland: Abstracts of the Forth Congress of European Microbiologists, Geneva, Switzerland, 2011.

23. Oteo J, Diestra K, Juan C et al. Extended-spectrum beta-lactamase-producing *Escherichia coli* in Spain belong to a large variety of multilocus sequence typing types, including ST10 complex/A, ST23 complex/A and ST131/B2. *Int J Antimicrob Agents* 2009; **34**: 173-6.

24. Cortés P, Blanc V, Mora A et al. Isolation and characterization of potentially pathogenic antimicrobial-resistant *Escherichia coli* strains from chicken and pig farms in Spain. *Appl Environ Microbiol* 2010; **76**: 2799-805.

25. Bergeron CR, Prussing C, Boerlin P et al. Chicken as reservoir for extraintestinal pathogenic *Escherichia coli* in humans, Canada. *Emerg Infect Dis* 2012; **18**: 415-21.

26. Valverde A, Cantón R, Garcillán-Barcia MP et al. Spread of $bla_{\text{CTX-M-14}}$ is driven mainly by IncK plasmids disseminated among *Escherichia coli* phylogroups A, B1, and D in Spain. *Antimicrob Agents Chemother* 2009; **53**: 5204-12.

27. Dahmen S, Haenni M, Madec JY. IncI1/ST3 plasmids contribute to the dissemination of the *bla*$_{\text{CTX-M-1}}$ gene in *Escherichia coli* from several animal species in France. *J Antimicrob Chemother* 2012. [Epub ahead of print]

28. Cloeckaert A, Praud K, Lefevre M et al. IncI1 plasmid carrying extended-spectrum-beta-lactamase gene *bla*$_{\text{CTX-M-1}}$ in *Salmonella enterica* isolates from poultry and humans in France, 2003 to 2008. *Antimicrob Agents Chemother* 2010; **54**: 4484-6.

29. Bielak E, Bergenholtz RD, Jorgensen MS et al. Investigation of diversity of plasmids carrying the *bla*$_{\text{TEM-52}}$ gene. *J Antimicrob Chemother* 2011; **66**: 2465-74.

30. Diestra K, Juan C, Curião T et al. Characterization of plasmids encoding *bla*$_{\text{ESBL}}$ and surrounding genes in Spanish clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae*. *J Antimicrob Chemother* 2009; **63**: 60-6.

31. Cullik A, Pfeifer Y, Prager R et al. A novel IS*26* structure surrounds *bla*$_{\text{CTX-M}}$ genes in different plasmids from German clinical *Escherichia coli* isolates. *J Med Microbiol* 2010; **59**: 580-7.

32. Schink AK, Kadlec K, Schwarz S. Analysis of *bla*$_{\text{CTX-M}}$-carrying plasmids from *Escherichia coli* isolates collected in the BfT-GermVet study. *Appl Environ Microbiol* 2011; **77**: 7142-6.

33. Fernández A, Gil E, Cartelle M et al. Interspecies spread of CTX-M-32 extended-spectrum beta-lactamase and the role of the insertion sequence IS*1* in down-regulating *bla*$_{\text{CTX-M}}$ gene expression. *J Antimicrob Chemother* 2007; **59**: 841-7.

34. Machado E, Coque TM, Cantón R et al. Leakage into Portuguese aquatic environments of extended-spectrum-beta-lactamase-producing *Enterobacteriaceae*. *J Antimicrob Chemother* 2009; **63**: 616-8.

3.3

Emergence of carbapenemase-producing *Enterobacteriaceae* in Portugal

First report of VIM-34, a new VIM-1 variant identified in a ST15 *Klebsiella pneumoniae* isolate in Portugal

Carla Rodrigues [1], *Ângela Novais* [1], *Elisabete Machado* [2] and *Luísa Peixe* [1*]

[1]REQUIMTE. Laboratório de Microbiologia, Faculdade de Farmácia, Universidade do Porto, Portugal;

[2]CEBIMED. Faculdade de Ciências da Saúde, Universidade Fernando Pessoa, Porto, Portugal.

Keywords: *Klebsiella pneumoniae*, VIM-34, ST15, SHV-12, class 1 integron

Manuscript Final Draft

Sir,

Metallo-β-lactamase (MBL)-producing *Enterobacteriaceae* have been increasingly reported in the past 10 years, being VIM-type MBLs the most frequently implicated in hospital outbreaks across Europe (1). The spread of bla_{VIM} genes has been frequently associated with plasmid or chromosomally encoded class 1 integrons linked to Tn*402* derivatives, and sporadically with particular clones (1, 7-10). In this study, we report the molecular epidemiology of VIM-34, a new VIM-1 variant identified in a *Klebsiella pneumoniae* clinical isolate in Portugal.

A carbapenem resistant *K. pneumoniae* clinical isolate was recovered in 2011 from an urine of a female outpatient (with history of previous multiple hospitalizations). Antimicrobial susceptibility tests showed that the isolate was susceptible to ertapenem and meropenem (MIC=0.25-0.38 µg/mL), while intermediate to imipenem (MIC=4 µg/mL) and resistant to cephalosporins (cephalothin, cefotaxime, ceftazidime, cefepime, cefpirome, cefoxitin), aztreonam and β-lactam/β-lactamase inhibitor combinations (amoxicillin-clavulanate, piperacillin-tazobactam, ticarcillin-clavulanate) (http://www.eucast.org/). Resistance to ciprofloxacin, nalidixic acid, kanamycin, tobramycin, chloramphenicol and sulphonamides was also observed (4). Standard disk diffusion tests, PCR and sequencing (3, 4) demonstrated the production of VIM-34 (GenBank accession number JX013656), a novel VIM-type enzyme differing from VIM-1 by one amino acid change (V113I), and also SHV-12 extended-spectrum β-lactamase (ESBL). The simultaneous production of VIM-1 and SHV- (SHV-5, -12 and -134) or CTX-M- (CTX-M-3) –type ESBLs has been frequently reported in *K. pneumoniae* isolates (6, 7).

Clonal analysis by multilocus sequence typing (MLST) (http://www.pasteur.fr/recherche/genopole/PF8/mlst/primers_Kpneumoniae.html) revealed that the isolate belonged to the ST15 *K. pneumoniae* clone, widely disseminated in Portugal (3) and other European countries (Spain, Hungary, Denmark), associated with the spread of different ESBLs (CTX-M-15; SHV-55, -106, -134) or MBLs (VIM-1, NDM-1) (2, 5, 7; Rodrigues *et al.*, unpublished results). Nevertheless, this study corresponds to the first description of MBL and SHV-12 in the ST15 *K. pneumoniae* clone in our country. Plasmid analysis performed by S1- and *I-Ceu*I-PFGE, and identification of incompatibility groups by PCR and hybridization (4), showed that the $bla_{\text{VIM-34}}$ gene was chromosomally located, as observed in other *Enterobacteriaceae* species (10).

The linkage of $bla_{\text{VIM-34}}$ to class 1 integrons and Tn*402* derivatives was investigated by PCR (*intI1*, 5´CS-3´CS region, *orf*5, *orf6*, IS*1326*, IS*1353*, IS*6100*) and sequencing (4, 8, 9). The $bla_{\text{VIM-34}}$ was located within a ca. 6 kb class 1 integron (GenBank accession number JX185132) with an original array of gene cassettes, comprising $bla_{\text{VIM-34}}$ followed by *aacA4*, *aphA15*, *aadA1* and *catB2* gene cassettes (encoding aminoglycoside acetyltransferase, phosphotransferase or adenyltransferase, and chloramphenicol acetyltranferase enzymes, respectively) (Figure 1). The absence of tni_{402} sequences and the high similarity detected with In70 (lacking *catB2*) and In113 (harbouring *dfrB1* instead of *aphA15*), primarily identified in a VIM-1-producing *Achromobacter xylosoxidans* from Italy or in *K. pneumoniae* and *Escherichia coli* associated with hospital outbreaks in Spain, suggests that this integron might have aroused by both recombination and *in vivo* evolution events (Figure 1) (8, 9).

In summary, we describe a novel integron type carrying the new $bla_{\text{VIM-34}}$ gene, a $bla_{\text{VIM-1}}$ variant, identified at the chromosome of the intercontinental ST15 *K.*

pneumoniae clone, co-producing SHV-12. Our results confirm further diversification of VIM MBLs and highlight the emergence and spread of multidrug resistance platforms containing bla_{MBL} genes among widespread *K. pneumoniae* clones, which needs to be further monitored.

ACKNOWLEDGEMENTS

We thank Valquíria Alves and Antónia Read (Hospital Pedro Hispano, Matosinhos, Portugal) for the strain's gift.

FUNDING

The present work was supported by Fundação para a Ciência e Tecnologia, which belongs to the Ministry of Science, Technology and Innovation of Portugal (through grant no. PEst-C/EQB/LA0006/2011), and Fundação Ensino e Cultura Fernando Pessoa. Ângela Novais was supported by a Marie Curie Intra European Fellowship within the 7th European Community Framework Programme (Ref. PIEF-GA-2009-255512).

TRANSPARENCY DECLARATIONS

No conflicts of interest to declare.

REFERENCES

1. **Cantón R, Akóva M, Carmeli Y, Giske CG, Glupczynski Y, Gniadkowski M, Livermore DM, Miriagou V, Naas T, Rossolini GM, Samuelsen Ø, Seifert H, Woodford N, Nordmann P; the European Network on Carbapenemases.** 2012. Rapid evolution and spread of carbapenemases among *Enterobacteriaceae* in Europe. Clin. Microbiol. Infect. **18**: 413-31.
2. **Damjanova I**, **Tóth A**, **Pászti J**, **Hajbel-Vékony G**, **Jakab M**, **Berta J**, **Milch H**, **Füzi M**. 2008. Expansion and countrywide dissemination of ST11, ST15 and ST147 ciprofloxacin-resistant CTX-M-15-type beta-lactamase-producing *Klebsiella pneumoniae* epidemic clones in Hungary in 2005 - the new 'MRSAs'? J. Antimicrob. Chemother. **62**:978-85.
3. **Novais A, Rodrigues C, Branquinho R, Antunes P, Grosso F, Boaventura L, Ribeiro G, Peixe L.** 2012. Spread of an OmpK36-modified ST15 *K. pneumoniae* variant during an outbreak involving multiple carbapenem resistant *Enterobactericeae* species and clones. Eur. J. Clin. Microbiol. Infect. Dis., *in press*.
4. **Novais A, Baquero F, Machado E, Cantón R, Peixe L, Coque TM**. 2010. International spread and persistence of TEM-24 is caused by the confluence of highly penetrating *Enterobacteriaceae* clones and an IncA/C_2 plasmid containing Tn*1696*::Tn*1* and IS*5075*-Tn*21*. Antimicrob. Agents Chemother. **54**:825-34.
5. **Poirel L, Benouda A, Hays C, Nordmann P.** 2011. Emergence of NDM-1-producing *Klebsiella pneumoniae* in Morocco. J. Antimicrob. Chemother. **66**:2781-3.
6. **Samuelsen Ø, Toleman MA, Hasseltvedt V, Fuursted K, Leegaard TM, Walsh TR, Sundsfjord A, Giske CG**. 2011. Molecular characterization of VIM-producing *Klebsiella pneumoniae* from Scandinavia reveals genetic relatedness with international clonal complexes encoding transferable multidrug resistance. Clin. Microbiol. Infect. **17**:1811-6.

7. **Sánchez-Romero I, Asensio A, Oteo J, Muñoz-Algarra M, Isidoro B, Vindel A, Álvarez-Avello J, Balandín-Moreno B, Cuevas O, Fernández-Romero S, Azañedo L, Sáez D, Campos J.** 2012. Nosocomial outbreak of VIM-1-producing *Klebsiella pneumoniae* isolates of multilocus sequence type 15: molecular basis, clinical risk factors, and outcome. Antimicrob. Agents Chemother. **56**:420-7.
8. **Tato M, Coque TM, Baquero F, Cantón R.** 2010. Dispersal of carbapenemase bla_{VIM-1} gene associated with different Tn*402* variants, mercury transposons, and conjugative plasmids in *Enterobacteriaceae* and *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **54**:320-7.
9. **Toleman MA, Biedenbach D, Bennett DM, Jones RN, Walsh TR**. 2005. Italian metallo-beta-lactamases: a national problem? Report from the SENTRY Antimicrobial Surveillance Programme. J. Antimicrob. Chemother. **55**:61-70.
10. **Vatapoulos A**. 2008. High rates of metallo-beta-lactamase-producing *Klebsiella pneumoniae* in Greece - a review of the current evidence. Euro Surveill. **24**:13(4).

3.3.2.

Spread of carbapenem resistance mediated by porin alterations in *Enterobacteriaceae* from Portuguese clinical settings

Eur J Clin Microbiol Infect Dis
DOI 10.1007/s10096-012-1665-z

ARTICLE

Spread of an OmpK36-modified ST15 *Klebsiella pneumoniae* variant during an outbreak involving multiple carbapenem-resistant *Enterobacteriaceae* species and clones

Â. Novais · C. Rodrigues · R. Branquinho · P. Antunes · F. Grosso · L. Boaventura · G. Ribeiro · L. Peixe

Received: 14 March 2012 / Accepted: 26 May 2012

Abstract We aim to characterise multiple ertapenem-resistant (ERT-R, *n*=15) *Enterobacteriaceae* isolates identified as presumptive carbapenemase producers in a Portuguese hospital in a short period of time (March–July 2010). Antibiotic susceptibility patterns, β-lactamases, genetic relatedness [pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST)], plasmid content and major enterobacterial porins were investigated. Ertapenem resistance was associated with deficiencies in major porins and, in some cases, extended-spectrum β-lactamase (ESBL) or AmpC β-lactamase production among outbreak and non-outbreak clones. Most isolates (*n*=8) corresponded to two ERT-R *Klebsiella pneumoniae* ST15 PFGE-types: (i) a sporadic variant (Kp-A-ERT, *n*=1) presenting a premature stop codon in *ompK36* and (ii) an epidemic variant (Kp-B-ERT, *n*=7) exhibiting a new OmpK36 porin variant, which differed additionally in plasmid and antibiotic susceptibility profiles. ST14 (*n*=1) and ST45 (*n*=1) *K. pneumoniae*, ST131 (*n*=1) and ST354 (*n*=1) *Escherichia coli*, *Enterobacter asburiae* (*n*=1), *Enterobacter cloacae* (*n*=1) and *Enterobacter aerogenes* (*n*=1) ERT-R clones were also sporadically detected. Porin changes in these isolates included non-sense mutations [*ompK35*, *ompK36*, *ompF*; minimum inhibitory concentration (MIC)=4–32 mg/l], IS-mediated porin disruptions (*ompK36*, *ompC*; MIC=12–>32 mg/l) or alterations in the L3 loop (*ompK36*; MIC=4–16 mg/l). We describe, for the first time in Portugal, the simultaneous emergence of multiple ERT-R *Enterobacteriaceae* species and clones in a short period of time. Moreover, our results support that a CTX-M-15-producing ST15 *K. pneumoniae* with an OmpK36-modified porin might successfully spread in the nosocomial setting.

Â. Novais · C. Rodrigues · R. Branquinho · P. Antunes · F. Grosso · L. Peixe (✉)
REQUIMTE, Laboratório de Microbiologia, Faculdade Farmácia,
Universidade do Porto,
Rua Jorge Viterbo Ferreira, 228,
4050-313 Porto, Portugal
e-mail: lpeixe@ff.up.pt

L. Boaventura · G. Ribeiro
Laboratório Microbiologia,
Hospitais da Universidade de Coimbra,
Coimbra, Portugal

P. Antunes
Faculdade de Ciências da Nutrição e Alimentação,
Universidade do Porto,
Porto, Portugal

Published online: 16 June 2012

Introduction

Nowadays, therapy of infections caused by extended-spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae* rely frequently on carbapenems, although their use is being seriously compromised by the acquisition and spread of class A (KPC), class B (VIM or NDM) and class D (OXA-48-like) carbapenemases [1, 2] or by the production of ESBL or AmpC-type enzymes combined with outer membrane permeability changes [3–6].

The emergence and spread of carbapenemase-producing *Enterobacteriaceae* is a great concern worldwide, but recent reports highlighted the implication of carbapenem non-susceptible non-carbapenemase producers in nosocomial outbreaks in different countries, mostly from *Klebsiella pneumoniae*, *Escherichia coli* or *Enterobacter* spp., and eventually belonging to particular clonal lineages [4, 7, 8]. These isolates are commonly selected in vivo during the course of carbapenem therapy, exhibit multidrug resistance (MDR) phenotypes

[6, 8–10] and are frequently assigned as possible carbapenemase producers by automated methods, hindering detection, antibiotic therapy and infection control measures [11, 12]. The expression of genes encoding major non-specific porins in *K. pneumoniae* (*ompK35* and *ompK36*) and their respective homologues in *E. coli*, *Enterobacter cloacae* (*ompF* and *ompC*) and *Enterobacter aerogenes* (*omp35* and *omp36*) might be affected by mutations causing protein structure changes, premature termination of translation or by gene disruption [3, 13], affecting most frequently ertapenem but also imipenem or meropenem susceptibility levels [5, 6].

In Portugal, the dissemination of particular ESBL types (mostly CTX-M-15, TEM-24, TEM-52 or SHV-12) and *Enterobacteriaceae* clones in the hospital setting might constitute an excellent substrate for the emergence of carbapenem resistance (L. Peixe, personal communication) [14]. Moreover, the recent identification of KPC-producing isolates increased the alert level throughout the country [15]. In this study, we aim to perform the molecular characterisation of multiple ertapenem-resistant (ERT-R) *Enterobacteriaceae* isolates identified in a Portuguese hospital within a 5-month period in 2010.

Materials and methods

Bacterial strains

We studied 15 ERT-R *Enterobacteriaceae* isolates [ten *K. pneumoniae*, two *E. coli*, one *Enterobacter asburiae*, one *E. aerogenes*, one *E. cloacae*; minimum inhibitory concentration (MIC) 2–>32 mg/l] from 15 patients at the Hospitais da Universidade de Coimbra (central region of Portugal, March–July 2010) identified as presumptive carbapenemase producers by the VITEK 2 (bioMérieux, Marcy-L'Etoile, France) semi-automated commercial system. They were obtained from urine (n=8; 53 %), blood (n=5; 33 %) and other samples (n=2; 13 %), mostly from immunocompromised patients located or previously admitted to the Urology (53 %), Internal Medicine (27 %) and Haematology (13 %) wards. Antimicrobial susceptibility patterns to cephalosporins (cefotaxime, ceftazidime), cephamycins (cefoxitin), carbapenems (ertapenem, imipenem and meropenem), amoxicillin–clavulanic acid, aminoglycosides (amikacin, gentamicin, kanamycin, netilmicin, streptomycin and tobramycin), ciprofloxacin, chloramphenicol, nalidixic acid, nitrofurantoin, tetracycline, trimethoprim and sulphonamides (Oxoid Ltd., Basingstoke, United Kingdom) were determined by disk diffusion methods and/or E-tests according to Clinical and Laboratory Standards Institute (CLSI) guidelines and breakpoints [16].

Clonal relationship

Relatedness among isolates was established by pulsed-field gel electrophoresis (PFGE) of *Xba*I-digested genomic DNA (10–40 s pulses for 21 h, 14 °C, 6 V/cm^2) and multi-locus sequence typing (MLST) for *K. pneumoniae* (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html) and *E. coli* (http://mlst.ucc.ie/mlst/dbs/Ecoli). *E. coli* phylogenetic groups were assessed by a multiplex polymerase chain reaction (PCR) assay, as previously described [17].

Characterisation of β-lactamases

ESBL, carbapenemases and AmpC β-lactamases were presumptively identified by isoelectric focusing (PhastSystem, Pharmacia Biotech, Uppsala, Sweden) and disk diffusion tests using specific combinations of β-lactams and β-lactamase inhibitors or the modified Hodge test, as previously recommended [14, 16, 18, 19]. The carbapenemase activity was evaluated by spectrophotometric assays with ertapenem (80 mg/l, 295 nm) and imipenem (33 mg/l, 295 nm) (Sigma Aldrich, Steinheim, Germany) in a spectrophotometer UV-1700 PharmaSpec (Shimadzu, Tokio, Japan), as previously reported [20]. The presence of genes encoding class A (bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{PER}, bla_{VEB}, bla_{GES}, bla_{KPC}), class B (bla_{NDM}, bla_{VIM}, bla_{IMP}, bla_{SPM}, bla_{GIM}, bla_{SIM}), class C (bla_{CMY}, bla_{MOX}, bla_{FOX}, bla_{LAT}, bla_{ACT}, bla_{MIR}, bla_{DHA}, bla_{MOR}, bla_{ACC}) and class D ($bla_{OXA-1-like}$, $bla_{OXA-2-like}$, $bla_{OXA-3-like}$, bla_{OXA-48}, bla_{OXA-23}, bla_{OXA-40}, bla_{OXA-58}, $bla_{OXA-143}$) β-lactamases was confirmed by PCR and sequencing [14, 17, 21–27].

Plasmid analysis

The conjugative transfer of *bla* genes was tested by filter-mating assays using *E. coli* K-12 strain BM21 (rifampicin- and nalidixic acid-resistant, plasmid-free) and *E. coli* Hb101 (kanamycin- and azide-resistant, plasmid-free) as recipient strains at 37 °C for 24 h, as previously described [17]. The location of *bla* genes was assessed by hybridisation of S1 or I-*Ceu*I digested genomic DNA from wild-type strains with intragenic β-lactamase probes. The characterisation of plasmids included the determination of plasmid content (number and size) and identification of the incompatibility group by PCR, sequencing and hybridisation with specific probes (FII, R, N and A/C replicons) as previously reported [17].

Investigation of porins

Outer membrane porins (OMPs) were obtained from overnight cultures in Tryptic Soy Broth (TSB), as previously described [28]. Samples were boiled and analysed by

Table 1 Epidemiological data of ertapenem-resistant (ERT-R) *Enterobacteriaceae* isolates recovered during an outbreak period at the Hospitais da Universidade de Coimbra in 2010

Species	MLST (*EC* PhG)[a]	PFGE type (no. of isolates)	Ward	Sample	Date (day/month)	ERT MIC (mg/l)	Changes in porin sequences		β-lactamases[c]	Plasmids carrying *bla* genes (size)[d]
							ompC-like[b]	*ompF*-like[b]		
K. pneumoniae	ST14	Kp-D-ERT (1)	Internal Medicine	Urine	01/March	12	–	+	SHV-1	ND
K. pneumoniae	ST15	Kp-A-ERT (1)	Gynaecology	Urine	02/March	32	+	–	CTX-M-15, OXA-1	IncR (70 Kb)
		Kp-B-ERT (7)	Urology (4), Urgency (2)[e], Internal Medicine (1)[e]	Urine (4), blood (3)	21/April–15/July	4–16	+	–	CTX-M-15	IncR (50 Kb)
K. pneumoniae	ST45	Kp-E-ERT (1)	Haematology[e]	Exudate	28/July	12	+	–	SHV-1	ND
E. coli	ST131 (B2)	Ec-G-ERT (1)	Nephrology[e]	Urine	09/June	>32	–	NA	CTX-M-15[cr]	ND
E. coli	ST354 (D)	Ec-J-ERT (1)	Surgery	Blood	03/July	4	NA	–	TEM-1	ND (90 Kb)
E. asburiae	–	Eas-ERT (1)	Haematology	Blood	16/March	>32	+	ND	ACT-4[cr]	ND
E. cloacae	–	Ecl-ERT (1)	Internal Medicine	Urine	29/March	4	ND	+	TEM-24	IncFIIs (150 Kb)
E. aerogenes	–	Eae-ERT (1)	Intensive Medicine	Unknown	17/April	2	NA	–	TEM-1	IncA/C (180 Kb)

ND = not determined; NA = no amplification

[a] *EC* PhG, *E. coli* phylogenetic group

[b] *ompC* and *ompF* in *E. coli* and their homologues, respectively, in *K. pneumoniae* (*ompK36* and *ompK35*) and in *Enterobacter* spp. (*omp36* and *omp35*)

[c] Conjugative transfer of *bla* genes encoding β-lactamases is indicated as underlined

[d] Plasmid size, content and type, and location of *bla* genes were assessed by the hybridisation of S1-nuclease and I-*Ceu*I-digested genomic DNA with specific probes (*bla* and *rep*)

[e] Patients had previously been admitted to the Urology/Nephrology, Haematology and Internal Medicine wards

[cr] *bla* located on chromosome

sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using the EzWay™ PAG system precast SDS-PAGE gel (10 % Tricine) (Koma Biotech, Seoul, Korea) and stained with Coomassie blue. Genes encoding major non-specific porins identified in *K. pneumoniae* (*ompK35*, *ompK36*), *E. coli* (*ompC*, *ompF*) and *Enterobacter* spp. (*omp35*, *omp36*) were investigated by PCR and sequencing, using primers previously described [3, 5, 10]. Wild-type *K. pneumoniae* strains (one non-ESBL producer and one CTX-M-15 producer belonging to ST15) were used as control strains for porin preparations. Strains used for the comparison of porin gene sequences were as follows: *K. pneumoniae* NTUH-K2044 (GenBank accession number BAH62652), *E. coli* K12 (GenBank accession number BAA15998), *E. cloacae* ATCC 13047 (GenBank accession number YP_003613214) and *E. aerogenes* ATCC 13048 (GenBank accession number AY487903).

GenBank accession numbers

Sequences corresponding to mutated porins identified in ST15 *K. pneumoniae* (*ompK36*, GenBank accession numbers JN128632 and JN128634), ST14 *K. pneumoniae* (*ompK35*, GenBank accession number JN128633) and *E. cloacae* (*ompF*, GenBank accession number JN571035) clones were submitted to the GenBank database.

Results

Relatedness among ERT-R isolates

ERT-R isolates belonged to different species and clones, including globally spread *K. pneumoniae* (ST14, ST15, ST45) and *E. coli* lineages (ST131) (Table 1). During the study period, an outbreak of ST15 *K. pneumoniae* strains involving patients admitted or with a previous record of admission to the Urology ward was detected. Two ST15 PFGE types were identified: (i) a sporadic ERT-R variant (n=1; Kp-A-ERT), detected at the beginning of the study and ii) an epidemic ERT-R variant (n=7; Kp-B-ERT), differing in ERT-R phenotypes and genotypes (see Table 1). *E. coli* isolates were identified as B2-ST131 and D-ST354. These patients had previous and, in some cases, prolonged hospital admissions related with severe renal or neoplasic syndromes, received antibiotherapy with carbapenems, extended-spectrum cephalosporins, aminoglycosides, fluoroquinolones and/or tigecycline, and most of them (67 %) died due to underlying diseases. Containment of ERT-R isolates' spread was achieved through the introduction of infection control measures such as patient isolation, screening of patients, change of antibiotic usage and re-evaluation of medical procedures in accordance with data obtained in this study.

Antibiotic resistance profiles

All ST15 *K. pneumoniae* strains were CTX-M-15 producers resistant to cefotaxime, ceftazidime, cefoxitin, amoxicillin–clavulanic acid, ertapenem, kanamycin, streptomycin, tobramycin, nalidixic acid, ciprofloxacin, sulphonamides, trimethoprim and nitrofurantoin, and most conferred resistance to chloramphenicol (88 %) and tetracycline (63 %), and less frequently to amikacin and netilmicin (38 % each). ESBL production was also detected for *E. coli* B2-ST131 (CTX-M-15) and *E. cloacae* (TEM-24) isolates, which were also resistant to different aminoglycosides, nalidixic acid, ciprofloxacin, tetracycline and trimethoprim. *K. pneumoniae* and *E. coli* were intermediate to meropenem and occasionally to imipenem (data not shown). Carbapenemase production was only detected for the *E. asburiae* isolate (data not shown), which produced an ACT-4 enzyme. This isolate was resistant to meropenem (MIC= 12 mg/l) and imipenem (MIC=>32 mg/l), kanamycin, streptomycin and nitrofurantoin, showed only one pI=8.9 band and was negative for all carbapenemase-encoding genes tested by PCR.

Analysis of enterobacterial porins

Two different porin modifications were identified within OmpK36 of ERT-R ST15 *K. pneumoniae* variants: (i) in Kp-A-ERT, a non-sense mutation leading to a premature stop codon (TGA) at position 248 was identified and (ii) in Kp-B-ERT, two amino acids (Asp137 and Gly138) were inserted in the L3 loop, leading to a new porin variant. Ertapenem resistance in non-outbreak isolates was associated with modifications of *ompK36* (disruption by IS*1*) in ST45 *K. pneumoniae*, *ompK35* (premature stop codon in position 211) in ST14 *K. pneumoniae*, *omp35* (premature stop codon in position 168) in *E. cloacae* or *omp36* (truncated by IS*903*) in *E. asburiae*. We could not obtain a PCR product using different primer combinations for *ompF* of *E. coli* Ec-G-ERT, *omp36* of *E. aerogenes* and *ompC* of *E. coli* Ec-J-ERT, probably indicating mutations in the primer binding sites or a larger insertion preventing amplification (Table 1).

Analysis of outer membrane proteins revealed that a protein band of ca. 37 kDa was present in ertapenem-susceptible (one non-ESBL-producing and one CTX-M-15-producing ST15 wild-type *K. pneumoniae* strains from our collection) and in Kp-B-ERT (isolates presenting a modified porin variant), whereas Kp-A-ERT variant or ST45 *K. pneumoniae* lacked this band (Fig. 1). We believe that this band corresponds to the OmpK36 porin, which is the only one expressed by most ESBL-producing isolates

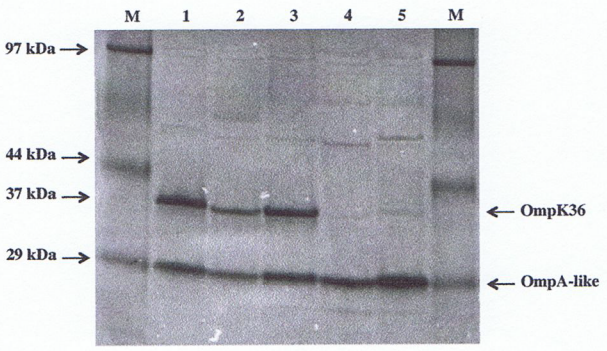


Fig. 1 Outer membrane profiles of representative *Klebsiella pneumoniae* isolates on a 10 % sodium dodecyl sulphate (SDS) polyacrylamide gel. Lanes M: protein marker (kDa); Lane 1: *K. pneumoniae* wild-type strain non-ESBL producer; Lane 2: Wild-type *K. pneumoniae* ST15; Lane 3: *K. pneumoniae* ST15 (Kp-B-ERT variant); Lane 4: *K. pneumoniae* ST15 (Kp-A-ERT variant); Lane 5: *K. pneumoniae* ST45 (Kp-E-ERT)

[13]. A putative OmpA homologue was observed in all OMP preparations.

Plasmid characterisation

Conjugative transfer of *bla* genes was only achieved in seven ST15 *K. pneumoniae* isolates (Table 1), where $bla_{\text{CTX-M-15}}$ was identified within 50–90-Kb IncR plasmids (the rep_{R} sequence being identical to that of plasmid pK245, GenBank accession number DQ449578) (Table 1), as previously described [29]. The $bla_{\text{ACT-4}}$ in *E. asburiae* was chromosomally located, whereas the *E. cloacae* isolate carried the $bla_{\text{TEM-24}}$ gene in a 150-Kb IncFIIs plasmid (Table 1).

Discussion

In this study, we describe the emergence of ERT-R isolates from different *Enterobacteriaceae* species and particular widespread clones associated with distinct modifications in major porins' sequences and, in most cases, ESBL (CTX-M-15 or TEM-24) or AmpC (ACT-4) production. Previous reports of the nosocomial emergence of ERT-R isolates are generally confined to a single species or even a particular clone, which has rarely been identified by MLST [4, 7, 8]. The identification of two ST15 ERT-R *K. pneumoniae* subtypes, and highly similar ERT-S ST15 isolates in the period analysed (data not shown), suggests spread prior to the period of study, with variants acquiring different ertapenem resistance mechanisms. What is alarming is the emergence of carbapenem resistance among globally spread high-risk clones (ST14 and ST15 *K. pneumoniae* or ST131 and ST354 *E. coli*) previously associated with the dissemination of ESBL (remarkably CTX-M-15), KPC and/or AmpC (CMY-2, DHA-1) in different settings, leaving few therapeutic options available and contributing to a great impact in infection control measures [30, 31]. Moreover, the preferential use of meropenem and/or imipenem amplifies the risk of further expansion of carbapenemase-producing bacteria [11, 30–33].

The highest ertapenem MIC levels were observed in isolates showing ESBL or AmpC production and concomitant porin changes preventing protein expression (Table 1) as expected [33], whereas lower level ERT-R was observed in isolates (Kp-B-ERT) showing an amino acid insertion (Asp-Gly) in the OmpK36 β-strand loop 3 (L3), similar to that described in an epidemic ST37 *K. pneumoniae* clone in Italy [4]. Variable potential for epidemicity was associated with Kp-A-ERT or Kp-B-ERT variants (non-epidemic vs. epidemic, respectively), suggesting that strains encoding altered, though still functional, porins might have an advantage over those with changes leading to a loss of function of the porin [4].

The identification of variable ertapenem MIC levels in isolates belonging to ST15 *K. pneumoniae* and presenting the same mutation type, and the identification of significant MIC levels in *K. pneumoniae* presumptively lacking ESBL or AmpC genes (Table 1) might be explained by other non-explored factors, such as the overexpression of efflux pumps [5, 9, 11]. The carbapenemase activity on ertapenem, meropenem and imipenem observed in the *E. asburiae* and the identification of only one band in isoelectric focusing gels suggests that the ACT-4 enzyme seems to be able to hydrolyse carbapenems, which is first reported in this study. The ACT-4 enzyme differs from ACT-1 (GenBank accession number U58495, [34]) by seven amino acid changes, and it has been previously identified in an *E. asburiae* strain from Korea (GenBank accession number EU427302, according to http://www.lahey.org/studies and wrongly designated as ACT-3 in the GenBank database).

In this study, we demonstrate that the selection of ERT-R bacteria might occur simultaneously in different species and clones in a short period of time, resulting from independent but coincidental occurrences, with serious impact on clinical outcomes and infection control measures. Moreover, we highlight that ERT-R isolates with particular porin changes can successfully spread in the nosocomial environment, which can constitute an additional problem in the hospital setting.

Acknowledgements This work was supported by a Marie Curie Intra European Fellowship within the 7th European Community Framework Programme (grant number PIEF-GA-2009-255512 to A.N.) and by Fundação para a Ciência e Tecnologia (grant number PEst-C/EQB/LA0006/2011). Raquel Branquinho and Filipa Grosso were supported by fellowships from Fundação para a Ciência e Tecnologia de Portugal (grant numbers SFRH/BD/61410/2009 and SFRH/BD/31647/2006, respectively).

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Pfeifer Y, Cullik A, Witte W (2010) Resistance to cephalosporins and carbapenems in Gram-negative bacterial pathogens. Int J Med Microbiol 300(6):371–379
2. Nordmann P, Naas T, Poirel L (2011) Global spread of carbapenemase-producing *Enterobacteriaceae*. Emerg Infect Dis 17(10):1791–1798
3. Doumith M, Ellington MJ, Livermore DM, Woodford N (2009) Molecular mechanisms disrupting porin expression in ertapenem-resistant *Klebsiella* and *Enterobacter* spp. clinical isolates from the UK. J Antimicrob Chemother 63(4):659–667
4. García-Fernández A, Miriagou V, Papagiannitsis CC, Giordano A, Venditti M, Mancini C, Carattoli A (2010) An ertapenem-resistant extended-spectrum-beta-lactamase-producing *Klebsiella pneumoniae* clone carries a novel OmpK36 porin variant. Antimicrob Agents Chemother 54(10):4178–4184
5. Kaczmarek FM, Dib-Hajj F, Shang W, Gootz TD (2006) High-level carbapenem resistance in a *Klebsiella pneumoniae* clinical isolate is due to the combination of *bla*(ACT-1) beta-lactamase production, porin OmpK35/36 insertional inactivation, and down-regulation of the phosphate transport porin phoE. Antimicrob Agents Chemother 50(10):3396–3406
6. Cuzon G, Naas T, Guibert M, Nordmann P (2010) In vivo selection of imipenem-resistant *Klebsiella pneumoniae* producing extended-spectrum beta-lactamase CTX-M-15 and plasmid-encoded DHA-1 cephalosporinase. Int J Antimicrob Agents 35(3):265–268
7. Chudácková E, Bergerová T, Fajfrlík K, Cervená D, Urbásková P, Empel J, Gniadkowski M, Hrabák J (2010) Carbapenem-nonsusceptible strains of *Klebsiella pneumoniae* producing SHV-5 and/or DHA-1 beta-lactamases in a Czech hospital. FEMS Microbiol Lett 309(1):62–70
8. Mena A, Plasencia V, García L, Hidalgo O, Ayestarán JI, Alberti S, Borrell N, Pérez JL, Oliver A (2006) Characterization of a large outbreak by CTX-M-1-producing *Klebsiella pneumoniae* and mechanisms leading to in vivo carbapenem resistance development. J Clin Microbiol 44(8):2831–2837
9. Gröbner S, Linke D, Schütz W, Fladerer C, Madlung J, Autenrieth IB, Witte W, Pfeifer Y (2009) Emergence of carbapenem-non-susceptible extended-spectrum beta-lactamase-producing *Klebsiella pneumoniae* isolates at the university hospital of Tübingen, Germany. J Med Microbiol 58(Pt 7):912–922
10. Lartigue MF, Poirel L, Poyart C, Réglier-Poupet H, Nordmann P (2007) Ertapenem resistance of *Escherichia coli*. Emerg Infect Dis 13(2):315–317
11. Leavitt A, Chmelnitsky I, Colodner R, Ofek I, Carmeli Y, Navon-Venezia S (2009) Ertapenem resistance among extended-spectrum-beta-lactamase-producing *Klebsiella pneumoniae* isolates. J Clin Microbiol 47(4):969–974
12. Endimiani A, Perez F, Bajaksouzian S, Windau AR, Good CE, Choudhary Y, Hujer AM, Bethel CR, Bonomo RA, Jacobs MR (2010) Evaluation of updated interpretative criteria for categorizing *Klebsiella pneumoniae* with reduced carbapenem susceptibility. J Clin Microbiol 48(12):4417–4425
13. Martínez-Martínez L (2008) Extended-spectrum beta-lactamases and the permeability barrier. Clin Microbiol Infect 14(Suppl 1):82–89
14. Machado E, Coque TM, Cantón R, Novais A, Sousa JC, Baquero F, Peixe L; Portuguese Resistance Study Group (2007) High diversity of extended-spectrum beta-lactamases among clinical isolates of *Enterobacteriaceae* from Portugal. J Antimicrob Chemother 60(6):1370–1374
15. Poirel L, Barbosa-Vasconcelos A, Simões RR, Da Costa PM, Liu W, Nordmann P (2012) Environmental KPC-producing *Escherichia coli* isolates in Portugal. Antimicrob Agents Chemother 56 (3):1662–1663
16. Clinical and Laboratory Standards Institute (CLSI) (2010) Performance standards for antimicrobial susceptibility testing; update. CLSI document M100–S20 June 2010 update. CLSI, Wayne, PA
17. Novais A, Baquero F, Machado E, Cantón R, Peixe L, Coque TM (2010) International spread and persistence of TEM-24 is caused by the confluence of highly penetrating *Enterobacteriaceae* clones and an IncA/C_2 plasmid containing Tn*1696*::Tn*1* and IS*5075*-Tn*21*. Antimicrob Agents Chemother 54(2):825–834
18. Doi Y, Potoski BA, Adams-Haduch JM, Sidjabat HE, Pasculle AW, Paterson DL (2008) Simple disk-based method for detection of *Klebsiella pneumoniae* carbapenemase-type beta-lactamase by use of a boronic acid compound. J Clin Microbiol 46(12):4083–4086
19. Galani I, Rekatsina PD, Hatzaki D, Plachouras D, Souli M, Giamarellou H (2008) Evaluation of different laboratory tests for the detection of metallo-beta-lactamase production in *Enterobacteriaceae*. J Antimicrob Chemother 61(3):548–553
20. Poirel L, Castanheira M, Carrër A, Rodriguez CP, Jones RN, Smayevsky J, Nordmann P (2011) OXA-163, an OXA-48-related class D beta-lactamase with extended activity toward expanded-spectrum cephalosporins. Antimicrob Agents Chemother 55 (6):2546–2551
21. Ellington MJ, Kistler J, Livermore DM, Woodford N (2007) Multiplex PCR for rapid detection of genes encoding acquired metallo-beta-lactamases. J Antimicrob Chemother 59(2):321–322
22. Antunes P, Coque TM, Peixe L (2010) Emergence of an IncIγ plasmid encoding CMY-2 β-lactamase associated with the international ST19 OXA-30-producing β-lactamase *Salmonella typhimurium* multidrug-resistant clone. J Antimicrob Chemother 65 (10):2097–2100. doi:10.1093/jac/dkq293
23. Higgins PG, Lehmann M, Seifert H (2010) Inclusion of OXA-143 primers in a multiplex polymerase chain reaction (PCR) for genes encoding prevalent OXA carbapenemases in *Acinetobacter* spp. Int J Antimicrob Agents 35(3):305
24. Poirel L, Héritier C, Tolün V, Nordmann P (2004) Emergence of oxacillinase-mediated resistance to imipenem in *Klebsiella pneumoniae*. Antimicrob Agents Chemother 48(1):15–22
25. Poirel L, Le Thomas I, Naas T, Karim A, Nordmann P (2000) Biochemical sequence analyses of GES-1, a novel class A extended-spectrum beta-lactamase, and the class 1 integron In52 from *Klebsiella pneumoniae*. Antimicrob Agents Chemother 44 (3):622–632
26. D'Andrea MM, Nucleo E, Luzzaro F, Giani T, Migliavacca R, Vailati F, Kroumova V, Pagani L, Rossolini GM (2006) CMY-16, a novel acquired AmpC-type beta-lactamase of the CMY/LAT lineage in multifocal monophyletic isolates of *Proteus mirabilis* from northern Italy. Antimicrob Agents Chemother 50(2):618–624
27. Bert F, Branger C, Lambert-Zechovsky N (2002) Identification of PSE and OXA beta-lactamase genes in *Pseudomonas aeruginosa* using PCR-restriction fragment length polymorphism. J Antimicrob Chemother 50(1):11–18
28. Carlone GM, Thomas ML, Rumschlag HS, Sottnek FO (1986) Rapid microprocedure for isolating detergent-insoluble outer membrane proteins from *Haemophilus* species. J Clin Microbiol 24 (3):330–332
29. Coelho A, González-López JJ, Miró E, Alonso-Tarrés C, Mirelis B, Larrosa MN, Bartolomé RM, Andreu A, Navarro F, Johnson JR, Prats G (2010) Characterisation of the CTX-M-15-encoding gene in *Klebsiella pneumoniae* strains from the Barcelona metropolitan area: plasmid diversity and chromosomal integration. Int J Antimicrob Agents 36(1):73–78
30. Woodford N, Turton JF, Livermore DM (2011) Multiresistant Gram-negative bacteria: the role of high-risk clones in the dissemination of antibiotic resistance. FEMS Microbiol Rev 35(5):736–755

31. Orsi GB, García-Fernández A, Giordano A, Venditti C, Bencardino A, Gianfreda R, Falcone M, Carattoli A, Venditti M (2011) Risk factors and clinical significance of ertapenem-resistant *Klebsiella pneumoniae* in hospitalised patients. J Hosp Infect 78(1):54–58
32. Lee MY, Ko KS, Kang CI, Chung DR, Peck KR, Song JH (2011) High prevalence of CTX-M-15-producing *Klebsiella pneumoniae* isolates in Asian countries: diverse clones and clonal dissemination. Int J Antimicrob Agents 38(2):160–163
33. Yan JJ, Wu JJ, Lee CC, Ko WC, Yang FC (2010) Prevalence and characteristics of ertapenem-nonsusceptible *Escherichia coli* in a Taiwanese university hospital, 1999 to 2007. Eur J Clin Microbiol Infect Dis 29(11):1417–1425
34. Mammeri H, Guillon H, Eb F, Nordmann P (2010) Phenotypic and biochemical comparison of the carbapenem-hydrolyzing activities of five plasmid-borne AmpC beta-lactamases. Antimicrob Agents Chemother 54(11):4556–4560

4. Conclusions

4. Conclusions

The main conclusions obtained in this work are:

• A **high diversity of ESBLs** has been found among recent nosocomial *Enterobacteriaceae* isolates, with CTX-M (mostly CTX-M-15) and SHV (mostly SHV-12) being the most predominant enzymes, confirming an **epidemiological shift** on ESBL-types previously described in other geographic locations.

• Despite the **high clonal diversity** detected observed among the most frequent ESBL-producing species detected (*Escherichia coli* and *Klebsiella pneumoniae*), particular clones (*E. coli* ST131; *K. pneumoniae* ST336, ST15, and ST147) have been responsible for the **amplification of specific ESBL-types (**CTX-M-15, different SHV-variants**)**, some of them being widespread in Portuguese hospitals.

• The variability of PFGE-types within the same clone or their ability to acquire different bla_{ESBL} genes suggest **intraclonal evolution** by both genomic and plasmid diversification events.

• A **piggery reservoir of $bla_{\text{TEM-52}}$ and $bla_{\text{CTX-M-1/-32}}$** genes associated with **widespread plasmids** (IncI1/ST3 and IncN, respectively) and **clones** (*E. coli* ST10 clonal complex) was detected. The identification of identical plasmids and clones between isolates from human and animal origins also highlights their potential **transmission throughout the food chain.**

• The identification of *Enterobacteriaceae* isolates belonging to widespread clones with **decreased susceptibility to carbapenems** (either due to production of a new carbapenemase type, VIM-34, or by production of ESBL/plasmid-mediated AmpC β-lactamases plus porin alteratios) in Portugal is worrisome and needs to be further monitored. Moreover, their characterization allowed the **implementation of appropriate and timely infection control measures**, and the reinforcement of continuous surveillance systems.

5. Appendix

5.1. Communications in international meetings

- Rodrigues C, Novais Â, Machado E, Peixe L, on behalf of The Portuguese Resistance Study Group. First report of a new VIM-1 variant identified in a ST15 *Klebsiella pneumoniae* clone co-producing SHV-12 in Portugal. In 35th International Congress of the Society for Microbial Ecology and Disease (SOMED), Abstracts Book, pp. 62.
- Rodrigues C, Novais Â; Cantón R, Coque T, Peixe L, Machado E. 2012. Spread of IncI-blaTEM-52 and IncN-blaCTX-M-1/-32 among *Escherichia coli* isolates from Portuguese piggeries. Clin Microbiol Infect. 18(suppl. 3): S449.
- Rodrigues C, Machado E, Montenegro C, Peixe L, Novais Â, on behalf of The Portuguese Resistance Study Group. 2012. High diversity of extended-spectrum beta-lactamases among clinical isolates of *Escherichia coli* from Portugal. Clin Microbiol Infect. 18(suppl. 3): S458.
- Rodrigues C, Machado E, Novais Â, Peixe L, on behalf of The Portuguese Resistance Study Group. 2012. Amplification of ST15, ST147 and ST336 *Klebsiella pneumoniae* clones producing different ESBLs in Portuguese hospitals. Clin Microbiol Infect. 18(suppl. 3): S529.
- Novais Â, Rodrigues C, Branquinho R, Antunes P, Peixe L. 2011. Outbreak of ertapenem-resistant widespread *Enterobacteriaceae* clones in a Portuguese Hospital. Clin Microbiol Infect. 17(suppl. 4): S720.

5.2. Communications in national meetings

- Novais Â, Machado E, Amaral S, Rodrigues C, Gonçalves T, Cantón R, Coque T, Peixe L. 2011. Epidemiological shift and expansion of widespread *Escherichia coli* clones from Portuguese hospitals. In MICROBIOTEC11 Book of Abstracts, PS3:36, pp.257.
- Novais Â, Rodrigues C, Branquinho R, Antunes P, Grosso F, Boaventura L, Ribeiro G, Peixe L. 2011. Emergence of multiple carbapenem

resistant clinical isolates from different *Enterobacteriaceae* species and widespread clones. In MICROBIOTEC'11 Book of Abstracts, PS3:50, pp.271.

- Machado E, Silva V, Rodrigues C, Novais Â, Costa S, Silva R, Cantón R, Coque T, Peixe L. 2011. Occurrence of ESBLs among *Enterobacteriaceae* isolates from swine and piggeries environment in Portugal. In MICROBIOTEC'11 Book of Abstracts, PS3:22, pp.244.

5.3. Sequences submitted to GenBank

- GenBank Acc. Nr.: JX185132. Rodrigues C, Novais Â, Machado E, and Peixe L. 2012. *Klebsiella pneumoniae* strain K43 class I integron DNA integrase (intI1) gene, complete cds, VIM-34 metallo-beta-lactamase (blaVIM-34), aminoglycoside 6'-N-acetyltransferase (aacA4), aminoglycoside phosphotransferase (aphA15), 3'-(9)-O-adenylyltransferase (aadA1), chloramphenicol acetyltransferase (catB2), and QacEdelta1 multidrug exporter (qacEdelta1) genes, complete cds, and dihydropteroate synthase(sul1) gene, partial cds.
- GenBank Acc. Nr.: JX050178. Rodrigues C, Machado E, Novais Â, Peixe L, on behalf of The Portuguese Resistance Study Group. 2012. *Proteus mirabilis* extended-spectrum beta-lactamase TEM-199 (blaTEM-199) gene, partial cds.
- GenBank Acc. Nr.: JX013655. Rodrigues C, Machado E, Novais Â, Peixe L, on behalf of The Portuguese Resistance Study Group. 2012. *Klebsiella pneumoniae* extended-spectrum beta-lactamase SHV-145 (blaSHV-145) gene, complete cds.
- GenBank Acc. Nr.: JX013656. Rodrigues C, Novais Â, Machado E, and Peixe L. 2012. *Klebsiella pneumoniae* strain K43 metallo-beta-lactamase VIM-34 (blaVIM-34) gene, complete cds.
- GenBank Acc. Nr.: JN128632. Novais Â, Rodrigues C, Branquinho R, Antunes P, Grosso F, Boaventura L, Ribeiro G, and Peixe L. 2011. *Klebsiella pneumoniae* strain ST15 porin variant (ompK36) gene, partial cds.

- GenBank Acc. Nr.: JN128633. Novais Â, Rodrigues C, Branquinho R, Antunes P, Grosso F, Boaventura L, Ribeiro G, and Peixe L. 2011. *Klebsiella pneumoniae* strain ST14 truncated porin (ompK35) gene, complete cds.
- GenBank Acc. Nr.: JN128634. Novais Â, Rodrigues C, Branquinho R, Antunes P, Grosso F, Boaventura L, Ribeiro G, and Peixe L. 2011. "*Klebsiella pneumoniae* strain ST15 truncated porin (ompK36) gene, complete cds.

5.4 Participation in scientific projects

- "Evaluation of adhesion and biofilm producing abilities of worldwide spread *Escherichia coli* uropathogenic clonal complexes". Universidade do Porto. Projecto Pluridisciplinar IJUP (2011-2012). REQUIMTE/FFUP. IP: Ângela Novais.