UNIVERSIDADE DE LISBOA

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Vera Mónica Martins Gonçalves Manageiro

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(Microbiologia)

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Para os meus amores...

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> *"Choose a job you love, and you'll never have to work a day in your life".*
>
> *Confucius*

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In accordance with Paragraph 1 of "Artigo 41, Capítulo V, do Regulamento de Estudos Pós-Graduados da Universidade de Lisboa, publicado no Diário da República – II Série No. 209, de 30 de Outubro de 2006", it is clarified that full scientific articles already published (4), and under revision (1) or submitted (3) for publication in peer-reviewed scientific journals, were used in the elaboration of this dissertation. Hence, the candidate states that was involved in the study design, execution of experimental work, in the analysis and interpretation of results, and in their preparation for publication, with the exception for the two publications "The Lys234Arg substitution in the enzyme SHV-72 is a determinant for resistance to clavulanic acid inhibition" and "Biochemical characterization of SHV-55, an extended-spectrum class A beta-lactamase from Klebsiella pneumoniae", in which the candidate was not the leading author.

PREFACE

The introduction of a large collection of β-lactam antibiotics into clinical practice, namely the third-generation cephalosporins, in response to the increased prevalence of pathogenic organisms-producing β-lactamases, lead to the emergence of an even larger variety of β-lactamases conferring resistance to those agents, both in clinical and community settings. Hence, the overall aim of this PhD thesis was to contribute to the knowledge of molecular epidemiology of β-lactamases, as the most important antibiotic resistance mechanism among Gram-negative isolates, and to the understanding of their diversity in a structural and functional level. To accomplish this aim, several studies with different approaches were performed.

After a general overview about antimicrobial resistance (Section I: Introduction), the results presented in this PhD thesis are branched into two chapters (presented in Section II, Results), including, overall, eight papers:

- ✓ Chapter 1 (Paper I to III), entitled "*β-lactamases: impact of antibiotic resistance, dissemination and co-resistance"* sheds light on the emergence of *Enterobacteriaceae* strains producing extended-spectrum β-lactamases and AmpC plasmid-mediated β-lactamases. Furthermore, the dissemination of multidrug-resistant *Acinetobacter baumannii* strains due to the expression of carbapenem-hydrolyzing class D β-lactamases was analyzed.
- ✓ Chapter 2 (Paper IV to VIII) entitled "*Class A β-lactamases: function meets structure*", reports the correlation of function and structure in novel clinical important β-lactamases identified throughout this PhD thesis, highlighting protein evolution and diversification as a mechanism of rapid adaptation of bacterial populations.

Each Paper I to VIII (Section II: Results) contains a specific background and a detailed discussion about the respective results. At the end, Section III (Concluding remarks), focus on an overall discussion regarding the main results of the different chapters.

The presentation of each paper in the present PhD dissertation does not necessarily reflect a chronological order, since some of the studies described below were done simultaneously and the results obtained during one particular work would influence the progress of the other and vice-versa.

Publications and Manuscripts included in the thesis:

Chapter 1:

Paper I. **Manageiro V**, Ferreira E, Jones-Dias D, Louro D, Pinto M, Diogo J, Caniça M (2011) Emergence of β-lactamase-mediated resistance to oxyimino-β-lactams in *Enterobacteriaceae* isolates in various services in a single centre: risk factors and contribution of the newly detected CTX-M-3 variant in Portugal. *Submitted to Int J Antimicrob Agents*.

Paper II. **Manageiro V**, Jones-Dias D, Ferreira E, Antimicrobial Resistance Surveillance Program in Portugal (ARSIP), Caniça M (2011) Diversity of plasmid-encoded AmpC β-lactamases among clinical isolates of *Enterobacteriaceae* lacking inducible chromosomal *ampC* gene from Portuguese Hospitals. *Submitted to Antimicrob Agents Chemother*.

Paper III. **Manageiro V**, Jones-Dias D, Ferreira E, Louro D, Antimicrobial Resistance Surveillance Program in Portugal (ARSIP), Caniça M (2011) Carbapenem-hydrolyzing class D β-lactamase-producing *Acinetobacter baumannii* isolated in community and health care facilities in Portugal. *Submitted to J Antimicrob Chemother*.

Chapter 2:

Paper IV. Mendonça N, **Manageiro V**, Robin F, Salgado MJ, Ferreira E, Caniça M, Bonnet R (2008) The Lys234Arg substitution in the enzyme SHV-72 is a determinant for resistance to clavulanic acid inhibition. *Antimicrob Agents Chemother* **52:** 1806-1811. doi:10.1128/AAC.01381-07.

Paper V. **Manageiro V**, Ferreira E, Albuquerque L, Bonnet R, Canica M (2010) Biochemical study of a new inhibitor-resistant beta-lactamase, SHV-84, produced by a clinical *Escherichia coli* strain. *Antimicrob Agents Chemother* **54:** 2271-2272. doi:10.1128/AAC.01442-09.

Paper VI. **Manageiro V**, Ferreira E, Albuquerque L, M. C, Bonnet R (2011) Characterization of the inhibitor-resistant SHV β-lactamase (SHV-107) in a clinical *Klebsiella pneumoniae* strain co-producing GES-7 enzyme**.** *Under review at Antimicrob Agents Chemother.*

Paper VII. Mendonça N, **Manageiro V**, Bonnet R, Caniça M (2008) Biochemical characterization of SHV-55, an extended-spectrum class A beta-lactamase from Klebsiella pneumoniae. *Antimicrob Agents Chemother* **52:** 1897-1898. doi:10.1128/AAC.01525-07.

Paper VIII.Ramdani-Bouguessa N*, **Manageiro V***, Jones-Dias D, Ferreira E, Tazir M, Canica M (2011) Role of SHV-beta-lactamase variants in resistance of clinical *Klebsiella pneumoniae* strains to beta-lactams in an Algerian hospital. *J Med Microbiol* **60:**983-987. doi:10.1099/jmm.1090.030577-030570.

* Authors with equal contribution

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LIST OF ABBREVIATIONS

In this thesis acronyms are expanded on first usage and whenever deemed necessary to improve clarity.

Aac(6')-Ib-cr PMQR determinant

Ab *Acinetobacter baumannii*

ARSIP Antibiotic Resistance Surveillance Program in Portugal

bla β-Lactamase coding gene

BSAC British Society for Antimicrobial Chemotherapy

CA-SFM Antibiogram Committee of the SFM

CHDL carbapenem-hydrolysing class D β-lactamase

CI 95% Confidence Interval

CLSI Clinical and Laboratory Standards Institute

CMS Complex Mutant SHV

CMT Complex Mutant TEM

DAP diaminopimelic acid

DDST Disk diffusion susceptibility test

EARS-Net European Antimicrobial Resistance Surveillance Network

EARSS European Antimicrobial Resistance Surveillance System

ECDC European Centre for Disease Prevention and Control

EDTA Ethylenediaminetetraacetic acid

EMBL European Molecular Biology Laboratory

ESAC Extended-spectrum AmpC cephalosporinase

ESBL Extended-Spectrum β-Lactamase

EUCAST European Committee on Antimicrobial Susceptibility Testing

HGT Horizontal Gene Transfer

IC_{50} Fifty Percent Inhibitory Concentration

IEF Isoelectric Focusing

IPTG isopropyl β-D-thiogalactoside

IR Inverted Repeat Sequences

IRS Inhibitor Resistant SHV

IRT Inhibitor Resistant TEM

IS Insertion Sequence

ISCR Insertion Sequence Common Region

k_{cat} Catalytic Activity Constant

K_i Inhibitor constant

k_{inact} Inactivation rate constant

K_m Michaelis Constant

MBL Metallo-β-lactamase

MDR Multidrug-resistance

MDR-Ab Multidrug-resistant *Acinetobacter baumannii*

MDS Molecular Dynamic Simulation

MGE Mobile Genetic Element

MIC Minimal Inhibitory Concentration

MLST Multilocus Sequence Typing

NAG N-acetylglucosamine

NAM N-acetylmuramic acid

NIH National Institute of Health

NRL-AR National Reference Laboratory of Antimicrobial Resistances

OMP Outer Membrane Protein

OPLSAA Optimized potentials for liquid simulations in all-atom

OR Odds Ratio

ORF Open Reading Frame

PBP Penicillin-Binding Protein

PCR Polymerase Chain Reaction

PDR Pandrug-resistant

PFGE Pulsed-Field Gel Electrophoresis

PMAβ Plasmid-mediated AmpC β-lactamase

PMQR Plasmid-mediated quinolone resistance

Qnr PMQR determinant

QRDR Quinolone-resistance determining region

RIVM National Institute for Public Health and the Environment

RMSD Root Mean Squared Deviation

Ser-β-lactamase β-lactamase with serine active site

SFM Société Française de Microbiologie

UPGMA Unweighted Pair Group Method

V_{max} Rates of hydrolys

WHO World Health Organization

β-Lactamase Abbreviations:

ACC Ambler class C

ACT AmpC type

BES Brazilian ESBLs

CFE derived from *Citrobacter freundii*

CMY Active on Cephamycins (cephamycinase)

CTX-M Active on Cefotaxime, First Isolated at Munich

DHA Discovered at Dhahran Hospital in Saudi Arabia

DIM Dutch imipenemase

FOX active on Cefoxitin

GES Guyana ESBLs

GIM German Imipenemase

IBC Integron-Borne Cephalosporinase

IMI Imipenem-hydrolyzing β-lactamase

IMP Active on Imipenem

KPC *K. pneumoniae* carbapenemase

LAT Active on Latamoxef

MIR Discovered at Miriam Hospital in Providence

MOX Active on Moxalactam

NDM New Delhi MBL

NMC-A *Enterobacter cloacae* carbapenemase

OXA Active on Oxacillin

PER *Pseudomona*s Extended Resistant

PSE *Pseudomonas*-Specific Enzyme

SFO *Serratia fonticola*

SHV Sulphydryl Reagent Variable

SIM Seoul imipenemase

SME *Serratia marcescens* carbapenemase

SPM São Paulo MBL

TEM Named after patient Temoniera

TLA Tlahuicas (Indian tribe)

VEB Vietnam ESBLs

VIM Verona Integron-encoded MBL

Amino Acid Abbreviations:

Ala, A Alanine

Arg, R Arginine

Asn, N Asparagine

Asp, D Aspartic Acid

Cys, C Cysteine

Gln, Q Glutamine

Glu, E Glutamic Acid

Gly, G Glycine

His, H Histidine

Ile, I Isoleucine

Leu, L Leucine

Lys, K Lysine

Met, M Methionine

Phe, F Phenylalanine

Ser, S Serine

Thr, Threonine

Tyr, Y Tyrosine

Val, V Valine

Xaa Variable amino acid

Latin abbreviations:

i.e. id est, that is to say

e.g. exempli gratia, for example

***et al*.** et alia, and other people

RESUMO DA TESE

A introdução de antibióticos β-lactâmicos na prática clínica, nomeadamente de oximino-β-lactâmicos e carbapenemos, em resposta ao aumento da prevalência de bactérias patogénicas produtoras de β-lactamases, tais como *E. coli* e *K. pneumoniae*, levou ao aparecimento de uma maior diversidade dessas enzimas, com uma actividade catalítica alargada. Actualmente existem mais de 860 β-lactamases descritas em bactérias clinicamente importantes (http://www.lahey.org/Studies/).

O trabalho apresentado nesta tese de doutoramento pretendeu responder a várias questões na área da resistência aos antibióticos, nomeadamente:

- ✓ Quais os actuais mecanismos de resistência aos oximino-β-lactâmicos e aos carbapenemos em isolados de *Enterobacteriaceae* e *Acinetobacter baumannii* e se, porventura emergentes, quais os mecanismos implicados na sua disseminação?
- ✓ Se propagados à comunidade, quais os mecanismos subjacentes?
- ✓ Quais os factores de risco associados?
- ✓ Que substituições aminoacídicas são responsáveis por alterações estruturais nas β-lactamases e qual a consequência a nível da actividade hidrolítica?

As respostas a estas questões são apresentadas em dois capítulos distintos desta dissertação, correspondendo aos resultados obtidos durante a realização da mesma (Secção II). O Capítulo 1, intitulado "β-lactamases: o impacto na resistência aos antibióticos, disseminação e co-resistência", permitiu avaliar a emergência de isolados produtores de β-lactamases de espectro alargado (ESBL; *extended-spectrum β-lactamases*) e de β-lactamases AmpC plasmídicas (PMAβ, *plasmid-mediated AmpC β-lactamases*) (Artigo I e II, respectivamente). A disseminação de isolados de *A. baumannii* multirresistentes foi também identificada e correlacionada com a expressão de β-lactamases de Class D capazes de hidrolisar carbapenemos (CHDL, *carbapenem-hydrolyzing class D β-lactamase*) (Artigo III).

O Capítulo 2 avalia a estrutura e função de cinco novas β-lactamases com importância clínica: três SHV resistentes aos inibidores (IRS, *inhibitor-resistant SHV*) (SHV-72, SHV-84 e SHV- 107), uma ESBL (SHV-55) e uma SHV parental

(SHV-99). Glovalmente, os resultados aqui apresentados contribuíram para o conhecimento da epidemiologia molecular das β-lactamases, assim como para a compreensão da actividade associada à sua estrutura secundária e terciária.

O primeiro estudo realizado (Artigo I) teve como principal objectivo avaliar a disseminação e a evolução dos mecanismos de resistência aos antibióticos oximino-β-lactâmicos, de entre os quais se destacam as cefalosporinas de terceira geração, em isolados de *Enterobacteriaceae*. Para tal, foram estudados 220 isolados, apresentando multirresistência a diferentes desses antibióticos, provenientes de um hospital da área de Lisboa (1999 e 2004-2008). Este estudo documenta a emergência e disseminação de genes bla_{ESBL} em Portugal, desde, pelo menos, o ano de 2004. No primeiro período do estudo, todas as β-lactamases detectadas eram não-ESBL, incluindo TEM-1, SHV-1 e enzimas da família CMY-2. Durante o segundo período, houve um aumento significativo ESBLs encontradas (8 em 2004, 58 em 2008), de entre as quais 94,8% eram da família CTX-M, sendo a variante mais prevalente a β-lactamase CTX-M-15 (80,5%). De notar, a presença de um novo clone epidémico local, transitório, de *K. pneumoniae*, produtor de CTX-M-3, associado a um aumento da frequência de ESBL da família CTX-M. Esta foi a primeira descrição de estirpes produtoras de CTX-M-3 em Portugal, a qual foi inicialmente reportada em 1995, na Polónia, em isolados de *Citrobacter freundii* e *Escherichia coli*. Para este hospital, foram identificados como factores de risco associados à resistência ao antibióticos oximino-β-lactâmicos, a idade superior a 65 anos, infecções nosocomiais e produção de β-lactamases da família CTX-M.

A resistência aos antibióticos oximino-β-lactâmicos no Artigo I, não foi, no entanto, atribuída apenas à produção de ESBLs. No decorrer do estudo foram detectadas não só β-lactamases não-ESBLs da família TEM e SHV, mas também PMAβs, nomeadamente CMY-2.

Em Portugal, à data de início da presente tese, pouco se conhecia acerca da existência e disseminação de PMAβ, quer em meio hospital, quer na comunidade. Assim, no Artigo II, colmatando a necessidade de conhecimento nesta matéria, foi pesquisada a eventual produção de PMAβ em isolados de *Enterobacteriaceae*, nos quais não existe expressão de *ampC* cromossómica, nomeadamente, *E. coli*,

Klebsiella spp. e *Proteus mirabilis*. Neste estudo, identificou-se uma prevalência de 2,8% de isolados produtores de PMAβ num total de 2570 isolados clínicos, obtidos em 28 hospitais portugueses, em diferentes períodos (1999 e 2004-2009). Entre os isolados produtores de PMAβ, 9.9% foram identificados em 1999 e 90,1% no segundo período. Globalmente, foi encontrada uma importante diversidade de β-lactamases, destacando-se não só PMAβs (DHA-1, CMY-2, CMY-39, MIR-1, MIR-3, FOX-5 e as novas CMY-46 e CMY-50), como também ESBLs da família CTX-M, e enzimas da família SHV e TEM. Foi também pesquisada a ocorrência de determinantes plasmídicos de resistência às quinolonas (PMQR, *plasmid-mediated quinolone resistance*), assim como a sua associação com ESBLs e PMAβs, o que era desconhecido até então. Os resultados obtidos demonstraram uma co-expressão de PMAβs com ESBLs (50,7%) e com PMQRs (78,9%), respectivamente. No decorrer deste estudo foi detectada a presença de QnrC em dois isolados, os quais representam a segunda descrição deste determinante PMQR, para além da primeira publicada na China, em 2009.

A elevada disseminação de enzimas PMAβ poderá estar associada à transferência horizontal dos respectivos genes codificantes, através de plasmídeos e/ou de elementos móveis, como IS*Ecp1*, IS*26* e IS*903*, encontrados com uma frequência de 23,5%, 17,6% e 46,0%, respectivamente. Acresce o facto de 91.5% dos isolados produtores de PMAβ do estudo possuírem o gene *intI*1, o que indica a presença de integrões de classe 1, e realça o papel importante que os elementos genéticos móveis têm na disseminação da resistência.

O estudo da clonalidade, realizada por electroforese em campo pulsado (PFGE), evidenciou heterogeneidade genética dos clones produtores de PMAβs, embora tenham sido identificados clones epidémicos locais, particularmente produtores de β-lactamases da família DHA. Em relação aos clones produtores de CMY, 66,7% não eram geneticamente relacionados, apesar de distribuídos por diferentes serviços hospitalares e pela comunidade.

O uso excessivo de antibióticos no tratamento de infecções bacterianas, nomeadamente de carbapenemos, tem contribuído para a emergência de β-lactamases capazes de hidrolisar esses antibióticos. A expressão de CHDLs em *A. baumannii* quer de origem cromossómica, através de uma sobre-expressão do

gene *bla*$_{\text{OXA-51-tipo}}$, quer através da aquisição de plasmídeos que codificam, por exemplo, genes da família *bla*$_{\text{OXA-23}}$ e/ou *bla*$_{\text{OXA-24/40}}$, é preocupante. Em Portugal, a resistência aos carbapenemos em isolados de *A. baumannii* tem sido associada à produção de OXA-24 (ou OXA-40), e atribuída à disseminação de um clone endémico, multirresistente. Para conhecimento da situação actual, em Portugal, foram avaliados 172 isolados provenientes de nove hospitais portugueses, de Abril de 2009 a Abril de 2010 (Artigo III). Os resultados obtidos demonstram uma alteração na epidemiologia molecular dos isolados de *A. baumannii* no país. Assim, os clones ST98, produtores de OXA-24/40, e ST92, produtores de OXA-23, os quais coexistiam numa situação endémica antes de 2009, verificou-se terem sido substituídos pelo clone ST118, produtor de OXA-23, identificado em todo o mundo como responsável por epidemias de isolados clínicos de *A. baumannii* multirresistentes.

Em suma, os resultados obtidos no capítulo 1 contribuíram para o conhecimento da epidemiologia molecular de diferentes β-lactamases, realçando clones específicos de *Enterobacteriaceae*, produtores de ESBL e/ou PMAβ, e de *A. baumannii*, produtores de CHDLs, com capacidade de persistir e disseminar em ambiente hospitalar e na comunidade, dando origem a uma situação endémica complexa.

No Capítulo 2, intitulado "Classe A β-lactamases: função vs estrutura", é descrita a relação estrutura-função de β-lactamases com importância clinica, levando a um maior conhecimento da proteína, enquanto mecanismo de resistência.

No decorrer da caracterização genotípica, foram detectados genes que codificam novas enzimas, algumas das quais envolvidas na resistência a cefalosporinas de terceira geração (ESBL) ou a inibidores de β-lactamases (IRS, Inhibitor Resistant SHV). A caracterização bioquímica de cinco novas β-lactamases permitiu compreender a sua actividade catalítica sobre os diferentes substratos, os antibióticos β-lactâmicos.

Numa primeira fase, foi efectuada a caracterização bioquímica de três novas β-lactamases (SHV-72, SHV-84 e SHV-107) expressando um aumento da resistência à combinação amoxicilina/ácido clavulânico (Paper IV ao VI). No total, com a identificação destas três novas enzimas, o número de β-lactamases da família SHV que conferem resistência aos inibidores (IRS) aumentou para sete (http//www.lahey.org/studies/webt.asp). Na β-lactamase SHV-72 identificaram-se as substituições aminoacídicas Ile8Phe, Ala146Val e Lys234Arg. As constantes cinéticas mostraram um aumento na concentração de ácido clavulânico para a inibição de 50% da actividade enzimática (em relação à parental SHV-1). De referir que as mutações observadas na β-lactamase SHV-72 não alteraram significativamente os valores de afinidade (K_m) e não conferiram uma diminuição da actividade catalítica (K_{cat}) para as penicilinas, como reportado para as outras IRS descritas, nomeadamente SHV-84 (Paper V) e SHV-59 (nas quais a substituição Lys234Arg é única ou associada a Leu35Gln, respectivamente). Assim, este estudo sugere que outras substituições aminoacídicas na β-lactamase SHV-72, que não Lys234Arg, conferem um aumento da afinidade da enzima para as penicilinas, assim como uma maior actividade catalítica. Simulações, efectuadas por dinâmica molecular, sugeriram que a substituição aminoacídica Lys234Arg, presente em SHV-72, era responsável pela estabilização da conformação da cadeia lateral de Ser130, impedindo a sua ligação com Ser70, o que poderá diminuir a susceptibilidade ao ácido clavulânico.

As propriedades catalíticas da enzima SHV-107, uma IRS apresentando as substituições aminoacídicas Leu35Gln e Thr235Ala, demonstraram que esta mutação não confere alterações significativas na resistência às penicilinas, apesar de a actividade catalítica ser inferior em SHV-1. Simulações, efectuadas por dinâmica molecular, para a substituição aminoacídica Thr235Ala, sugeriram que esta modifica a acomodação do ácido clavulânico no sítio activo da enzima e, consequentemente, altera a sua função de inibição. Em conclusão, os resultados obtidos no estudo bioquímico do mecanismo de resistência ao ácido clavulânico, permite-nos inferir que o motivo Lys234-Ser/Thr235-Gly236, conservado em β-lactamases de classe A, é um ponto essencial para a inibição das β-lactamases, o que significa que novos inibidores poderão ser desenhados, tendo em consideração as características estruturais e funcionais da β-lactamase nesse motivo.

As propriedades enzimáticas da nova β-lactamase SHV-55 (com as mutações Tyr73Phe, Gly238Ser e Glu240Lys) evidenciaram a sua maior afinidade para as cefalosporinas de espectro alargado, característica das ESBLs, em contraste com a enzima parental SHV-1. Por outro lado, a β-lactamase SHV-99, que apresentava a substituição aminoacídica Asp104Gly, evidenciou um aumento na afinidade

para o aztreonam, sugerindo que este resíduo é importante para a ligação e reconhecimento da enzima ao substrato. Assim, mesmo não conferindo um fenótipo ESBL, este estudo mostra a importância da caracterização de novas β-lactamases, uma vez que, sob pressão de selecção, a evolução das ESBL e IRS terá o seu início nas β-lactamases parentais.

Em conclusão, o trabalho apresentado nesta dissertação, permite a elucidação da dinâmica das β-lactamases produzidas por bactérias de Gram negativo, em Portugal. A ocorrência de *Enterobacteriaceae* produtoras de ESBL, assim como a disseminação de novos clones epidémicos, nomeadamente de *A. baumannii* multi- ou pan-resistentes, é de grande preocupação. De facto, os resultados apresentados reforçam a necessidade de desenvolver estratégias de intervenção, no sentido, quer de prevenir o desenvolvimento de bactérias resistentes aos antibióticos, quer de reduzir a sua disseminação nos hospitais e destes para a comunidade.

Palavras chave: Bactérias, Gram negativo, Resistência antimicrobiana, β-lactamases, Epidemiologia molecular, Estrutura/Função, Dinâmica molecular

ABSTRACT

β-Lactamase production is the most important resistance mechanism among Gram-negative bacteria. The overall aim of this PhD thesis was to contribute to the knowledge of molecular epidemiology of β-lactamases and to the understanding of their diversity in a structural-functional level. To accomplish this aim, several studies with different approaches were performed.

The emergence of β-lactamase-producing isolates, as well as the appearance of new epidemic clones, is of great concern. The studies presented in the first chapter of results, have clearly shown that specific extended-spectrum β-lactamase (ESBL)-, plasmid-mediated AmpC β-lactamase (PMAβ)- and carbapenem-hydrolyzing class D β-lactamase (CHDL)-producing clones are able to persist in clinical settings for long periods, resulting in a complex β-lactamase endemic situation. A high diversity of β-lactamases was encountered, specifically: CTX-M family which is the most prevalent ESBL, and PMAβ (e.g., DHA-1, CMY-2, CMY-39, MIR-1, MIR-3, FOX-5 and the novel CMY-46 and CMY-50), both in *Enterobacteriaceae, as* well as CHDLs OXA-23 and OXA-24/40 in *Acinetobacter baumannii*. The results obtained in this thesis also highlight different strategies for bacterial spread of resistance that can occur through either clonal spread or horizontal gene transfer of mobile genetic elements.

In the second chapter of results, structure/function correlation of five novel clinical important β-lactamases, namely three inhibitor-resistant SHV (SHV-72, SHV-84 and SHV-107), one ESBL (SHV-55) and one parental SHV (SHV-99), are presented. One of the key findings we can infer from results is that the conserved motif Lys234-Thr/Ser235-Gly236, present in class A β-lactamases, is a hot-spot for β-lactamase inhibition, meaning that new compounds can be designed to address this structural feature.

In summary, the work performed in this thesis allows the elucidation on the dynamics of β-lactamases in Gram-negative bacteria, in Portugal. Molecular characterization together with biochemical data is essential for understanding the emergence of new resistance mechanisms and their spread.

Keywords: Gram-negative bacteria, Antimicrobial resistance, β-lactamases, Molecular epidemiology, Structure/function, Molecular dynamics

SECTION I. INTRODUCTION

Chapter 1. An overview on antibiotic resistance

1.1. Importance of antibiotic-resistant Gram-negative bacteria in public health

Infectious diseases are responsible, at the European level, for several million patients hospitalized each year and more than 2 million of these patients acquire nosocomial infections, resulting in around 175000 deaths per year (Chopra *et al*, 2008; ECDC, 2009).

Antibiotic resistance, a natural biological phenomenon that can be accelerated by repeated exposure to antibiotics, presently constitutes one of the major factors influencing infectious diseases and the outcome of infections in antibiotic-exposed patients. Indeed, the study of antibiotic resistant bacteria has been recognized as a high priority intervention area (ITFAR, 2003; WHO, 2001).

Infections caused by multidrug-resistant (MDR) Gram-negative bacteria, i.e. organisms that have acquired resistance to multiple unrelated classes of antibiotics, which emerged as major concerns both in and out of the hospital environment, not only have a profound impact on healthcare systems as a whole, but also on patients, society and the general economy. Specifically, they lead to substantial morbidity and mortality, especially in critically ill patients, to longer hospital stays, increasing the exposure of others to drug-resistant isolates, and to higher hospital costs, when compared with infections associated with susceptible isolates (Cassell, 1997; Evans *et al*, 2007; Giske *et al*, 2008; Slama, 2008; Sydnor & Perl, 2011; Wilson *et al*, 2004).

1.2. Monitoring of Resistance

The occurrence of antibiotic-resistant bacteria is a major public health threat. Information on the prevalence of resistance to specific drugs is necessary to understand the magnitude of the problem and to establish baselines for taking action. Hence, the objective of surveillance of antibiotic resistance is to provide the information necessary to the management of communicable diseases in order to minimize morbidity and mortality while also containing the emergence of pathogens resistant to antibiotics (WHO, 2001). Surveillance studies on antibiotic resistance allow us to gather information regarding: a) existing trends in pathogen incidence and antibiotic resistance mechanisms; b) the appearance of novel resistance types; and c) the prediction of future trends in antibiotic resistance. Used in conjunction with disease prevention and infection control procedures and data on antibiotic usage, strategies can be developed to protect the public health now and in the future.

With respect to this particular thesis, two surveillance programs should be mentioned: the Antibiotic Resistance Surveillance Program in Portugal (ARSIP) and the European Antimicrobial Resistance Surveillance Network (EARS-Net), at national and international levels, respectively.

ARSIP, managed and coordinated by the National Reference Laboratory of Antimicrobial Resistances (NRL-AR), at the National Institute of Health (NIH), is a voluntary Portuguese surveillance program that continually monitors the *in vitro* activity of antibiotics against several pathogens of clinical importance, such as carbapenem- and extended-spectrum-resistant *Enterobacteriaceae*, *Acinetobacter baumannii* and *Pseudomonas spp.* isolates, collected from different healthcare institutions over the country. This surveillance program works in order to investigate the development of antibiotic resistance, the ways in which it may be disseminated and the biochemical mechanisms responsible. For that, all isolates collected from hospital laboratories are analyzed by phenotypic, molecular and/or biochemical techniques to identify those mechanisms of antibiotic resistance.

EARS-Net, managed and coordinated by the European Centre for Disease Prevention and Control (ECDC), is an European network of national surveillance systems, comprising about 900 public-health laboratories serving over 1400 hospitals in Europe, which maintains a comprehensive surveillance and information system with European reference data on antibiotic resistance for public health purposes (http://www.ecdc.europa.eu/en/activities/surveillance/EARS-Net/). The coordination of EARS-Net was transferred from the Dutch National Institute for Public Health and the Environment (RIVM) to the ECDC in January 2010. The results contribute to greater public awareness and scientific understanding of antibiotic resistance and its importance in public health. EARS-Net performs surveillance of antibiotic susceptibility of seven bacterial pathogens causing invasive infections in humans, namely, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Enterococcus faecium*, *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* (EARS-Net, 2010).

Chapter 2. Antibiotic Agents

Most microbiologists distinguish between two groups of antimicrobial agents used in the treatment of infectious diseases: i) antibiotics, which are natural substances produced by certain groups of microorganisms, and ii) chemotherapeutic agents, which are chemically synthesized. In this thesis, and as initially proposed by Selman Waksman (Waksman & Flynn, 1973), the generic term “antibiotic” will be used to denote any class of organic molecule that kills bacteria (bactericidal) or inhibits their growth (bacteriostatic) through specific interactions with a bacterial target, while antimicrobial agents will be considered as a general term for substances that either kill or slow the growth of microbes, including antibiotics, antiviral agents, antifungal agents, and antiparisitic drugs.

2.1. History of antibiotics

In 1928, Alexander Fleming (1881–1955) discovered the bacteria-killing property of penicillin (Fleming, 1929). Although he had made an incredible discovery, he was unable to produce penicillin in a form useful to physicians. In 1941, two English scientists, Howard Florey (1898–1968) and Ernst Chain (1906–1979), developed a form of penicillin that could be used to fight bacterial infections in humans. In 1945, penicillin was available for widespread use. Fleming, Florey, and Chain shared the Nobel Prize in Medicine in 1945 for their work on penicillin (Davies & Davies, 2010).

While Fleming was working on penicillin, Gerhard Domagk (1895–1964) discovered, in 1935, the first sulfa drug, sulfonamide. However, these drugs had some serious problems, especially kidney failure, and scientists continued their search for other antibiotics. The next breakthrough was in 1944, when Selman Waksman discovered a drug called streptomycin. It was originated from microbes found naturally in soil and it was proved to be a cure for many intestinal diseases. In the late 1940s and early 1950s, new antibiotics were introduced, including chloramphenicol and tetracycline, and the age of antibiotic chemotherapy came into full being (Figure 1).

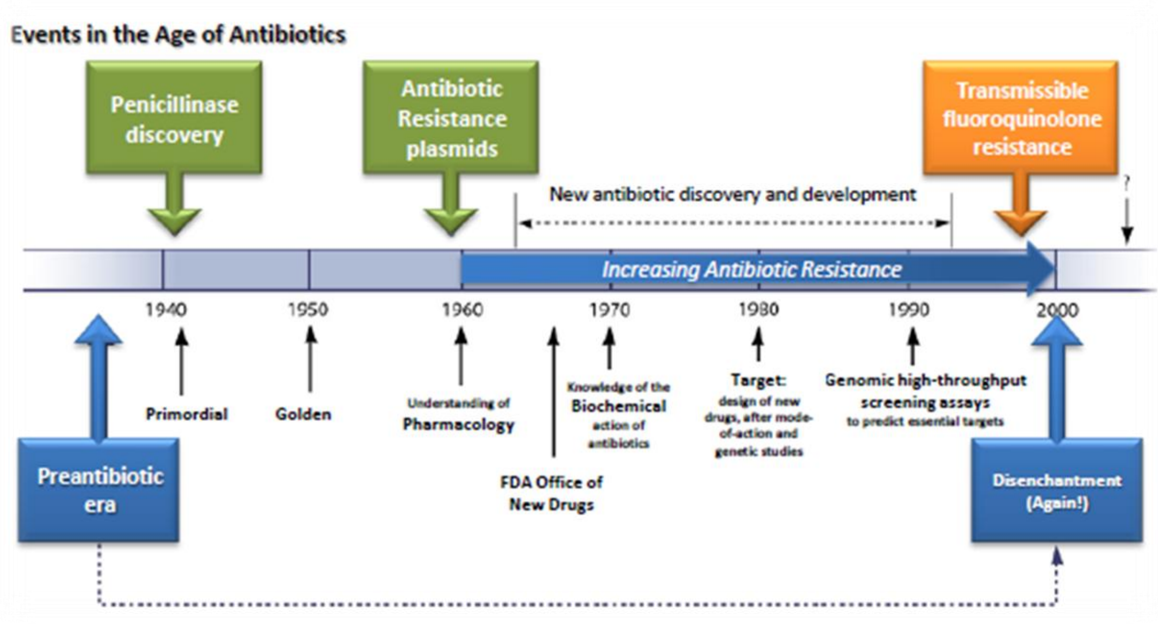


Figure 1 - History of antibiotic discovery and concomitant development of antibiotic resistance. Adapted from Davies & Davies (2010).

2.2. Antibiotic Targets

Antibiotics generally target processes or structures that are essential for bacterial growth, survival or both simultaneously; the most successful compounds are those that interfere with the construction of the bacterial cell wall, the synthesis of protein, or the replication and transcription of DNA. Relatively few clinically useful agents act at the level of the cell membrane or by interfering with specific metabolic processes of the bacterial cell (Figure 2).

Their specificity for bacterial targets means that eukaryotic cells in the host are not greatly affected (Betina, 1983). Antibiotics can be classified according to their antimicrobial spectrum of activity, mechanism of action, source (e.g., produced by *Streptomyces* spp.), mechanism of production (natural, synthetic, or semi-synthetic drugs), or chemical structure.

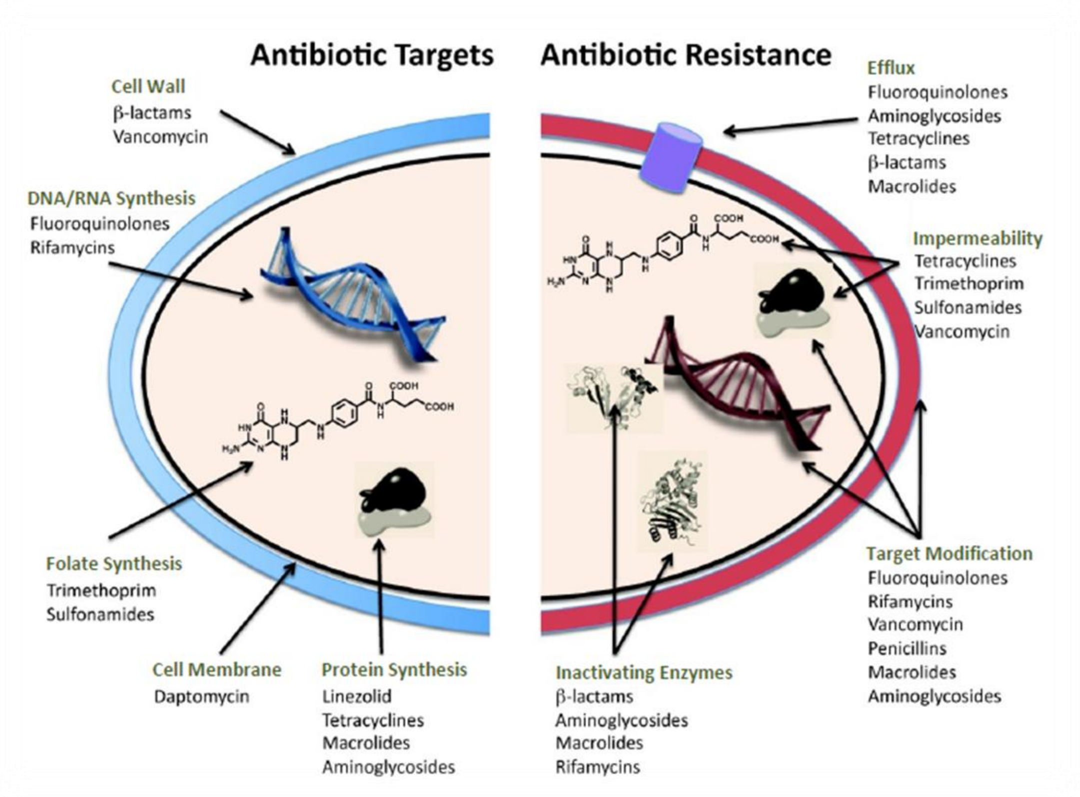


Figure 2 - Antibiotic targets and mechanisms of resistance (Wright, 2010).

2.2.1. *Inhibition of Cell Wall Synthesis*

Bacterial cell wall contain murein or peptidoglycan, which is essential in maintaining cell wall structure. Cell wall synthesis inhibitors, such as β-lactams or glycopeptides, block the ability of microorganisms to synthesize their cell wall by inhibiting the synthesis of peptidoglycan. The interruption of the normal crosslinking can be mediated by penicillins, that inhibit the transpeptidase activity, or by vancomycin, that sequester the substrate by complexation of the D-Ala-D-Ala termini of peptidoglycans; these antibiotics show synergy when used in combination.

In Chapter 4, the inhibition of cell wall synthesis mechanism will be described in more detail upon the elucidation of the mechanism of action of β-lactamases.

2.2.2. *Inhibition of Protein Synthesis*

Many antibiotics work by binding to bacterial ribosomes, inhibiting the protein synthesis machinery (translation) in the cell. Examples of antibiotics that bind to the 30S ribosomal subunit are aminoglycosides and tetracyclines, which prevent the binding of tRNA (Brodersen *et al*, 2000; Carter *et al*, 2000). Macrolide antibiotics, such as erythromycin, bind to the 50S ribosomal subunit and block the exit tunnel of the bacterial ribosome (Schlünzen *et al*, 2001).

2.2.3. *Alteration of Cell Membranes*

Polymyxins consists of a cationic cyclic peptide with a fatty acid chain. The interaction between the cationic peptide and the bacterial cell membrane causes disruption of the membrane and increases the permeability of cell components (Daugelavicius *et al*, 2000; Newton, 1956). These antibiotics are mostly effective on Gram-negative bacteria because they contain a definite cell membrane.

On the other hand, the biological activity of ionophore antibiotics is related to their ability to disrupt the flow of ions either into or out of cells, i.e. they can disrupt the ionic imbalance by allowing ions to penetrate the cell membrane as ion-ionophore complexes or via the formation of ion channels (Bergen & Bates, 1984; Bilgili & Kart, 2008). Gram-positive bacteria appear to be particularly sensitive to the effect of ionophores perturbing normal ion transport.

2.2.4. *Inhibition of Nucleic Acid Synthesis*

Differences between the enzymes used to synthesize nucleic acids in prokaryotes and eukaryotes provide the means for selective action of antibiotics that take their effect by inhibiting nucleic acid synthesis. Antibiotics of the rifamycin family inhibit RNA synthesis by binding to the RNA polymerase, which is responsible for transcribing bacterial DNA to RNA (Campbell *et al*, 2001; Floss & Yu, 2005). Antibiotics of the (fluoro)quinolone group interfere with DNA synthesis by inhibiting the activity of topoisomerase II (DNA gyrase), an enzyme involved in DNA replication(Hooper & Rubinstein, 2003; Shen *et al*, 1989).

2.2.5. *Competitive Inhibitors*

Also referred to as anti-metabolites or growth factor analogs, these antibiotics competitively inhibit the important metabolic pathways occurring inside the bacterial cell. The most important in this class are sulfonamides and trimethoprim.

Amino acid and purine synthesis in bacteria is dependent on tetrahydrofolate, which is a folic acid derivative. Bacteria are unable to absorb preformed folic acid and need to synthesize it by means of two key enzymes involved in folate synthesis (DHFR, a dihydrofolate reductase and DHFS, a dihydrofolate synthase) which are inhibited by sulfamethoxazole and trimethoprim, respectively. Overproduction of these enzymes causing resistance to these drugs is recognized in several pathogenic organisms. These agents act at separate stages in the pathway of folic acid synthesis and therefore they have a synergistic effect (Murray *et al*, 2005; Walsh, 2000).

2.3. The β-lactam antibiotics

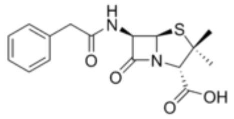
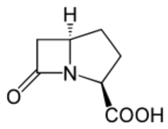
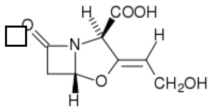
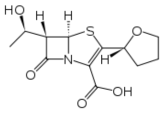
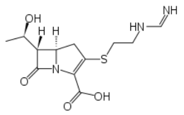
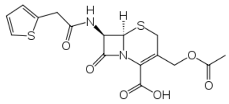
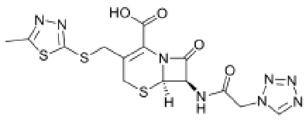
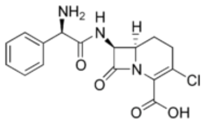
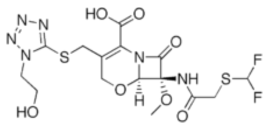
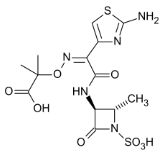
2.3.1. *Structure*

β-Lactam antibiotics are among the most commonly prescribed drugs. They are composed of an isolated ring (monobactam), or associated in bicyclic ring structures in other classes (Table 1). Overall, side chain modifications within groups alter the pharmacokinetic and antibacterial properties of different β-lactam antibiotics. For example, modifications at position 7 of cephalosporins increase penetration into the periplasmatic space and stability against β-lactamases, but may reduce antibiotic activity (Donowitz & Mandell, 1988a; Donowitz & Mandell, 1988b).

β-Lactam antibiotics are indicated for the prophylaxis and treatment of bacterial infections caused by susceptible organisms. Although there are several classification schemes for antibiotics based on bacterial spectrum (broad versus narrow) or type of activity (bactericidal vs. bacteriostatic), the most useful is based on chemical structure (Table 1).

β-Lactams can range from very narrow spectrum to very broad spectrum depending on the subgroups. The ones with the broadest spectrum, third and fourth generation cephalosporins, can inactivate both Gram-negative and Gram-positive bacteria (Murray *et al*, 2005).

Table 1 - Classification scheme for β-lactam antibiotics, based on chemical structure.

Class	Subclass	Example	Chemical struture
Penams	Penams - Penicillin - Aminopenicillins - Ureidopenicillin - Carboxypenicillin - Penicillinase-stable penicillins	**Benzylpenicillin**, the gold standard type of penicillin	
	Carbapenams	**(3S,5R)-carbapenam**; carbapenams exist primarily as biosynthetic intermediates to the carbapenem antibiotics	
	Oxapenams or clavams - β-lactam/β-lactamase inhibitor combinations	**Clavulanic acid**	
Penems	Penems	**Faropenem**	
	Carbapenems	**Imipenem**	
Cephems	Cephamycins[1]	**Cefoxitin**	
	Cephalosporins (oral and parenteral) - 1st generation - 2nd generation[3] - 3rd generation[2,3] - 4th generation[2,3]	**Cefalotin** (1st generation)	
	Carbacephems (oral)	**Loracarbef**	
	Oxacephems (parenteral)	**Flomoxef**; oxacephems are synthetic compounds and have not been discovered in nature.	
Monobactams[3]		**Aztreonam**; monobactams have a second thiazole ring which is not fused to the β-lactam ring	

[1] Cephamycins contain a methoxyl group and are thus also called 7-α-methoxy-cephalosporins.

[2] Cephalosporins from 3rd and 4th generations are also referred to as “extended-spectrum cephalosporins”.

[3] Cephalosporins from 2nd (cefuroxime), 3rd (cefotaxime, ceftazidime, ceftriaxone) and 4th (cefepime) generations, and aztreonam are also oxyimino-β-lactams. With the exception of aztreonam, these antibiotics are also called oxyimino-cephalosporins.

Among β-lactams, carbapenems are antibiotics with a broad spectrum of antibacterial activity, compared to other β-lactams such as penicillins and cephalosporins. These antibiotics were originally developed from thienamycin, a naturally-derived product of *Streptomyces cattleya* (Kahan *et al*, 1983), and they are often used for treatment of resistant isolates of *P. aeruginosa* and *A. baumannii* that have become increasingly resistant to the broad-spectrum cephalosporins used in hospital settings (Livermore & Woodford, 2006). Carbapenems (imipenem, meropenem, ertapenem, and doripenem) have proven to be more stable against the action of many β-lactamases, due to their unusual stereochemistry across the C5-C6 bond and the presence of the α-hydroxyethyl group (Table 1).

Clavulanic acid, discovered as a natural product of the bacterium *Streptomyces clavigularis*, was the first β-lactamase inhibitor to be introduced into clinical practice (Reading & Cole, 1977). Modifications to its structure were later made in improvement of its activity, producing two additional inhibitors, sulbactam and tazobactam. They are called “suicide inhibitors” because they irreversibly bind to β-lactamase (Bonomo & Rice, 1999; Knowles, 1985).

2.3.2. *Mechanism of action*

β-lactam antibiotics inhibit the growth of sensitive bacteria by inactivating enzymes that are involved in the third stage of cell wall synthesis and are located in the bacterial cell membrane. In Gram-positive bacteria, peptidoglycan is the main constituent of the cell wall, appearing as a heteropolymer of complex composition that provides strength to the wall, to which the cytoplasmic membrane is juxtaposed. It is in these structures, in the outer leaflet, that the targets of β-lactam antibiotics – the PBP (Penicillin-Binding Proteins) – are inserted (Figure 3) (Murray *et al*, 2005; Scheffers & Pinho, 2005). By contrast, Gram-negative cell walls have a more complex structure than do those of Gram-positive organisms. On the outside of the cytoplasmic membrane, in whose outer leaflet PBPs are located, there is the periplasm, which contains a thin layer of peptidoglycan, with less cross-linking than in Gram-positive cells; Gram-negative bacteria are thus mechanically weaker than Gram-positive cells. Beyond the peptidoglycan of the Gram-negative cell wall lies an outer membrane, which has protein channels -

porins - that act as transporters through its surface (Figure 3).

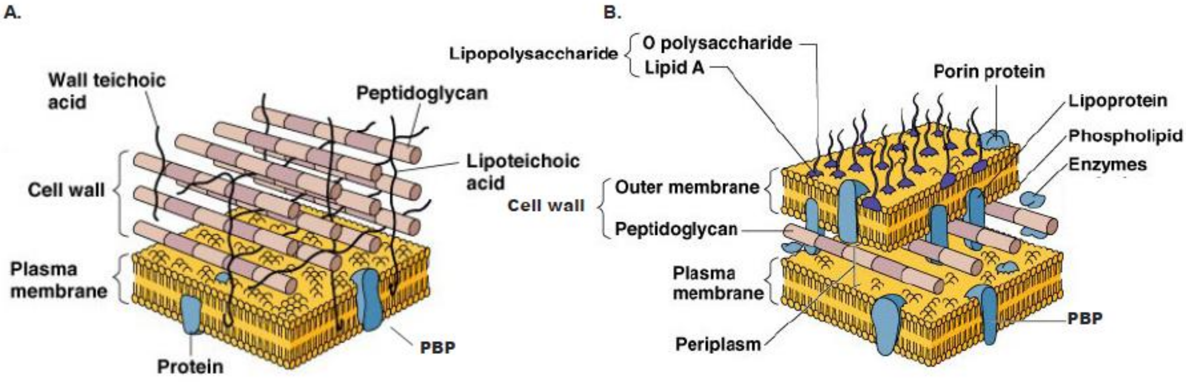


Figure 3 – Graphical illustration of Gram-positive (A) and Gram-negative (B) cell wall structures. The Gram-positive cell wall is thicker than that of Gram-negative bacteria, compensating for the absence of a second (outer) bilayer membrane. Adapted from Tortora *et al* (2010)

Peptidoglycan is composed of alternating chains of *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM) linked by β-(1,4)-glycoside units (Figure 4).
The carboxyl group of muramic acid is frequently replaced by an amino acid chain composed of four amino acids. The most common are L-alanine, D-alanine, D-glutamic acid, D-glutamine and L-lysine or diaminopimelic acid (DAP). This is the only biological structure that contains D-amino acids and it is the target of numerous antibacterial antibiotics.

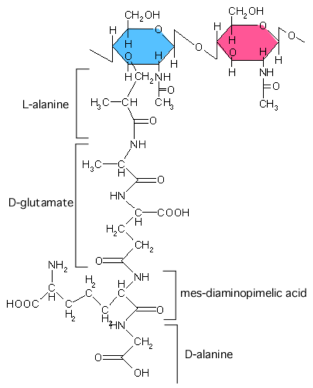


Figure 4 - N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) chains forming the structure of peptidoglycan (Paustian & Roberts, 2011).

Synthesis of peptidoglycan occurs in three phases: assembly of precursor in the cytoplasm, transport across the inner membrane, and polymerization. Peptidoglycan synthesis starts in the cytoplasm by binding the peptide chains to one of the sugar molecules. After translocation to the outside of the cell, peptide chains from adjacent glycan chains are cross-linked to each other by a peptide bond exchange (transpeptidation) between the free amine of the amino acid in the third position of the pentapeptide (e.g., lysine) and the D-alanine at the fourth position of the other peptide chain, releasing the terminal D-alanine of the precursor (Figure 5a). These reactions are catalyzed by transpeptidase and carboxypeptidase enzymes, which are only sensitive to β-lactam antibiotics (Araki *et al*, 1966).

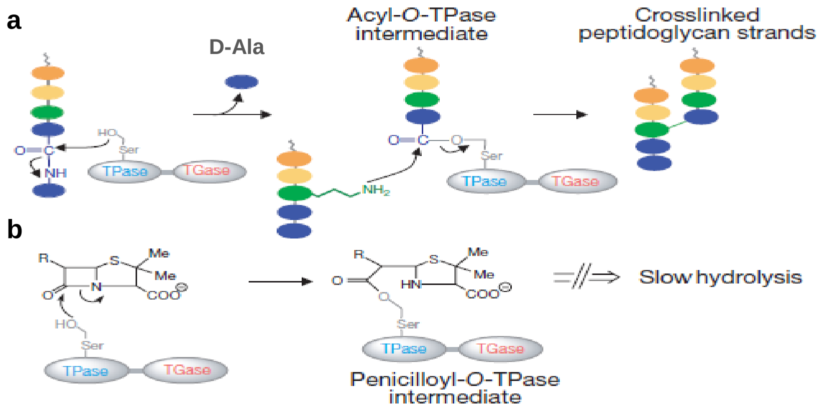


Figure 5 – Schematic representation of the biosynthesis of the cell wall. a) The final step comprises the cross-linking between two peptidoglycan isolates and release of the terminal D-Alanine (D-Ala) of the precursor; and b) the inhibition of transpeptidase activity by penicillins through the formation of a slowly hydrolyzing covalent acyl-enzyme intermediate. Adapted from Walsh (2000).

β-lactam antibiotics inhibits the cell wall synthesis because the highly reactive CO-N bond in the β-lactam ring of the β-lactam molecule lies in exactly the same position as the CO-N bond in D-alanyl-D-alanine, which is the target of transpeptidation (Tipper & Strominger, 1965) (Figure 6). The β-lactam-enzyme complex acts as a competitor to the formation of the normal acylated enzyme (Figure5b). The β-lactam-enzyme complex is very stable, and its formation culminates with the inactivation of the PBP's functions (Georgopapadakou & Liu, 1980; Ghuysen, 1988). This interference with the normal cross-linking in the cell wall results in cellular lysis.

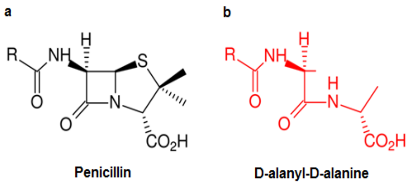


Figure 6 – β-lactams (a) acts as a substrate analog, binding to the substrate-anchoring site normally occupied by D-alanyl-D-alanine (b).

Chapter 3. Antibiotic Resistance in Gram-negative Bacteria

> *"The greatest possibility of evil in self-medication is the use of too small doses so that instead of clearing up infection, the microbes are educated to resist penicillin".*
> *Alexander Fleming, the discoverer of penicillin*

Chance has always played a part in the evolution of bacteria, and the emergence of antibiotic resistance is one of the many aspects of this phenomenon (Courvalin, 2005). Hence, once an antibiotic is proven to be effective and enters widespread human therapeutic use, its days are numbered. Clinically significant antibiotic resistance appears in periods of months to years (Davies, 1996; Davies & Davies, 2010; Demain & Sanchez, 2009).

Gram-negative bacteria are usually more resistant to the antibiotic action than Gram-positive (Russell & Day, 1996). This is due to the presence of a double membrane structure that prevents antibiotics, like penicillin G, from accessing the target in the cell wall. Generally, Gram-negative microorganisms exhibit two types of antibiotic resistance mechanisms, i.e. intrinsic and acquired resistance (Livermore, 2003):

- ✓ *Innate or intrinsic resistance* is an inherent feature of a species, resulting in the lack of activity of an antibiotic or antibiotic class; it is usually controlled by the microorganism’s own genetic background, i.e. all bacteria of the same specie may lack the appropriate antibiotic target or possess natural antibiotic resistance mechanisms that avoid the agent to reach the target (Table 2). In this case, the microorganism is resistant to an antibiotic without record of previous exposure; for example, most Gram-negative organisms are intrinsically resistant to vancomycin and teicoplanin, because their outer membrane is impermeable to large glycopeptide molecules (Arthur & Courvalin, 1993; Reynolds, 1989).

Table 2 – Examples of intrinsic resistance phenotypes.

Organism	***Intrinsic Resistance***
All *Enterobacteriaceae*	penicillin G, macrolides, lincosamides, streptogramins, glycopeptides, fusidic acid, linezolid, mupirocin
Klebsiella* spp.** ***Citrobacter koseri	aminopenicillins, carboxypenicillins
Proteus vulgaris ***Proteus penneri***	aminopenicillins, carboxypenicillins, cefuroxime, colistin, nitrofurantoin, tetracyclines
Proteus mirabilis	colistin, nitrofurantoin, tetracyclines
Serratia marcescens	aminopenicillins, amoxicillin-clavulanic acid, 1st and 2nd gen cephalosporins, colistin
***Enterobacter* spp.**	aminopenicillins, amoxicillin-clavulanic acid, 1st gen cephalosporins, cefoxitin
Citrobacter freundii	aminopenicillins, amoxicillin-clavulanic acid, 1st gen cephalosporins, cefoxitin, nitrofurantoin
Pseudomonas aeruginosa	aminopenicillins, carboxypenicillins, amoxicillin-clavulanic acid, 1st and 2nd gen cephalosporins, cefotaxime, ceftriaxone, nalidixic acid, aminoglycosides, colistin, nitrofurantoin, fosfomycin, chloramphenicol, imipenem and trimethoprim
***Salmonella* spp.**	1st and 2nd gen cephalosporins, cefuroxime (active *in vitro*, not active *in vivo*), aminoglycosides (*in vivo*)
Morganella morgannii	aminopenicillins, amoxicillin-clavulanic acid, 1st and 2nd gen cephalosporins, cefoxitin, tetracyclines, fosfomycin, colistin, nitrofurantoin
Acinetobacter baumannii ***Acinetobacter calcoaceticus***	aminopenicillins, 1st and 2nd gen cephalosporins, nitrofurantoin, fosfomycin, chloramphenicol and trimethoprim
Stenotrophomonas maltophilia	ureidopenicillins, carboxypenicillins, 1st and 2nd gen cephalosporins, imipenem, cefotaxime, aztreonam, aminoglycosides, tetracyclines, fosfomycin

- ✓ *Extrinsic or acquired resistance* is the result of a series of genetic changes, through the horizontal acquisition of foreign genetic information from bacteria cohabitating the same environment (mediated by plasmids and/or transposons, which may contain integron sequences) or by mutation in structural or regulatory housekeeping genes, such as *gyrA*, *parC* and *rpoB*, (Courvalin, 2005). Sometimes, genetic changes result in diminished activity, but not complete loss of antibiotic effectiveness.

3.1. Mechanisms of antibiotic resistance

Bacteria employ several basic strategies for evading the effects of antibiotics (Davies & Davies, 2010; Peleg & Hooper, 2010; Walsh, 2000). Figure 2 (Chapter 2.2) shows the main strategies, such as altered target sites, decreased uptake, active efflux systems, and enzymatic inactivation or modification.

3.1.1. *Altered target sites*

Alterations of antibiotic target sites are usually caused by point mutations in the regions of a specific gene necessary for antibiotic activity, resulting in lowered binding affinity between the antibiotic and its target. Two examples that characterize this resistance mechanism are listed below:

- ✓ Modification of PBPs, the targets of β-lactams; this can be achieved by one of three mechanisms: i) amino acid substitutions, ii) the acquisition of new PBPs-encoding genes, iii) the recombination of genes coding for PBPs, resulting in mosaic genes encoding proteins with decreased affinity to β-lactams (Georgopapadakou & Liu, 1980).
- ✓ Point mutations in the *gyrA* and *gyrB* genes, coding for DNA topoisomerase II (also called DNA gyrase) or in topoisomerase IV, which also acts as target proteins for the action of antibiotics (Hooper, 1995; Tankovic *et al*, 1996), can alter the binding efficiency of (fluoro)quinolone antibiotics, thereby reducing their efficacy. In addition, not only single mutations, but multiple point mutations may also occur, eventually leading to higher levels of resistance, as observed for the quinolone resistance determining region (QRDR) of DNA topoisomerase II (*gyrA*, *gyrB*) and IV (*parC* and *parE*) genes. Although, resistance to quinolones can also be mediated by Qnr proteins, that protect the complex of DNA and DNA gyrase or topoisomerase IV enzymes from the inhibitory effect of those antibiotics (Strahilevitz, 2009). These plasmid-mediated quinolone resistance (PMQR) determinants have emerged as a significant concern in recent years. Aac(6')-Ib-cr, a variant aminoglycoside acetyltransferase with two amino acid alterations, which allows to inactivate ciprofloxacin through the acetylation of its piperazinyl substituent, and QepA, which encode efflux pumps that extrude quinolones, are the more recent PMQR determinants described (Strahilevitz, 2009).

3.1.2. *Decreased uptake*

Antibiotic resistance may occur through a decrease in the permeability of the outer membrane of Gram-negative bacteria (Poole, 2002). The flow of molecules into the cell is ensured by complex membrane proteins, known as OMPs (Outer Membrane Proteins), which provide channels for the entry of molecules into the cell membrane (including antibiotics) based on charge, shape, and size. Loss of function of one of these porins due to a mutation event may possibly lead to antibiotic resistance (Delcour, 2009). For example, loss of the D2 porin due to mutation causes imipenem resistance in *P. aeruginosa* (Livermore, 1992). In *E. coli*, proteins OmpF, OmpC and OmpE are commonly associated with antibiotic resistance (Nikaido, 2003).

3.1.3. *Active efflux system*

The efflux of antibiotics provides a residual level of resistance; i.e., it is usually not enough for the expression of clinical resistance. However, together with other mechanisms, it may lead to treatment failures. Resistance is frequently caused by increased synthesis of protein machinery, due to, for example, mutations that occur in the transcriptional repressors of these proteins, eventually leading to increased efficiency of transport of antibiotics to the outside of the cell (Poole, 2004). An example of active efflux mediated antibiotic resistance is the high intrinsic resistance of *P. aeruginosa* to penem antibiotics due to the composition of its cellular membrane and an active efflux system (Okamoto *et al*, 2001).

3.1.4. *Enzymatic inactivation or modification*

Enzymatic inactivation or modification of antibiotic agents is a common mechanism of resistance that reduces or eliminates antibiotic activity (Morar & Wright, 2010). The primary resistance mechanism to β-lactams, is enzymatic degradation. In β-lactams, hydrolysis of the β-lactam ring is mediated by β-lactamases (see Chapter 4). Enzymatic modification is the most common type of aminoglycoside resistance. One classical example of such modification involves aminoglycoside acetyltransferase, that acetylates and inactivates aminoglycosides such as gentamicin (Wright, 2005). In addition, this mechanism is also involved in the chloramphenicol resistance (Suzuki & Okamoto, 1967).

Given the importance of β-lactamases in this thesis, the mode of action and the resistance mechanism mediated by these enzymes are described in detail in Chapter 4.

3.2. Mechanisms of dissemination of resistance genes

The evolution and spread of antibiotic resistance in bacteria is a complex process involving a variety of different mechanisms. The dissemination of resistance can occur in different ways, either through clonal spread, plasmid epidemics or by other mobile genetic elements (MGEs) (Courvalin, 2005). MGEs are segments of DNA that encode enzymes and other proteins, which move within genomes (intracellular mobility) or between bacterial cells by conjugation, transformation or transduction (intercellular mobility) (Frost *et al*, 2005).

Although both chromosomal mutations and genetic transfer can be responsible for the acquisition of resistance, it is the horizontally transmitted resistance which causes the greatest threat, as it usually involves much larger changes that allow the bacteria to carry out new functions and adapt to different environments (de la Cruz & Davies, 2000; Martinez, 2009; Martinez & Baquero, 2002). Horizontal gene transfer (HGT) can be mediated by various MGEs, such as plasmids, pathogenicity islands, bacteriophages, as well as transposons, integrons and insertion sequences (IS) (Normark & Normark, 2002) (Table 3).

Plasmids may harbor a variety of MGEs, including transposons, conjugative transposons, IS elements and integrons (Norman *et al*, 2009). One of the most important process in the acquisition of resistance determinants is performed by transposons carrying multiple antibiotic resistance genes, which have the ability to enter conjugative plasmids or chromosomes (Norman *et al*, 2009). It has been proven that these elements can provide a significant advantage for microorganisms, during particular circumstances, involving changes in environment (Ochman *et al*, 2000). Integrons, one of the established mechanisms by which Gram-negative bacteria acquire resistance genes, are mobile DNA elements with specific structures consisting of two conserved segments flanking a central region – gene cassette (Table 3 and Figure 7). These gene cassettes may encode various functions, such as resistance and can be inserted in that region.

Table 3 – Characteristics of the most important MGEs.

Gene Transfer Element	***Characteristics of DNA transfer elements***
Plasmid	*Plasmids* are transferable genetic elements capable of autonomous replication within a suitable host. Plasmids can be either *self-transmissible* (conjugative) or *mobilisable* (non-self-transmissible). Whereas the first group encodes a complete conjugative DNA transfer apparatus (Tra functions), the second group usually bears only the functions required for initiation of its own transfer DNA replication (Mob functions).
Insertion sequences	*Insertion sequences* (IS) are the simplest transposable elements; by definition, IS carry only the genetic information necessary for insertion functions and no accessory genes (for example, drug resistance). IS elements are small genetic elements that are flanked by short terminal inverted-repeat sequences (IR) of 10-40bp and are able to insert at multiple sites in target DNA.
ISCR	*Insertion Sequence Common Region* (ISCR) elements are IS that have similarities to the IS*91* family in both structure and function. These elements are known to move by a process called rolling-circle replication, and a function of this process is the concomitant movement of additional sequences found upstream of their transposase genes.
Transposon	*Transposons* are genetic elements that physically transpose from one genetic position to another, within the chromosome or plasmid in which they reside. Some transposons carry one or more antibiotic resistance genes in their central regions. *Complex transposons* contain IS with short IR at their termini; undergo replicative transposition. *Conjugative transposons*, also called integrated conjugative elements, are integrated DNA elements that excise themselves to form a covalently closed circular intermediate. This circular intermediate can either reintegrate in the same cell or transfer by conjugation to a recipient and integrate into the recipient's genome.
Integron	*Integrons* are DNA elements, not self transmissible, with the ability to capture genes, by site-specific recombination. Integrons have an integrase gene (*int*) to mediate excision and orientation-specific integration of gene cassettes, a nearby recombination site (*attI)*, and a promoter, Pc, which ensures expression of the operon. There are three main classes of integrons based upon the type of integrase gene they possess: class 1 and class 2 integrons are the most common, whereas class 3 are rare.
Gene cassette	*Gene cassettes* are genetic elements that may exist as free, circular, non-replicating DNA molecules when moving from one genetic site to another, but which are normally found as linear sequences that constitute part of a larger DNA molecule, such as a plasmid or bacterial chromosome. The genes carried on gene cassettes usually lack promoters and are expressed from a promoter on the integron.
Bacteriophage	*Bacteriophage* (phage) are obligate intracellular parasites that multiply inside bacteria by making use of some or all of the host biosynthetic machinery (i.e., viruses that infect bacteria). They mediate the transfer of resistance genes.

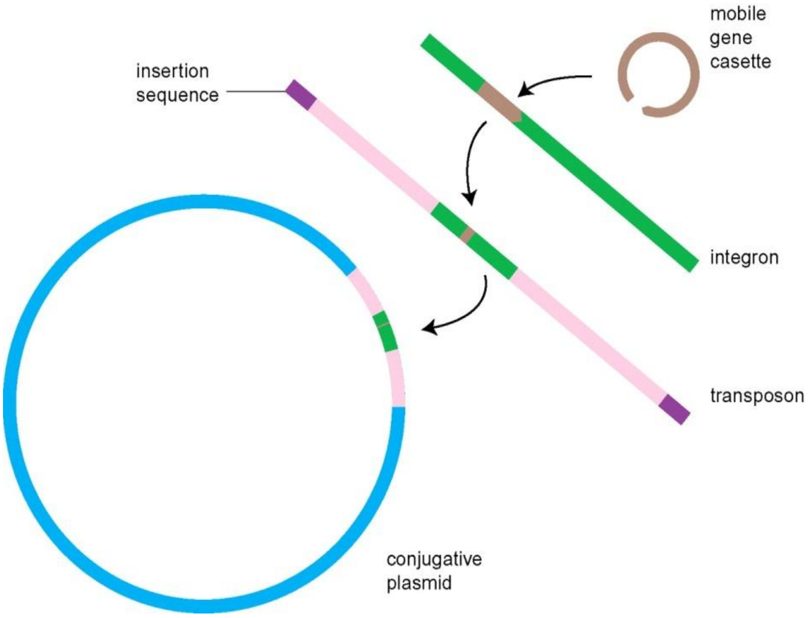


Figure 7 - The modular and hierarchical composition of MGEs. Gene cassettes are inserted into integrons by integrase-mediated site-specific recombination. Integrons may be inserted into composite transposons, which in turn may be inserted into a dispersive element like a conjugative plasmid (Norman *et al*, 2009).

Bacteria can accumulate and link resistance genes within the same MGE units allowing resistance to a specific antibiotic to be maintained via a co-selection process. Indeed, adjacent genes encoding virulence determinants, biodegradation pathways, and resistance to heavy metals, disinfectants or detergents provide a selective advantage to the organism (Baker-Austin *et al*, 2006; Normark & Normark, 2002).

Chapter 4. β-lactamases: the most important antibiotic resistance mechanism in Gram-negative bacteria

4.1. Overview and classification schemes

The first β-lactamase (EC 3.5.2.6) to be identified was located in an *E. coli* isolate, even before the widespread use of penicillin in clinical practice (Abraham & Chain, 1940). Indeed, Abraham and Chain described a substance in *E coli* that could inactivate penicillin. This penicillinase was the first bacterial product isolated that mediated resistance to an antibacterial agent, hence, these authors foreshadowed the era of widespread antibiotic resistance that occurs 15 years after.

Many Gram-negative bacteria express a naturally occurring, chromosomally mediated β-lactamase. The origin of these proteins appears to be linked to their own PBPs, from a structural and an evolutionary point of view, suggesting that these protein might have common ancestors in primitive antibiotic-producing bacteria (Ghuysen, 1991; Hall & Barlow, 2004). In fact, class A β-lactamases conserve the 3D structure and biochemical mechanism of PBPs (Massova & Mobashery, 1998). Indeed, β-lactamase-producing soil bacteria are frequently found, since they need to produce their own antidote to avoid antibiotic pressure selection caused by other organisms.

Various classifications have been proposed for the increasing number of β-lactamases that appeared since the late 1960s, which show a great diversity of characteristics (Table 4). Each classification has arisen because of the need to fill gaps left by previous classifications in order to characterize and integrate new β-lactamases. These classifications involve two major approaches: i) based on the biochemical and functional characteristics, namely subtract specificity, of the enzyme (Bush & Jacoby, 2010; Bush *et al*, 1995) and ii) based on the amino acid sequence and molecular structure of the enzyme (Ambler *et al*, 1991).

The latest classification scheme proposed by Bush and Jacoby in 2010 is widely accepted, as is Ambler’s proposal for molecular structure. As such, this work also follows these classification schemes (Table 5).

Table 4 - Evolution of the molecular and functional classification of β-lactamases.

Year	Basis of classification of β lactamases	Author
1968	Used cephalosporins versus penicillins as substrates	Sawai *et al*, 1968
1973	Expanded substrate profile and suggested five major groups (Ia-d, II, III, IV, V)	Richmond & Sykes, 1973
1976	Extended the last scheme to include plasmid-mediated β-lactamases differentiated by isoelectric focusing	Sykes & Matthew, 1976
1980	Molecular structure classification was first proposed including classes A and B	Ambler, 1980
1981	Added class C to the molecular structure classification	Jaurin & Grundström, 1981
	Added the category "cefuroxime-hydrolyzing β-lactamase" to the "penicillinase and cephalosporinase" classification	Mitsuhashi & Inoue, 1981
1987/ 1988	Class D, constituting oxacillin-hydrolyzing enzymes, were segregated from the other Ser-β-lactamases, in the molecular structure classification.	Huovinen *et al*, 1988; Ouellette *et al*, 1987
1989	Expanded further the substrate profile, added the reaction with EDTA, correlated between functional and molecular classification	Bush, 1989a; Bush, 1989b; Bush, 1989c
1995	Expanded the Bush scheme. β-Lactamases were classified into four groups (1-4) on the basis of the spectrum of activity and other functional characteristics, namely substract profiles, biochemical properties, molecular structure, and amino acid sequence.	Bush *et al*, 1995
2010	Updated scheme of 1995, including group 1 cephalosporinases (class C); group 2 broad-spectrum, inhibitor-resistant and extended-spectrum β-lactamases and serine carbapenemases (classes A and D); group 3 metallo-β-lactamases (class B); group 4 have been omitted in the present scheme due to incomplete characterization. Several new subgroups of each of the major groups are described, based on specific substract activity of individual enzymes.	Bush & Jacoby, 2010

Table 5 - Classification schemes for the major families of β-lactamases of clinical importance in Gram-negative bacteria. Adapted from Bush & Jacoby (2010).

Ambler Class	Functional group or subgroup[1]	Enzyme families	Preferred substract(s) and inhibitor profile	Examples of Representative enzymes
A	2b	TEM, SHV	Penicillins, early cephalosporins (inhibited by β-lactamase inhibitors)	TEM-1, TEM-2, TEM-13, SHV-1, SHV-11, SHV-89
	2be (ESBL)	TEM, SHV	Extended-spectrum cephalosporins, monobactams (inhibited by β-lactamase inhibitors)	TEM-3, TEM-7, TEM-10, TEM-12, TEM-26, SHV-2, SHV-3, SHV-115
		CTX-M		CTX-M-1 to CTX-M-119
		VEB		VEB-1 to VEB-7
		PER		PER-1 to PER-7
	2br (IRT/IRS)	TEM, SHV	Penicillins (resistance to β-lactamase inhibitors)	TEM-30 , TEM-31 , TEM-163, SHV-10, SHV-72, SHV-84, SHV-107
	2ber (CMT)	TEM	Extended-spectrum cephalosporins, monobactams (resistance to β-lactamase inhibitors)	TEM-50, TEM-158
	2f	GES	Increased hydrolysis of carbapenems, oxyimino-β-lactams and cephamycins (variable resistance to β-lactamase inhibitors)	GES-2 to GES-17
		KPC		KPC-2 to KPC-11
		SME		SME-1 to SME-3
B	3a (MBL)	IMP	Broad-spectrum hydrolysis including carbapenems but not monobactams (resistance to β-lactamase inhibitors)	IMP-1 to IMP-30
		VIM		VIM-1 to VIM-30
		NDM		NDM-1 to NDM-6
C	1 (PMAβ)	CMY	Cephalosporins, cephamycins (resistance to β-lactamase inhibitors)	CMY-1 to CMY-72
		DHA		DHA-1 to DHA-8
	1e (ESAC)	CMY	Cephalosporins, cephamycins, with increased hydrolysis of ceftazidime. (resistance to β-lactamase inhibitors)	GC1, CMY-10, CMY-19, CMY-37
D	2d	OXA	Cloxacillin (variable resistance to β-lactamase inhibitors)	OXA-1, OXA-2, OXA-10
	2de (ESBL)	OXA	Cloxacillin, Extended-spectrum cephalosporins (variable resistance to β-lactamase inhibitors)	OXA-11, OXA-14, OXA-15
	2df (CHDL)	OXA	Cloxacillin, Carbapenems (variable resistance to β-lactamase inhibitors)	OXA-23, OXA-24, OXA-51, OXA-58

[1] Subgroups 2a, 2c, 2ce, 2e and 3b are missing in this table, because they represent enzymes not commonly encountered in clinical practice.

ESBL, extended-spectrum β-lactamase; IRT/IRS, inhibitor resistant TEM or SHV, respectively; CMT, complex mutant TEM; MBL, metallo-β-lactamase; PMAβ, plasmide-mediated AmpC β-lactamase; ESAC, extended-spectrum AmpC cephalosporinase; CHDL, carbapenem-hydrolyzing class D β-lactamase,

4.2. β-lactamase active site and hydrolytic mechanisms

The four classes defined by molecular structure, A to D, have essential differences in their active sites, responsible for the different hydrolytic properties to destroy the lactam ring (Ambler *et al*, 1991): an active site serine in classes A, C and D and a zinc or another heavy metal in class B.

4.2.1. *Serine β-lactamases*

Ser-β-lactamases contain an active site consisting of a groove between the α/β domain and an all α domain, with a cavity on its floor (the oxyanion pocket), which have conformational flexibility in terms of substrate binding (Figure 8).

Compared to class A, class C enzymes possess a larger active centre, which conformational flexibility facilitates the hydrolysis of oxyimino-β-lactams by providing more space for water molecules to attack the acyl-enzyme intermediate (Crichlow *et al*, 1999). Class D enzymes share a practically perfect symmetry with class A for all amino acids that constitute the catalytic mechanism for acylation, such as Ser67, Lys70, Ser115 and Lys205 in OXA-10, and Ser70, Lys73, Ser130, and Lys234 in TEM-1 (Figure 8).

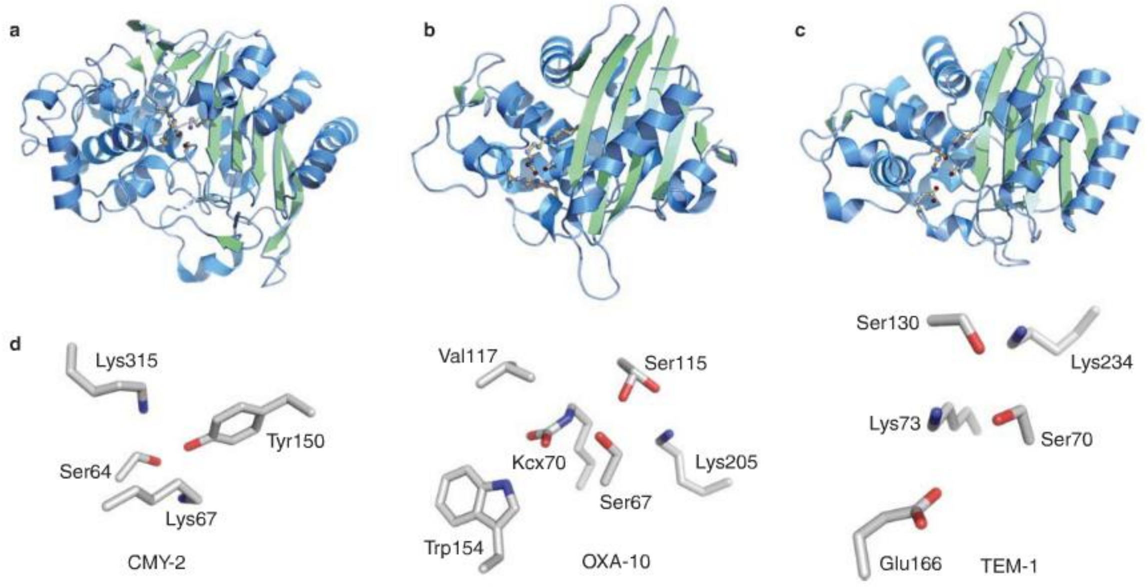


Figure 8 – Ser-β-lactamases structures: a) Class C (CMY-2); b) Class D (OXA-10) and c) Class A (TEM-1); the helices are represented in blue, strands in cyan and loops in grey. d) Representation of the most important amino acids in the respective active sites; for OXA-10, Kcx is the carboxylated Lys residue; oxygen atoms are represented in red, while nitrogen atoms are in blue (Bebrone *et al*, 2010).

All Ser-β-lactamases of known structure share the same mixed α/β fold (Ghuysen, 1991). These enzymes also feature four conserved motifs in the active site that are located in similar positions (Table 6):

(i) **Ser-Xaa-Xaa-Lys** - includes the active-site serine nucleophile; in class D β-lactamases, a carbamyl group (Figure 8) is present on the conserved lysine of this motif (Golemi *et al*, 2001; Schneider *et al*, 2009).

(ii) **Ser(Tyr)-Xaa-Asn(Val)** - the serine has been suggested to have a role in proton transfer during opening of the β-lactam ring and may act as a nucleophile in a secondary reaction with some β-lactams (Frère *et al*, 1999). Tyr has been suggested to act as a general base in both acylation and deacylation or only in promotion of the deacylation reaction (Dubus *et al*, 1994a).

(iii) **Lys-Thr(Ser)-Gly** - the Lys has been proposed to be involved in activation of Ser/Tyr from element 2, in substrate recognition (Dubus *et al*, 1994b).

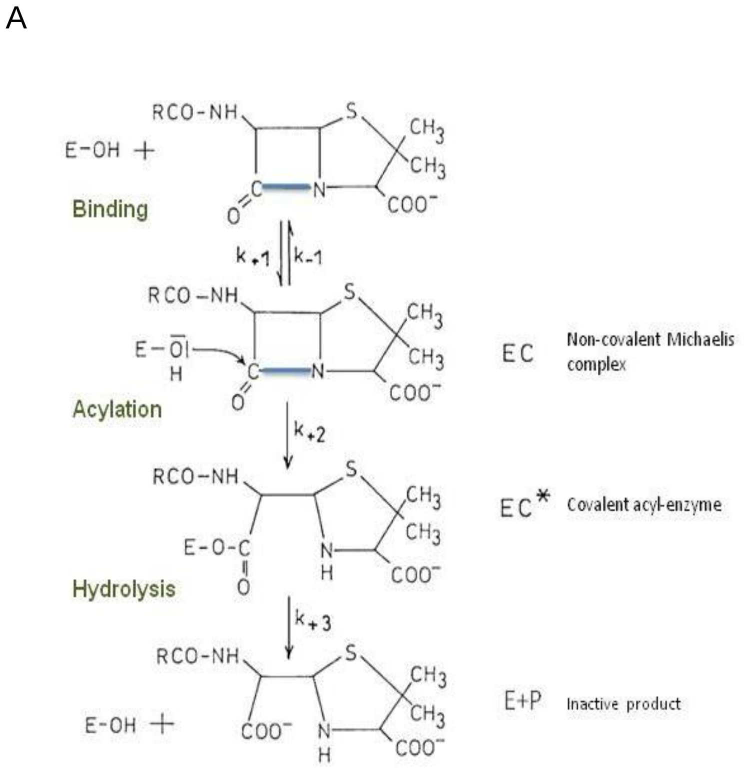
(iv) **Ω loop** - this conserved motif is present in class A and D (Strynadka *et al*, 1992); in class A includes the residues 163 to 178 and known to form a part of the active site, being implicated in the deacylation step by the Glu166 residue. In class D, the indole group of Trp154, carried by the omega loop, is essencial for the stability of Lys70 (first element) (Baurin, 2009). This motif is not evident in class C enzymes, but its function could be performed by the tyrosine of the second element.

Table 6 – Conserved motifs (elements) in the active site of Ser-β-lactamases.

Elements	***Class A***	***Class C***	***Class D***
1st	^{70}Ser-Xaa-Xaa-Lys	^{64}Ser-Xaa-Xaa-Lys	^{67}Ser-Xaa-Xaa-Lys
2nd	^{130}Ser-Asp-Asn	^{150}Tyr-Ala-Asn	^{115}Ser-Xaa-Val
3rd	^{234}Lys-Thr(Ser)-Gly	^{315}Lys-Thr-Gly	^{205}Lys-Thr(Ser)-Gly

4.2.1.1. Mechanism of hydrolysis of Ser-β-lactamases

The mechanism of hydrolysis mediated by Ser-β-lactamases and their respective steady state kinetic parameters are shown in Figure 9.



B

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{:}S \xrightarrow{k_2} E - S \xrightarrow{k_3,\ H_2O} E + P$$

C

$$K_m = k_3K_s/(k_2 + k_3)$$

$$k_{cat} = k_2k_3/(k_2 + k_3)$$

$$V_{max} = k_{cat}[E]$$

Figure 9 – Action of Ser-β-lactamases (Matagne *et al*, 1998). A: Amide bond of the β-lactam ring is represented in blue. B: In this scheme, E is a β-lactamase, S is a β-lactam substrate, E:S is the pre-acylation Michaelis complex, E - S is the acyl-enzyme, and P is the product of antibacterial activity. The rate constants for each step are represented by k_1 (association rate constant), k_{-1} (dissociation rate constant), k_2 (acylation rate constant), and k_3 (deacylation rate constant). C: Steady state kinetic parameters are also indicated (Copeland, 2005; Drawz & Bonomo, 2010; Galleni & Frere, 2007).

Ser-β-lactamases first associates noncovalently with the antibiotic to yield the reversible noncovalent Michaelis complex EC (k_1 and k_{-1}). The β-lactam ring is then attacked by the free hydroxyl on the side chain of a serine residue at the active site of the β-lactamase, yielding a covalent acyl ester EC* (k_2). Ser-β-lactamases are also able to efficiently hydrolyze the β-lactam (k_3), using strategically positioned water molecules (Minasov *et al*, 2002). This effectively leaves the β-lactam inactive (P) before it can reach the PBPs, and the β-lactamase is regenerated (E) and able to inactivate additional β-lactam molecules.

4.2.1.2. β-Lactamase inhibitors and pathway of inhibition by clavulanic acid

β-lactamase inhibition is used as a clinical strategy to combat the increasing problem of β-lactam resistance. The inhibitors do not have anti-bacterial activity themselves (Finlay *et al*, 2003); however, when co-administered with a β-lactam antibiotic, an inhibitor can restore the activity of the β-lactam by binding to a β-lactamase and inactivating it (Figure 10). Three β-lactamase inhibitors - tazobactam, clavulanic acid, and sulbactam - are currently in clinical use in five different β-lactam/β-lactamase inhibitor combinations: ampicillin/sulbactam, amoxicillin/clavulanic acid, cefoperazone /sulbactam, piperacillin/tazobactam and ticarcillin/clavulanic acid (Cantón *et al*, 2008a; Drawz & Bonomo, 2010; Rotschafer & Ostergaard, 1995).

Irreversible "suicide inhibitors", such as clavulanic acid, can permanently inactivate the β-lactamase in the periplasmatic space through different chemical reactions in the active site of the enzyme, making it possible that the associated antibiotic can reach its target (Figure 10). Although these inhibitors share structural similarity with β-lactam antibiotics, they differ in that they display a proficient leaving group (an enol ether oxygen) at position C1 of the second, 5-membered, ring (Figure 11). This leads to ring opening and enzyme modification through a pathway illustrated in Figure 11.

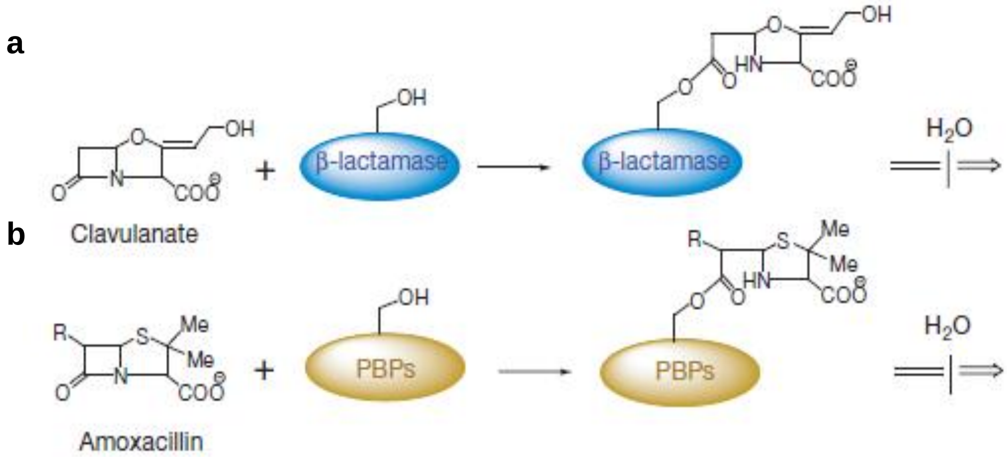


Figure 10 – Mechanism of action of clavulanate and amoxicillin. a) Combination of clavulanate with β-lactamase inactivates the enzyme. b) Combination of amoxicillin with PBPs blocks the cell wall-crosslinking transpeptidase; both form a slowly hydrolyzing covalent acyl-enzyme intermediate (Walsh, 2000)."Suicide inhibitors" form stable complexes between the bacterial β-lactamase and the β-lactamase inhibitor in a multistep chemical reaction.

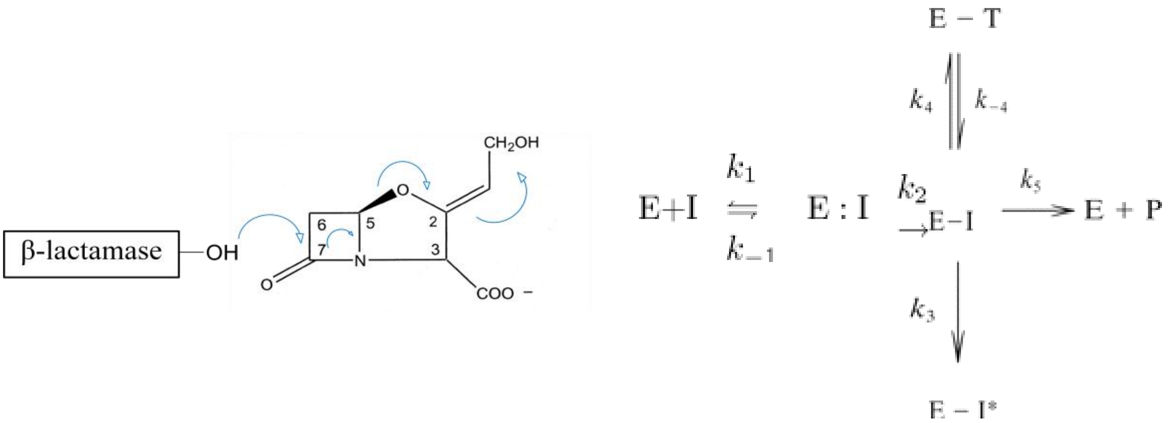


Figure 11 – Representation of the general mechanism of action of irreversible inhibitors. Inhibitors of class A β-lactamases undergo complex reaction schemes with multiple branch points after formation of the acyl-enzyme (E:I), namely the generation of a transiently inhibited enzyme (E–T), a permanent inactivated covalent acyl-enzyme (E–I*) and the regeneration of the active β-lactamase (E+P). I, inhibitor; E, enzymes (Bonomo & Rice, 1999; Drawz & Bonomo, 2010).

The reaction begins with formation of an acyl-enzyme (E:I) (Brown *et al*, 1996; Chen & Herzberg, 1992; Padayatti *et al*, 2005). After acylation, i.e. opening of the five-membered ring, the acyl-enzyme intermediate can:

(i) Undergo a reversible change that generates a transiently inhibited enzyme, a tautomer (E–T); the transient imine intermediate is likely the common intermediate preceding the chemical conversions that lead to transient enzyme inhibition (Imtiaz *et al*, 1993a; Kalp *et al*, 2009). The duration of transient inhibition is determined, in part, by the stability of the intermediate species (Kalp *et al*, 2007). After rearrangement to form enamine intermediates (Kalp *et al*, 2009; Padayatti *et al*, 2005), in either the trans or cis conformations (Chen & Herzberg, 1992), the reaction will ultimately proceed to deacylation or irreversible inactivation, depending on the properties of the enzyme and inhibitor;

(ii) lead to permanent inactivation as a covalent acyl-enzyme (E–I*);

(iii) in case of deacylation of the enamine intermediate (E+P), undergo decarboxylation and ester bond hydrolysis of clavulanic acid (P), regenerating the active β-lactamase (E), albeit very slowly.

Overall, these reactions are characterized by the **k_{inact}**, the rate of inactivation achieved with an "infinite" concentration of inactivator; by **K_i**, the concentration of inhibitor which yields an inactivation rate that is half the value of $k_{inact;}$ and by **IC_{50}**, the 50% inhibitory concentration, which measures the amount of inhibitor required to decrease enzyme activity to 50% of its uninhibited velocity; IC_{50} can reflect an inhibitor's affinity or k_{cat}/k_{inact} ratio, although these parameters are not always congruent.

The functional inhibition of the enzyme is determined by the relative rates (k_3, k_4, k_{-4}, and k_5) of each of these pathways and in particular by the formation of the E–I* species (Bush, 1988). Stabilizing or prolonging the acyl-enzyme intermediate (E-I) is central to successful inactivation of class A β-lactamases by the currently available inhibitors (Sulton *et al*, 2005).

4.2.1.3. Class A β-lactamases: the catalytically important residues

Although resistant mutants may appear before exposure to antibiotics (D'Costa *et al*, 2006; Lederberg & Lederberg, 1952), overuse and misuse of these drugs has placed tremendous selective pressure on bacteria, which has resulted in resistance to β-lactams, as well as to the β-lactamase inhibitors (e.g. clavulanic acid). This new resistance commonly arises from point mutations in existing enzymes, such as TEM-1 and SHV-1, altering one to five key amino acids (Bradford, 2001; Gniadkowski, 2008; Paterson & Bonomo, 2005).

Mutations leading to changes in substract specificity of class A β-lactamases include (Figure 12):

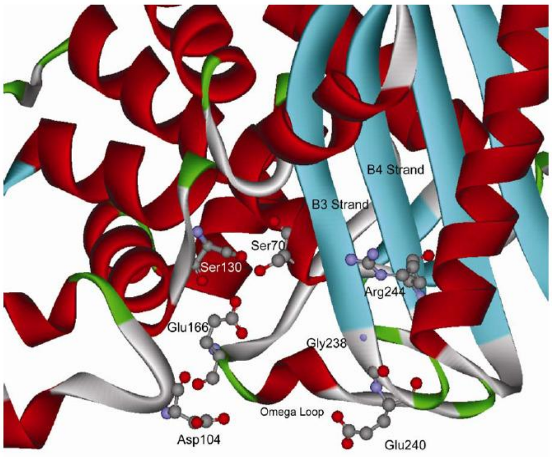


Figure 12 - The SHV β-lactamase active site pocket.

✓ **Met69** - Mutations at this position likely distort the oxyanion hole, an electron poor group of side chains that polarize the β-lactam oxygen for nucleophilic attack by Ser70 and, in the case of the inhibitors, by Ser130 as well (Knox, 1995). Hence, substitutions at position 69 are associated with an inhibitor-resistant phenotype, appearing either as a single substitution or in combination with additional amino acid replacements (http//www.lahey.org/studies/ webt.asp). Met69Leu, for example, appears to be associated with other amino acid substitutions conferring also an extended-spectrum phenotype (e.g., TEM-50 or CMT-1).

- ✓ **Ser70** - This is the catalytic residue of class A β-lactamases. Most mutants with substitutions at this position are inactive, and have not been identified in nature (Chen *et al*, 1996; Mazzella *et al*, 1991). The Ser70 flexibility could even have a role in allowing the carbapenems and cephamycins to readjust to accommodation of the α-substituent group during the initial Michaelis-Menten complex (Ke *et al*, 2007).

- ✓ **Lys73 and/or Glu166** - These residues are the catalytic base essential for activating the active site water molecule for both acylation and deacylation. A leading theory holds that Glu166 acts as the activating base of the hydrolytic water in deacylation (Strynadka *et al*, 1992); additional evidence suggests that this acylation scheme may be in competition with a mechanism where Lys73 acts as the general base to activate Ser70 (Meroueh *et al*, 2005).

- ✓ **Glu/Asp104** - Located on the left side of the active site pocket from TEM and SHV enzymes, respectively, this residue is important for recognition of substrates with large/complex R1 side-chains. The Glu104Lys substitution is one of the most common in the TEM family (Guo *et al*, 1999; Petit *et al*, 1995), having been found in 46 variants (http://www.lahey.org/Studies/). Certain substitutions in this position increase binding to extended-spectrum cephalosporins, enhancing the ESBL activity. At the time the present work started, this substitution had not yet been found in natural isolates of SHV (Bethel *et al*, 2006).

- ✓ **Ser130** - This residue has been implicated as the catalytic amino acid necessary for protonating the β-lactam nitrogen-leaving group, promoting the opening of the β-lactam ring during substrate hydrolysis (Atanasov *et al*, 2000; Lamotte-Brasseur *et al*, 1991). Ser130 mutants are resistant to β-lactamase inhibitors and have reduced affinity for substrates by preventing the final, cross-linking step in β-lactamase inhibition (Drawz & Bonomo, 2010)

- ✓ **Lys234 -** This residue stabilizes Ser130 through hydrogen bonding involving a water molecule (Matagne *et al*, 1998). Mutagenesis studies show that Lys234 is an important active-site residue involved in both ground state and transition-state binding (Ellerby *et al*, 1990; Lenfant *et al*, 1991). A recent study of clinical inhibitor-resistant SHV β-lactamases with Lys234Arg substitutions imply this residue in resistance to clavulanic acid changes in the position of Ser130 (see Section II: Results).

- ✓ **Gly238 and Glu240** - These residues are located adjacent to each other in the β3 strand, on the right side of the active site pocket; there is no residue 239 for alignment purposes among class A β-lactamases (Jacoby, 2006). Substitutions at residue Gly238 result in active site pocket enlargement, enabling the enzyme to hydrolyze oxyimino-cephalosporins. The Glu240 amino acid is assumed to be important for cephalosporin recognition. Mutations in both residues, alone or in combination, lead to an ESBL phenotype (Chaïbi *et al*, 1999; Knox, 1995). In general, the substitution at position 238 confers a large increase in resistance to cefotaxime, while the additional substitution at position 240 confers a large increase in resistance to ceftazidime (Du Bois *et al*, 1995; Page, 2008).

- ✓ **Arg244 -** Located in the B4 β-strand of the active site pocket, it is implicated in recognition of the C3 carboxylate of penicillins and inhibitors and the C4 carboxylate of cephalosporins. Substitutions at Arg244 in TEM result in an inhibitor-resistant phenotype (Chaïbi *et al*, 1999; Drawz & Bonomo, 2010). No substitution in this position has been found in natural isolates of SHV. However, mutagenesis of SHV at Arg244 revealed that the 244Leu and 244Ser substitutions also confer resistance to amoxicillin-clavulanic acid (Thomson *et al*, 2006).CTX-M enzymes do not contain the equivalent Arg244 residue (Bonnet, 2004). The Arg276 residues of CTX-M enzymes were predicted to be a “substitute” for these residue (Ibuka *et al*, 1999). Indeed, a recent study supports that the absence of a "Arg244 equivalent" is compensated by the positive charge at Arg276, a residue with a very flexible side chain that can present in multiple conformations (Bethel *et al*, 2011).

- ✓ **Arg275 and Asp276** - Similar to observations regarding Arg244, the variants Arg275 and Asn276, have been identified only in TEM enzymes. In these β-lactamases, amino acid 275 and 276 are located on the enzyme's C-terminal α-helix H11, which lies behind the β-sheet including Arg244. Substitutions at these residues disrupt local electrostatic interactions and displace the water molecule that is the key to inactivation by clavulanic acid (Chaïbi *et al*, 1999; Swarén *et al*, 1999).

4.2.2. *Metallo-β-lactamases*

Class B metallo-β-lactamases (MBLs) require a zinc or another type of heavy metal ion to hydrolyze β-lactam antibiotics, and their activity is inhibited by chelating agents like EDTA (Rasmussen & Bush, 1997). In general, MBLs use the -OH group from a water molecule, coordinated by Zn^{2+}, to hydrolyze the amide bond of a β-lactam (Garau *et al*, 2005; Wang *et al*, 1999; Xu *et al*, 2006). This method of attack and hydrolysis is unique among β-lactamases and is clinically critical, as the MBL does not form a stable or even a pseudo-stable covalent intermediate, i.e., it does not physically bind to the β-lactam substrate and therefore escapes the action of β-lactamase inhibitors such as clavulanic acid and sulbactam (Walsh *et al*, 2005).

Based on a combination of structural functions, zinc affinities for the two binding sites and hydrolysis characteristics, the MBLs are classified into three different groups (Rasmussen & Bush, 1997):

- ✓ subclass B1, which are fully active with either one or two Zn^{2+} ions (Figure 13a and d);
- ✓ subclass B2, which employ one Zn^{2+} ion and are inhibited by binding of an additional ion (Figure 13b and d);
- ✓ and subclass B3 , which require two Zn^{2+} ions (Figure 13c and d).

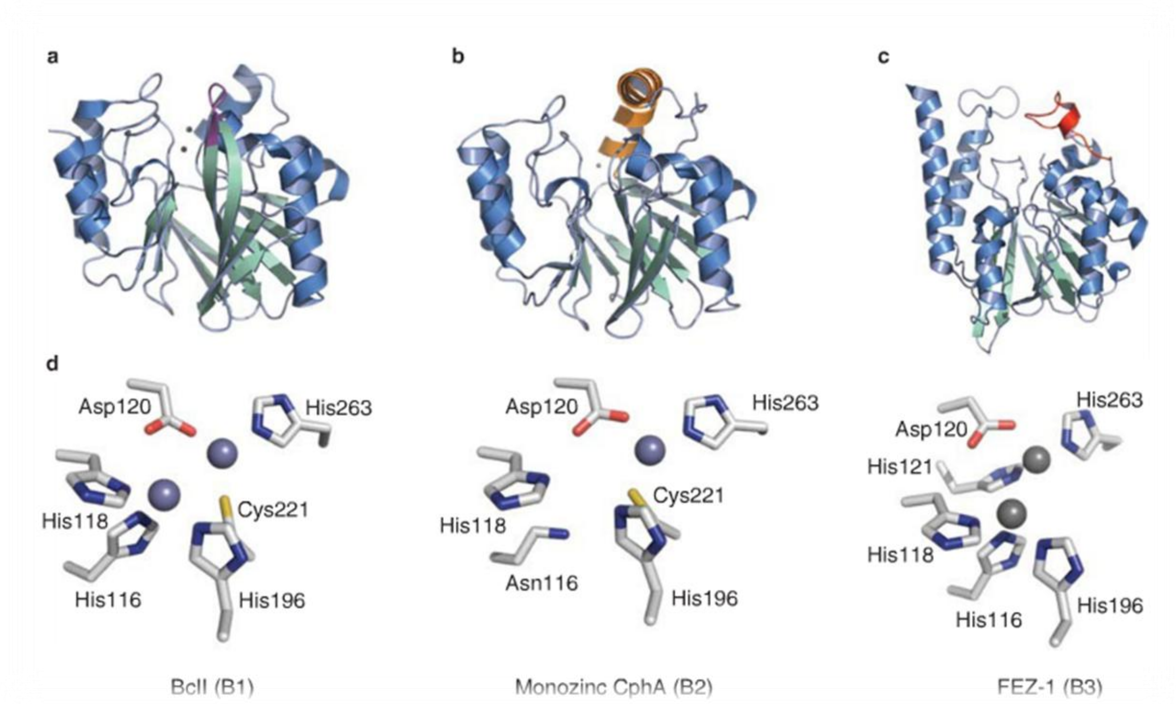


Figure 13 – Structures of B1 (BcII), B2 (CphA) and B3 (FEZ-1) MBLs subclasses. The helices are represented in blue, strands in cyan and loops in grey. Representation of the most important amino acid in the respective active site; Zinc binding sites are indicated by spheres; oxygen, nitrogen and sulphur atoms are represented in red, blue and yellow, respectively (Bebrone *et al*, 2010).

4.3. β-lactamase mediated-resistance

The first plasmid-mediated β-lactamase in Gram-negative bacteria was discovered in Greece in 1965 (Datta & Kontomichalou, 1965); it was named TEM due to the name of the patient from whom it was isolated (Temoniera). Subsequently, a closely related enzyme was discovered, in *P. aeruginosa*, and named TEM-2 (Bush *et al*, 1995). It was identical in its biochemical properties to the more common TEM-1, but differed by the single amino acid substitution Gln39Lys, resulting in a change in the isoelectric point of the enzyme. Meanwhile, another plasmid-mediated β-lactamase, known as SHV-1 (because sulfhydryl reagents had a variable effect on substrate specificity) and sharing 68% of its amino acids with TEM-1 (Kuzin *et al*, 1999), was found in *K. pneumoniae* and *E. coli* (Pitton, 1972). The SHV-1 β-lactamase is chromosomally encoded in the majority of isolates of *K. pneumoniae*, but it is usually plasmid mediated in *E. coli*. TEM-1, TEM-2 and SHV-1 hydrolyze penicillins and narrow spectrum cephalosporins, such as cefalotin or cefazolin.

At present, more than 860 β-lactamases are identified in important bacteria (http://www.lahey.org/Studies/). The most prevalent enzymes, and the most clinically important in Gram-negative bacteria, are represented in

Table 5. The role played by these enzymes in β-lactam resistance is determined by their substrate/inhibitor profiles. Resistance to third generation cephalosporins, for example, can have its origin in class A ESBLs (such as TEM or SHV derivatives and the CTX-M family), class D OXA ESBLs, class C AmpC β-lactamases, or in class A or B including carbapenem hydrolyzing β-lactamases (Bradford, 2001).

4.3.1. *Extended-spectrum β-lactamases*

The term ESBL, was first used for derivatives of TEM and SHV enzymes that were able to hydrolyze oxyimino-β-lactams and were inhibited by clavulanic acid (Bonnet, 2004; Bradford, 2001; Bush & Jacoby, 2010; Bush *et al*, 1995; Paterson & Bonomo, 2005). These all belonged to β-lactamase functional group 2be (Figure 5). Subsequently, the term has been expanded to include:

(i) enzymes with similar hydrolytic profiles and inhibitor susceptibility to those of TEM and SHV mutants, but with dissimilar evolutionary histories (e.g., the “new” ESBL CTX-M, and the “minor ESBL” VEB);

(ii) TEM and SHV mutants that have increased cephalosporinase activity and a greater ability to confer resistance, but fail to meet the criterion of hydrolyzing any individual oxyimino-cephalosporin at 10% or more of the rate for benzylpenicillin (e.g., TEM-7 and TEM-12), according to Bush & Jacoby classification (Bush & Jacoby, 2010);

(iii) β-lactamases conferring wider resistance to extended-spectrum cephalosporins than their parent types, such as those belonging to class C and D β-lactamases (group 1e and 2de), but not meeting the definition for group 2be, as they are inherently resistant to clavulanic acid;

(iv) some enzymes, particularly in the GES family, meeting the criteria for inclusion in class 2be, due to increased hydrolysis of oxyimino-β-lactams, but, in addition, being able to slowly hydrolyze carbapenems (included in group 2f).

For the purpose of this thesis, ESBLs are defined as those β-lactamases belonging to Ambler classes A or D and Bush groups 2be or 2de, respectively, as previously suggested (Paterson & Bonomo, 2005). Class C cephalosporinases (groups 1 and 1e) and class A and D carbapenemases (including those from groups 2f, 2df and 3a) will be discussed separately below (4.3.3 and 4.3.4, respectively).

The first ESBL (SHV-type) reported to cause the inactivation of oxyimino-β-lactams was reported in Germany, in 1983 (Knothe *et al*, 1983). Sequencing data showed that this β-lactamase differed from SHV-1 by a replacement of glycine with serine at position 238, which earned it the designation of SHV-2. In 1987, *K. pneumoniae* isolates collected in France three years prior were described as harboring a novel plasmid-mediated β-lactamase, which was named CTX-1, due to its enhanced activity against cefotaxime (Brun-Buisson *et al*, 1987; Sirot *et al*, 1987); this enzyme differed from TEM-2 by two amino acid substitutions, and it is now called TEM-3 (Sirot *et al*, 1987). However, as early as in 1982, in England, a *K. oxytoca* strain harboring a plasmid carrying *bla*$_{\text{TEM-E2}}$, a gene encoding ceftazidime resistance, has been isolated (Du Bois *et al*, 1995; Payne *et al*, 1990). Since 1995, reports on the emergence and rates of dissemination of ESBLs among different bacterial isolates have increased rapidly in most parts of the world (Bonnet, 2004; Bradford, 2001; Jacoby & Munoz-Price, 2005; Paterson & Bonomo, 2005).

Initially, resistance to oxyimino-cephalosporins in Gram-negative bacteria was mainly caused by the dissemination of TEM- and SHV-type ESBLs. During the last years, ESBLs from CTX-M family have become increasingly predominant (Bonnet, 2004; Bradford, 2001; Cantón & Coque, 2006; Hawkey & Jones, 2009; Livermore *et al*, 2007) (Figure 14).

CTX-M is a “new” class of β-lactamases (Jacoby & Munoz-Price, 2005), which are not very closely related to TEM or SHV β-lactamases, showing less than 40% sequence identity with these two enzymes (Tzouvelekis *et al*, 2000). CTX-M enzymes are much more active against cefotaxime than against ceftazidime and cefepime, contrasting with ESBLs from TEM and SHV families in this regard (Bonnet, 2004; Tzouvelekis *et al*, 2000). However, CTX-M enzymes harboring the

Asp240Gly substitution, namely CTX-M-15, the most prevalent worldwide, and CTX-M-16, exhibit an unusually high activity against ceftazidime (Bonnet *et al*, 2001; Poirel *et al*, 2002a).

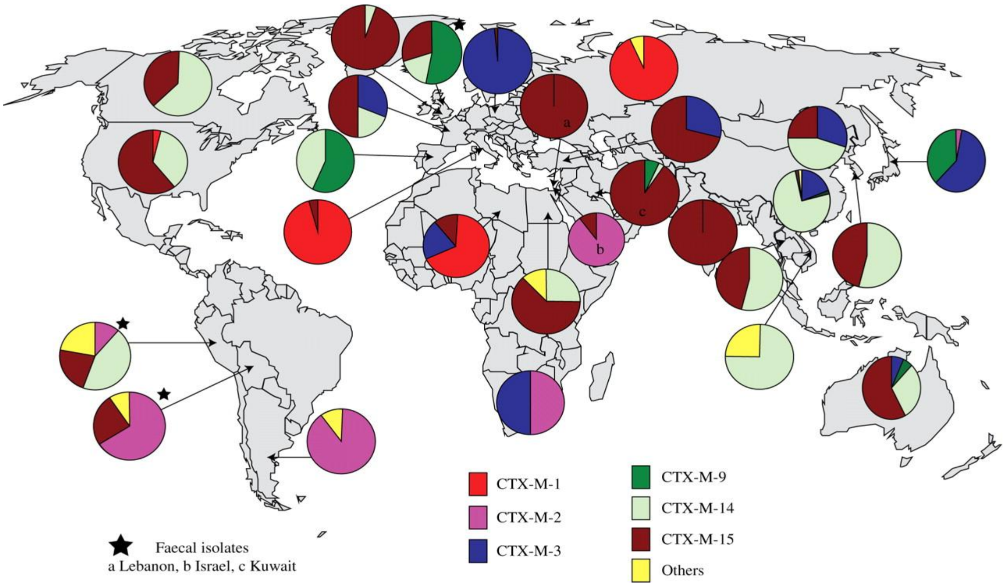


Figure 14 - Global distribution of CTX-M β-lactamases. Adapted from Hawkey & Jones (2009).

Different studies suggest that CTX-M-encoding genes have their origins in the chromosomes of different *Kluyvera* species, having subsequently spread among pathogenic and non-pathogenic relevant bacteria (Decousser *et al*, 2001; Humeniuk *et al*, 2002; Poirel *et al*, 2002b; Rodriguez *et al*, 2004). Firstly detected in Japan, in 1986, and designated as CTX-M-1 in 1989, in Germany (Bauernfeind *et al*, 1989; Matsumoto *et al*, 1988), the rapid growing of CTX-M family of β-lactamases now consists of over 110 variants (http://www.lahey.org/Studies). The CTX-M enzymes can be further subclassified into six groups, based on amino acid similarities and sequence homology: groups 1, 2, 8, 9, 25 and 45 (Bonnet, 2004; Rossolini *et al*, 2008).

CTX-M dissemination is changing the epidemiology of ESBLs and, in several settings, the CTX-M β-lactamases are now the most prevalent ESBLs encountered in *E. coli* and *K. pneumoniae*. Indeed, these enzymes are not restricted to the nosocomial setting, but also involves the community (Pitout *et al*, 2005).

Although the majority of Ambler class A ESBLs identified worldwide in family *Enterobacteriaceae* are mostly of the TEM, SHV, and CTX-M types, some “minor” ESBLs have been reported, such as the VEB, PER, GES, BES-1, SFO-1, and

TLA-1 enzymes (Naas *et al*, 2008). Among these, VEB, PER and GES variants are of particular interest due to their widespread dissemination. The VEB-1 ESBL was first identified in 1996, in an *E. coli* isolate from Vietnam and widespread to other species and in Southeast Asia. (Poirel *et al*, 1999). PER-1, now widespread in *Acinetobacter* spp. and *P. aeruginosa* and reported in various continents, was restricted to Turkish hospitals for years, after the first identification in a isolate of *P. aeruginosa*, in 1993 (Nordmann *et al*, 1993). The GES-1 β-lactamase was first detected in a *K. pneumoniae* isolate obtained in France, in 1998 (Poirel *et al*, 2000), from a child transferred from Cayenne, French Guiana. The *bla*$_{\text{GES-1}}$ gene was subsequently detected in *P. aeruginosa*, also in France (Dubois *et al*, 2002), and in *K. pneumoniae*, in Portugal (Duarte *et al*, 2003; Mendonça *et al*, 2009a).1. A worrying aspect is that some of these enzymes may extend their spectrum of hydrolysis to include carbapenems through single point mutations, as illustrated, for example, by GES-2, with Gly170Asn substitution (Poirel *et al*, 2001) and GES-7, with Ala126Leu substitution (Giakkoupi *et al*, 2000), being GES-2 also poorly inhibited by clavulanic acid.

4.3.2. *Inhibitor-resistant class A β-lactamases*

Since 1992, over thirty-five inhibitor resistant TEM (IRT) and eleven complex mutant TEM (CMT) enzymes were identified. However, only seven inhibitor-resistant mutants from the SHV β-lactamase family (IRS) have been detected clinically so far, mainly in *K. pneumoniae* isolates (Drawz & Bonomo, 2010; see also Section II: Results): SHV-10, SHV-26, SHV-49, SHV-56, SHV-72, SHV-84, SHV-107 (http://www.lahey.org/studies/webt.htm). The IRT and IRS enzymes belong to subgroup 2br of the Bush-Jacoby classification (Bush & Jacoby, 2010), being described as broad-spectrum β-lactamases that have acquired resistance to clavulanic acid ($IC_{50} \geq 1 \mu m$) and related inhibitors. Subgroup 2ber includes CMT enzymes that confer combined resistance to extended-spectrum cephalosporins and relative resistance to clavulanic acid inhibition. No complex mutant SHV has been encountered so far, which is surprising since SHV enzymes are usually more susceptible to inactivation by clavulanic acid than TEM enzymes, due to differences in the enzyme’s active sites (Bonomo & Rice, 1999; Drawz & Bonomo, 2010).

4.3.3. *Plasmid-mediated AmpC β-lactamases*

AmpC β-lactamases are a major group of clinically important enzymes, together with those from class A (Rice & Bonomo, 2000). These enzymes belong to class C according to the Ambler classification (Ambler *et al*, 1991) and to Bush-Jacoby group 1 and 1e, in terms of functional classification (Bush & Jacoby, 2010). AmpC enzymes, in contrast with ESBLs, hydrolyze both oxyimino- and 7-α-methoxy-cephalosporins (cefoxitin and cefotetan), and are not inhibited by β-lactamase inhibitors, such as clavulanic acid (Sanders, 1987). AmpC β-lactamases are usually either not active or very poorly active on aztreonam and on the zwitterionic oxyimino-cephalosporins (such as cefepime and cefpirome). AmpC-producing isolates are typically susceptible to carbapenems. However, resistance to these antibiotics can still be expressed by the production of an AmpC enzyme in combination with diminished porin expression (Bradford *et al*, 1997; Livermore, 1992; Stapleton *et al*, 1999).

AmpC enzymes are typically encoded on the chromosome of many species of Gram-negative bacteria including *C. freundii*, *S. marcescens, Enterobacter cloacae* and *P. aeruginosa*, where its expression is usually inducible. In organisms such as *E. coli* and *Shigella* spp., AmpC is also present but not inducible (Bergström *et al*, 1982; Honoré *et al*, 1986; Philippon *et al*, 2002).

The continuous use of 7-α-methoxy-cephalosporins and the clinical introduction of β-lactamase inhibitor combinations led to the emergence of plasmid-mediated AmpC β-lactamases (PMAβ) (Medeiros, 1997). Transmissible plasmids have acquired genes coding for AmpC enzymes, which consequently now also appear in bacteria either lacking or poorly expressing a chromosomal *ampC* gene, such as *E. coli, K. pneumoniae, Salmonella* spp. and *P. mirabilis*. Consequently, these important clinical pathogens are supplied with new and additional resistance phenotypes, once the expression of such PMAβ is identical to that of a derepressed chromosomally-encoded AmpC (Jacoby, 2009; Philippon *et al*, 2002; Rice & Bonomo, 2000; Walther-Rasmussen & Høiby, 2002). First described in 1989 (Bauernfeind *et al*, 1989), PMAβ descend from chromosomal *ampC* genes (*Hafnia alvei*, *Morganella morgannii*, *C. freundii*, *E. cloacae*, *Aeromonas hydrophila*, *Aeromonas caviae* and *Enterobacter asburiae*) falling into six phylogenetic groups (Table 7) (Jacoby, 2009; Pérez-Pérez & Hanson, 2002).

Table 7 - Chronology and homology of plasmid-mediated AmpC β-lactamases. Adapted from Jacoby (2009) and Pérez-Pérez & Hanson (2002).

AmpC β-lactamase	***Country of origin***	***Publication year***	***Species of first isolate***	***Likely source of AmpC gene***	***Similarity (%)***
CMY-1	South Korea	1989	*K. pneumoniae*	*A. hydrophila*	82
MOX-1	Japan	1993	*K. pneumoniae*	*A. hydrophila*	80
LAT-1	Greece	1993	*K. pneumoniae*	*C. freundii*	95
CMY-2	Greece	1996	*K. pneumoniae*	*C. freundii*	96
CFE-1	Japan	2004	*E. coli*	*C. freundii*	99
MIR-1	United States	1990	*K. pneumoniae*	*E. cloacae*	99
ACT-1	United States	1997	*K. pneumoniae*	*E. asburiae*	98
FOX-1	Argentina	1994	*K. pneumoniae*	*A. caviae*	99
DHA-1	Saudi Arabia	1997	*S. enteriditis*	*M. morgannii*	99
ACC-1	Germany	1999	*K. pneumoniae*	*H. alvei*	99

Just as amino acid substitutions, insertions and deletions in the parental TEM and SHV β-lactamases have given rise to ESBLs with broader substrate specificities, amino acid changes have also been described for AmpC β-lactamases, enhancing their catalytic efficiency toward oxyimino-β-lactam substrates, making them called extended-spectrum AmpC β-lactamases (ESAC) (Nordmann & Mammeri, 2007). Such changes have been described in both plasmid-determined and chromosomally mediated AmpC enzymes (Jacoby, 2009).

Infections caused by pathogenic PMAβ-producing Gram-negative bacteria have increased worldwide, with the majority being described in *K. pneumoniae* and *E. coli* (Jacoby, 2009; Walther-Rasmussen & Høiby, 2002). The identification of isolates containing AmpC-encoding plasmids is epidemiologically and clinically relevant due to the limitation of treatment options (Pai *et al*, 2004).

4.3.4. *The challenge of carbapenem resistance*

Carbapenems are considered “slow substrates” or even inhibitors of Ser-β-lactamases, as these structural differences contribute to undergo acylation by these antibiotics, and to resist to the rapid hydrolysis of the Ser-acyl enzyme intermediate, yielding a ring-opened inactive drug (De Pascale & Wright, 2010). Indeed, carbapenems are often drugs of last resort against ESBL- and PMAβ-producing organisms, which are also increasingly resistant to quinolones, aminoglycosides, trimethoprim-sulfamethoxazole and other class of antibiotics. However, the introduction of carbapenems in antibiotic chemotherapy resulted in the emergence of carbapenem-hydrolyzing β-lactamases (carbapenemases), first in *P. aeruginosa* and *Acinetobacter* spp., later in *Enterobacteriaceae* (Queenan & Bush, 2007), and presently in nearly every Gram-negative pathogenic species. These enzymes are often responsible for therapeutic failure, because they mediate multidrug-resistance. Table 5 (Chapter 4.1) outlines the common carbapenemases produced by pathogenic bacteria.

These enzymes have been described in all four Ambler classes of β-lactamases, but the epidemiologically most relevant carbapenemases fall into three: A, B, and D classes, according to Ambler (Ambler *et al*, 1991) and into 2f, 2df, 3a and 3b, according to Bush and Jacoby (Bush & Jacoby, 2010; Queenan & Bush, 2007; Walsh, 2008; Walsh, 2010). In class C (group 1e), the best example is CMY-10, which have hydrolytic activity towards imipenem (Kim *et al*, 2006).

Class A carbapenemases can be broadly divided into four major groups based mainly on phylogeny: GES, KPC, SME, and the chromosomally encoded IMI/NMC-A; these groups share amino acid sequence identities ranging from 32% to 70% (Walther-Rasmussen & Høiby, 2007). Imipenem and cefoxitin induce the expression of chromosomal carbapenemases, conferring a unique susceptibility profile characterized by resistance to carbapenems, penicillins, and aztreonam but susceptibility to oxyimino-cephalosporins. These enzymes are more susceptible to the inhibition by clavulanic acid, than sulbactam (Mariotte-Boyer *et al*, 1996).

Class A KPC enzymes are currently the most common acquired carbapenemases, having been first detected in North Carolina, USA, in 1996 and having since spread worldwide (Nordmann *et al*, 2009). These enzymes are generally broadly active against all β-lactams despite the fact that they may appear susceptible to some carbapenems, by phenotypic methods.

Class B carbapenemases (MBLs), have been reported in several bacteria including *P. aeruginosa* and *Acinetobacter* spp. species. Zinc β-lactamases have a wide spectrum of activity against carbapenems, penicillins and oxyimino-cephalosporins, but not aztreonam, including also resistance to β-lactamase inhibitors (Walsh *et al*, 2005). The emergence of MBL-encoding genes is worrisome, since they are usually carried by highly mobile genetic structures. The first report of an acquired MBL (IMP-1) was in an isolate of *S. marcescens,* in 1991, in Japan (Osano *et al*, 1994), where it is now also scattered in strains of *P. aeruginosa*. Currently, the most prevalent and widespread types of acquired MBLs are the IMP- and VIM-type enzymes, of which about 60 variants are known (IMP-1 to IMP-30 and VIM-1 to VIM-30). However, four additional types of acquired MBLs (SPM-1, GIM-1, DIM-1, and SIM-1) have recently been identified (Lee *et al*, 2005; Poirel *et al*, 2010b; Walsh *et al*, 2005).

The most significant development in the last few years is the emergence of MBLs in *Enterobacteriaceae*. From a global perspective, the two regions that are particularly affected are the Mediterranean basin (particularly Greece and Turkey) and the South Asian subcontinent, the former owing to the emergence and spread of VIM-1 and VIM-4 (Walsh, 2010). Recently, a new MBL emerged out of India and Pakistan; NDM-1, isolated first from a Swedish patient previously hospitalized in New Delhi, in 2007, has now been identified mostly in *E. coli* and *K. pneumoniae* and, to a lesser extent, in other *Enterobacteriaceae* species (Kumarasamy *et al*, 2010; Nordmann *et al*, 2011; Yong *et al*, 2009). Furthermore, the $bla_{\text{NDM-1}}$ gene has been identified in isolates that also carry resistance to macrolides, aminoglycosides, rifampicin, sulfamethoxazole and aztreonam, contributing to their multidrug-resistance patterns. The highly resistant phenotype, coupled with the increased prevalence of these enzymes among clinical isolates makes them a major concern for public health (Grundmann *et al*, 2010; Nordmann *et al*, 2011; Walsh, 2005).

Carbapenem-hydrolyzing class D β-lactamases (CHDLs) are mostly found in *Acinetobacter* spp., although there are reports on *Enterobacteriaceae*, in particular OXA-48-producing *K. pneumoniae* that hydrolyze carbapenems and penicillins, but not oxyimino-cephalosporins. CHDLs can be divided into the following clusters, based on sequence homology: OXA-23, OXA-24, OXA-51 and OXA-58 (Poirel *et al*, 2010a). Other minor groups, such as OXA-72 and OXA-143, are now emerging and increasing in clinical importance (Poirel *et al*, 2010a); Section II: Results). CHDLs hydrolyze carbapenems poorly, but are able to confer resistance and are partially inhibited by clavulanic acid.

The emergence and global spread of carbapenemase-producing isolates is of great concern to health services worldwide (Grundmann *et al*, 2010; Struelens *et al*, 2010). Although current criteria to screen for these enzymes and the methods for their confirmation are useful, the ability to limit the spread of these pathogens will require effective laboratory screening methods to rapidly identify patients infected with these organisms (Miriagou *et al*, 2010).

SECTION II. RESULTS

Chapter 1. β-lactamases: impact in the antibiotic resistance, dissemination and co-resistance

Paper I. Emergence of β-lactamase-mediated resistance to oxyimino-β-lactams in *Enterobacteriaceae* isolates in various services in a single centre: risk factors and contribution of the newly detected CTX-M-3 variant in Portugal

This chapter was submitted as:

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Abstract

We studied 220 *Enterobacteriaceae* isolates (130 *Escherichia coli*, 84 *Klebsiella pneumoniae* and 6 *Proteus mirabilis*) recovered in various healthcare settings from a tertiary-level Portuguese Hospital. All but one of the 27 isolates collected in the first period (year 1999) showed susceptibility to all oxyimino-β-lactams tested due to the expression of non-extended-spectrum β-lactamases (ESBLs); the 26 isolates carried *bla*$_{\text{TEM-1}}$ or *bla*$_{\text{SHV-1}}$ genes, and the exception carried a plasmid-mediated AmpC β-lactamase (PMAβ), encoded by the *bla*$_{\text{CMY-2-type}}$ gene. All the 193 isolates collected in the second period (2004-2008) presented diminished susceptibility to at least one oxyimino-cephalosporin and 97.6% to aztreonam. In 1999, there were no isolates expressing ESBL, whereas in 2004 there were eight and in 2008 there were 58, mainly CTX-M family enzymes (*bla*$_{\text{CTX-M-1, -3, -9, -14, -15, -32}}$); in 2008 there were also two PMAβ enzymes (*bla*$_{\text{CMY-2-type}}$ and *bla*$_{\text{DHA-1-type}}$). CTX-M-3 producers, detected in Portugal for the first time, emerged in *E. coli* and *K. pneumoniae* in 2006, and were associated with a microepidemic in 2007 in *K. pneumoniae* clones, mostly in internal medicine wards; genetic relatedness analysis indicated that they formed a cluster (KpII). CTX-M-15-producing isolates clustered mainly into cluster EcI in *E. coli*, and cluster KpI in *K. pneumoniae*. Production of CTX-M-type enzymes, age ≥65 years and nosocomial infection were risk factors in this hospital for higher nonsusceptibility associated with β-lactamase-mediated resistance to oxyimino-β-lactams in *Enterobacteriaceae* isolates; additionally, 38.3% of the CTX-M-producing isolates were from cases of community-acquired infection, showing that CTX-M-3 and CTX-M-15 clones are crossing the hospital-community barrier.

Keywords. Oxyimino-β-lactams, *Enterobacteriaceae*, Portugal, ESBL, CTX-M-3, CTX-M-15

1. Introduction

The most common cause of bacterial resistance to β-lactam antibiotics is the production of β-lactamases. Some groups of these enzymes, such as the extended-spectrum β-lactamases (ESBLs) and plasmid-mediated AmpC β-lactamases (PMAβ), are of particular significance in the failure of treatment for infections [1, 2, 3].

The prevalence and geographic spread of CTX-M enzymes has increased rapidly, and these enzymes are currently the main cause of resistance to extended-spectrum cephalosporins [4, 5, 6, 7]. First detected in 1986 [8], CTX-M are typical Ambler class A ESBL and 2be Bush group members [9, 10]. More than 100 different enzymes this family of β-lactamases have been reported (http://www.lahey.org/studies/webt.htm), classified into five major groups, typified by CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25 [4]. β-lactamases of CTX-M family were first described in the Iberian Peninsula in 2000, with the identification of isolates of *Escherichia coli* producing CTX-M-9 recovered between 1996-1998 [11, 12]. In Portugal, the first isolate carrying a CTX-M enzyme, CTX-M-14, was collected from a healthy subject in 2003 [13], but CTX-M-15 has been described as being the most prevalent [14, 15].

A recent study described the acquisition of the PMAβ $\textit{bla}_{\text{CMY-2}}$ by an isolate belonging to the multidrug-resistance (MDR) OXA-30-producing *Salmonella* Typhimurium clone; this clone has been present for several years in Portugal, Spain and the UK and is a cause of foodborne infections [16]. However, the dissemination of PMAβ in Portuguese hospitals and health institutions, as well as its association with ESBL production, has not previously been reported.

The aim of this study was to characterize the spread of resistance mechanisms to extended-spectrum β-lactam antibiotics in *Enterobacteriaceae* isolates in one Portuguese hospital providing diverse healthcare facilities, during two different periods, and assess the risk factors. The contribution of the newly detected CTX-M-3 variant in Portugal is also evaluated.

2. Materials and methods

2.1. Bacterial strain collection

Over two periods, 220 *Enterobacteriaceae* isolates were collected consecutively at Hospital Garcia de Orta, in Almada, Portugal. It is a 600-bed tertiary-level hospital (including a 28-bed intensive care unit), with a catchment population of about 390,000 citizens. All isolates identified between the first semesters of 2004 and 2008 by the hospital laboratory as producing ESBL (n=193, including 109 *Escherichia coli*, 81 *Klebsiella pneumoniae* and 3 *Proteus mirabilis*) were sent to the National Reference Laboratory of Antimicrobial Resistances at the National Institute of Health, in Lisbon. Twenty-seven amoxicillin-resistant *Enterobacteriaceae* isolates (21 *E. coli*, 3 *K. pneumoniae* and 3 *P. mirabilis*), also collected consecutively in the same hospital between January and June of 1999, were included for comparison. The origin of infection (nosocomial or community acquired) was identified according to Centers of Disease Control and Prevention criteria [17].

2.2. Antimicrobial susceptibility testing

A disk diffusion method was used to test the antimicrobial susceptibility of clinical isolates and the results were interpreted according to the clinical breakpoints of the AntibioGram Committee of the French Society for Microbiology (CA-SFM; http://www.sfm-microbiologie.org/). An isolate was scored multidrug-resistant (MDR) if it had reduced susceptibility to three or more structurally unrelated antibiotics. Minimal inhibitory concentrations (MICs) for transformants and their clinical isolates were determined by a microdilution method and the results were also interpreted according to the same guidelines; MICs of ticarcillin, cefalotin, cefepime, cefoxitin, imipenem, meropenem, ciprofloxacin, gentamicin and trimethoprim were tested alone, and MICs of amoxicillin, cefotaxime and ceftazidime were determined alone and in combination with 2 mg/L clavulanic acid.

2.3. Screening of β-lactamases

Clinical isolates expressing resistance to amoxicillin and nonsusceptibility to one or more extended-spectrum β-lactams (cefotaxime, ceftazidime and/or ceftriaxone) but for which clavulanic acid showed synergy were inferred to produce ESBLs. All isolates, which showed nonsusceptibility to cefotaxime, were presumed to produce ESBL of the CTX-M family and were subjected to PCR testing for the presence of *bla*$_{\text{CTX-M}}$ genes [15]. All isolates susceptible to cefotaxime, and those for which the PCR test was negative, but which hydrolyzed oxyimino-β-lactams and/or cefoxitin, were screened for the presence of non-CTX-M-ESBL genes, in particular *bla*$_{\text{GES}}$, *bla*$_{\text{OXA}}$, *bla*$_{\text{SHV}}$ and *bla*$_{\text{TEM}}$, and for PMAβ genes, using specific primers as previously described [15, 18]. All genes amplified by PCR, detected in clinical isolates and transformants, were purified with ExoSAP IT, and sequenced directly on both strands as previously described [15]. The presence of β-lactamases in all non-CTX-M-positive isolates, other than those tested for by PCR, was excluded by isoelectric focusing (IEF) as previously described [19].

2.4. Transfer of resistance

We tested whether the ESBL phenotypes of isolates producing CTX-M-1, CTX-M-3, CTX-M-9, CTX-M-14, CTX-M-15, and CTX-M-32 enzymes were transferable. Direct transfer of the β-lactam resistance phenotype was attempted by liquid and solid mating assays at 37°C using nalidixic acid, rifampicin and/or streptomycin-resistant *E. coli* K12 C600 as the recipient strain, according to the antibiotic susceptibilities of the clinical isolate used as donor. Transconjugants were selected on MacConkey agar plates containing 100 mg/l of nalidixic acid, 100 mg/l of rifampicin or 250 mg/l of streptomycin plus 5 mg/l of cefotaxime. Transformants were subjected to MIC testing, and PCR and DNA sequencing of *bla* genes as described above.

2.5. PFGE

The clonal relationship between 49 *E. coli* and *K. pneumoniae* isolates representative of the different CTX-M β-lactamase groups in this study was studied by pulsed-field gel electrophoresis (PFGE) as previously described [14]. *Bionumerics* version 3.5 cluster analysis (Applied Maths, Sint-Martens-Latem, Belgium) was used for genomic analysis. Dice coefficients and the unweighted pair group method (UPGMA) with the arithmetic averages clustering method were used for analysis of grouping. Isolates with a Dice band-based similarity coefficient value of ≥80% were considered to belong to the same cluster.

2.6. Statistical analysis

OpenEpi software, version 2.3.1 [20], was used for statistical analysis. Fisher exact test was used to assess differences in antibiotic resistance between different groups. Two-sided *P* values of ≤ 0.05 were considered to be statistically significant. Associations were determined by calculation of odds ratios (OR) with 95% confidence intervals (CI). The null hypothesis was rejected for P values of ≤ 0.05.

3. Results

3.1. Clinical isolates and patient characteristics

One hundred and sixteen of the 220 isolates (52.7%) were identified as nosocomial, 92 (41.8%) were community acquired, and 12 (5.4%) were of unknown origin; 146 isolates were isolated from urine (66.4%), 32 from blood (14.5%), 19 from wounds (8.6%), 19 from other biological samples (8.6%), and four were of unknown origin (1.8%). Of the 121/220 (55%) isolates from women, 69 (57.0%) were from patients over 65 years old, 25 (20.7%) from patients between 41 and 64 years old, 21 (17.3%) from patients between 19 and 40 years old and 6 (5.0%) from under 18 years old. Of the 96/220 (43.6%) isolates from men, 57 (59.4%) were from patients over 65 years old, 21 (21.9%) from patients between 41 and 64 years old, 8 (8.3%) from patients between 19 and 40 years old and 10 (10.4%) from under 18 years old. Information regarding the age and sex of the patient was lacking for three isolates. As concerns the wards, 68/220 (30.9%) isolates were collected in the internal medicine service; 41/220 (18.6%) were from outpatients (emergency room or ambulatory), 20/220 (9.1%) from surgery, 14/220 (6.4%) from the intensive care unit, 11/220 (5.0%) from observation, 9/220 (4.1%) from hematology/oncology, 8/220 (3.6%) from nephrology, 7/220 (3.2%) from obstetrics/gynecology, 6/220 (2.7%) from neurology, 6/220 (2.7%) from urology, 5/220 (2.3%) from gastroenterology, and 25/220 (11.4%) were isolated in other hospital wards (Table 1).

3.2. Antimicrobial susceptibility of isolates

Data on antimicrobial susceptibility for all the 220 isolates are provided in Table 2. All but one of the isolates (a PMAβ-producer) from the first period of the study showed susceptibility to all oxyimino-cephalosporins tested and to aztreonam. All *Enterobacteriaceae* isolated during the second period (including PMAβ-producers) presented diminished susceptibility to at least one oxyimino-cephalosporin and 97.6% to aztreonam. All isolates from both periods were susceptible to carbapenems.

In this set of strains, 64.5% (142/220) were MDR, and 95.1% of these were nonsusceptible to at least one oxyimino-cephalosporin and 90.8% to aztreonam. Ninety-two percent of the MDR isolates were nonsusceptible to fluoroquinolones (ciprofloxacin and/or norfloxacin) and to aminoglycosides (gentamicin and/or amikacin), and 73.2% to SXT. Forty-six of the 142 MDR isolates (32.4%) were from the community and 89 (62.7%) were nosocomial; and information about the site of infection was not available for seven.

3.3. Non-ESBL β-lactamase genes and antimicrobial resistance

Fifty clinical isolates (22.7%), 26 from 1999 and 24 from 2004-2008, were not ESBL producers (excluding PMAβ), and expressed *bla*$_{\text{SHV-1}}$ (n=20), *bla*$_{\text{SHV-11}}$ (n=1), *bla*$_{\text{SHV-77}}$ (n=1), *bla*$_{\text{SHV-83}}$ (n=1), *bla*$_{\text{TEM-1}}$ (n=18), *bla*$_{\text{OXA-30}}$ (n=1) and *bla*$_{\text{TEM-1}}$ plus *bla*$_{\text{OXA-30}}$ (n=2) genes (Table 1). No β-lactamase genes were found in six isolates. The presence of other β-lactamase genes was excluded by IEF (data not shown).

In this group of non-ESBL producers, nonsusceptibility to amoxicillin plus clavulanic acid was more prevalent in the period 2004-2008 than in 1999 (87.5% and 34.6%, respectively); the same was true for non susceptibility to piperacillin plus tazobactam (66.7% and 7.7%, respectively), ciprofloxacin (20.8% and 7.7%, respectively) and gentamicin (20.8% and 3.8%, respectively) (Table 2). Nonsusceptibility to oxyimino-cephalosporins in non-ESBL-producing isolates was only found during the second period, and ceftazidime nonsusceptibility was more frequent (91.7%) than cefotaxime nonsusceptibility (25.0%).

Three non-ESBL PMAβ-producing isolates were identified: two *E. coli* expressing the *bla*$_{\text{CMY-2-type}}$ gene and one *K. pneumoniae* expressing the *bla*$_{\text{DHA-1-type}}$ gene. PMAβ-producing isolates were nonsusceptible to almost all β-lactams tested, except the combination of piperacillin plus tazobactam, cefepime, and carbapenems. Regarding oxyimino-β-lactams, the three isolates were resistant to ceftazidime, but only one of them was nonsusceptible to cefotaxime and two to aztreonam. The PMAβ-producing group showed a higher percentage of resistance than the other groups to cefoxitin (100% and ≤4.5%, respectively).

3.4. ESBL β-lactamase genes and antimicrobial resistance

Overall, the presence of ESBLs was confirmed in 167 of 193 (86.5%) isolates from second period (102 *E*. *coli*, 62 *K*. *pneumoniae* and three *P*. *mirabilis*) nonsusceptible to penicillins, cephalothin and one or more oxyimino-β-lactams (Table 2). There were 171 isolates nonsusceptible to cefotaxime, and the *bla*$_{\text{CTX-M}}$ genes were detected in 154 (90.1%) of them, all from the second period; 95.5% (147/154) of these isolates were nonsusceptible also to ceftazidime. Sequencing identified *bla*$_{\text{CTX-M-1}}$ in six (3.9%), *bla*$_{\text{CTX-M-3}}$ in 13 (8.4%), *bla*$_{\text{CTX-M-9}}$ in two (1.3%), *bla*$_{\text{CTX-M-14}}$ in four (2.6%), *bla*$_{\text{CTX-M-15}}$ in 123 (79.9%) and *bla*$_{\text{CTX-M-32}}$ in six (3.9%) isolates. Among MDR isolates, 82.4% belonged to the CTX-M-group (73 *E. coli*, 42 *K. pneumoniae* and 2 *P. mirabilis*).

In the non-CTX-M-ESBL group (n=13), we detected β-lactamase genes of the SHV (*bla*$_{\text{SHV-2a}}$, n=3, *bla*$_{\text{SHV-12}}$, n=7, *bla*$_{\text{SHV-115}}$, n=1), TEM (*bla*$_{\text{TEM-10}}$, n=1, *bla*$_{\text{TEM-52}}$, n=1, *bla*$_{\text{TEM-107}}$, n=1) and GES (*bla*$_{\text{GES-1}}$, n=1) families, alone, or associated with each other, with non-ESBL genes (*bla*$_{\text{SHV-11}}$, *bla*$_{\text{TEM-1}}$ and *bla*$_{\text{OXA-30}}$) or with inhibitor-resistant SHV genes (*bla*$_{\text{SHV-26}}$) (Table 1). All isolates of this group were nonsusceptible to ceftazidime, but only 76.9% were nonsusceptible to cefotaxime (Table 2).

Table 1 - Distribution of 220 β-lactamase-producing *Enterobacteriaceae* isolates collected in a single hospital: by ward, year (1999 and 2004-2008) and PFGE profile (49/220).

Hospital Ward	PFGE Profile	β-lactamase by year (nº. of isolates)						Total no. of isolates
		Y 1999	Y 2004	Y 2005	Y 2006	Y 2007	Y 2008	
Gastroenterology	Ec0010(1)					KpCTX-M-15(1)	EcCTX-M-15(3) KpSHV-1(1)	5
Hematology/ Oncology	Ec0010(1), Kp0001(1)	EcTEM-1(2) KpSHV-77(1)		KpCTX-M-15(1)	EcCTX-M-15(1) KpCTX-M-15(1)	KpCTX-M-15(1)	EcCTX-M-15(2)	9
Intensive care unit	Ec0010(1), Kp0006(1)	EcTEM-1(1)		EcCTX-M-15(1) KpCTX-M-15(1) KpSHV-1(1)	EcSHV-2(1) KpCTX-M-3(1) KpCTX-M-15(1)	EcCTX-M-15(1)	EcCTX-M-15(2) KpCTX-M-15(3) KpSHV-1(1)	14
Internal medicine	Ec0002(1), Ec0003(1), Ec0006(1), Ec0010(1), Ec0011(1), Ec0013(1), Ec0017(1), Kp0001(4), Kp0002(1), Kp0003(1), Kp0006(8)	EcCMY-2-type+OXA-30(1)		EcCTX-M-32(1) KpCTX-M-15(1)	KpCTX-M-15(2) KpTEM-1+SHV-2 (1)	Ec (1) EcCTX-M-1(1) EcCTX-M-3(2) EcCTX-M-15(14) EcCTX-M-32(2) EcSHV-12(1) EcTEM-1+OXA-30(1) EcTEM-1+CMY-2-type(1) KpCTX-M-3(9) KpCTX-M-15(18) KpSHV-11(1) PmCTX-M-15(1) PmCTX-M-32(1)	EcCTX-M-14(1) EcCTX-M-15(8)	68
Nephrology	Ec0007(1), Ec0018(1), Kp0007(1)		EcCTX-M-15(1)	Ec (1) EcTEM-52(1)	EcCTX-M-15(1)		EcCTX-M-1(1) EcCTX-M-15(1) EcGES-1+TEM-1+TEM-107(1) KpCTX-M-1(1)	8
Neurology		EcTEM-1(1) Pm (1)		EcCTX-M-15(1)	KpCTX-M-15(1)		EcCTX-M-15(1) KpOXA-30+DHA-1-type(1)	6

Observation	Ec0015(1), Kp0004(1)	Ec (1)			EcCTX-M-1(1) EcCTX-M-15(6) KpCTX-M-15(1) KpTEM-1+SHV-12(1)		KpCTX-M-15(1)	11
Obstetrics/ Gynecology		EcTEM-1(3) Ec (1)				KpSHV-1(1)	KpSHV-1(2)	7
Surgery	Ec0001(1), Ec0010(1), Ec0013(1), Ec0014(1), Kp0005(1)	Ec (1) EcTEM-1(1) KpSHV-1(1) KpSHV-83(1)		EcCTX-M-1(1) EcCTX-M-15(1) EcCTX-M-32(1) KpCTX-M-15(1)	EcCTX-M-15(1) KpCTX-M-15(1)	KpCTX-M-9(1)	EcCTX-M-15(4) KpCTX-M-15(1) KpSHV-1(4)	20
Urology	Ec0010(1), Ec0012(1), Ec0016(1)		EcCTX-M-14(1)	EcCTX-M-15(1)	KpTEM-1+OXA-30(1) KpCTX-M-15(1)		EcCTX-M-1(1) EcCTX-M-15(1)	6
Outpatient	Ec0004(1), Ec0009(1) , Ec0010(1), Kp0001(1)	EcOXA-30(1) EcSHV-1(3) EcTEM-1(5) PmTEM-1(1)	EcCTX-M-15(1) KpTEM-1+SHV-12(1)	EcCTX-M-15(3) EcTEM-1(1)	EcSHV-12+OXA-30(1) KpTEM-1+SHV-12(1)	EcTEM-1(1) KpCTX-M-15(3)	EcCTX-M-9(1) EcCTX-M-14(1) EcCTX-M-15(13) KpCTX-M-15(1) KpSHV-1(2) KpTEM-1+SHV-2+ SHV-115(1)	41
Other Ward	Ec0005(1), Ec0006(1), Ec0008(1), Ec0010(2), Kp0001(1)	PmTEM-1(1)	EcCTX-M-15(2) KpCTX-M-15(1) KpTEM-1+SHV-12 +SHV-11(1)	EcCTX-M-15(1) KpSHV-1(1)	EcCTX-M-3(1) EcCTX-M-15(2)	EcCTX-M-15(1) EcTEM-1(1) KpSHV-1(1) KpTEM-10+SHV-26(1)	EcCTX-M-14(1) EcCTX-M-15(5) EcCTX-M-32(1) KpSHV-1(2) KpSHV-12(1) PmCTX-M-15(1)	25

* Isolates were non β-lactamase-producers.

Y, year; Ec, β-lactamase-producing E. *coli*; Kp, β-lactamase-producing *K. pneumoniae*; Pm, β-lactamase-producing *P. mirabilis*.

Table 2 - Antimicrobial susceptibility of the 220 *Enterobacteriaceae* ESBL-, non-ESBL-, and/or PMAβ-producing isolates, collected in two periods (1999 and 2004-2008)

Antibiotic (Disk content, μg)	Year 1999		Years 2004-2008										Years 1999 and 2004-2008	
	non-ESBL[*] (n=26)		non-ESBL[*] (n=24)		CTX-M (n=154)		Non-CTX-M-ESBL[†] (n=13)		Total ESBL (n=167)		Total (n=191)		PMAB (n=3)	
	R (%)	IR (%)	R (%)	IR (%)	R (%)	IR (%)	R (%)	IR (%)	R (%)	IR (%)	R (%)	IR (%)	R (%)	IR (%)
Amoxicillin (25)	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Amoxicillin plus Clavulanic acid (20+10)	7.7	34.6	58.3	87.5	40.9	84.4	23.1	53.8	39.5	82.0	41.88	82.7	100.0	100.0
Ticarcillin (75)	88.5	88.5	91.7	91.7	100.0	100.0	100.0	100.0	100.0	100.0	99.0	99.0	100.0	100.0
Piperacillin (75)	57.7	65.4	87.5	91.7	98.7	100.0	92.3	100.0	98.2	100.0	96.9	99.0	66.7	100.0
Piperacillin plus Tazobactam (75+10)	3.8	7.7	62.5	66.7	16.2	53.9	23.1	38.5	16.8	52.7	22.5	54.5	0.0	0.0
Cephalothin (30)	7.7	26.9	66.7	95.8	100.0	100.0	92.3	100.0	99.4	100.0	95.3	99.5	100.0	100.0
Cefuroxime (30)	7.7	7.7	20.8	20.8	100.0	100.0	61.5	61.5	97.0	97.0	87.4	87.4	100.0	100.0
Cefepime (30)	0.0	0.0	12.5	25.0	69.5	98.1	7.7	61.5	64.7	95.2	58.1	86.4	0.0	0.0
Cefoxitin (30)	0.0	11.5	8.3	12.5	4.5	13.0	0.0	0.0	4.2	12.0	4.7	12.0	100.0	100.0
Ceftazidime (30)	0.0	0.0	25.0	91.7	81.8	95.5	92.3	100.0	82.6	95.8	75.4	95.3	66.7	100.0
Cefotaxime (30)	0.0	0.0	16.7	25.0	100.0	100.0	61.5	76.9	97.0	98.2	86.9	89.0	33.3	33.3
Ceftriaxone (30)	0.0	0.0	25.0	29.2	100.0	100.0	84.6	100.0	98.8	100.0	89.5	91.1	0.0	0.0
Aztreonam (30)	0.0	0.0	12.5	20.8	88.3	98.1	69.2	92.3	86.8	97.6	77.5	88.0	33.3	66.7
Imipenem (10)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Meropenem (10)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Ciprofloxacin (5)	7.7	7.7	16.7	20.8	80.5	81.8	69.2	69.2	79.6	80.8	71.7	73.3	100.0	100.0
Norfloxacin (5)	11.5	15.4	12.5	16.7	81.8	81.8	69.2	69.2	80.8	80.8	72.3	72.8	100.0	100.0
Gentamicin (15)	0.0	3.8	12.5	20.8	56.5	57.8	76.9	76.9	58.1	59.3	52.4	54.5	33.3	33.3
Amikacin (30)	0.0	0.0	0.0	0.0	5.8	21.4	7.7	23.1	6.0	21.6	5.2	18.8	0.0	0.0
Trimethoprim-sulfamethoxazole (1.25+23.75)	34.6	34.6	29.2	50.0	63.0	63.6	38.5	46.2	61.1	62.3	57.1	60.7	66.7	100.0

[*] Excluding PMAβ-producing isolates

[†] Non-CTX-M-ESBL, including enzymes of the SHV, TEM and GES families.

3.5. Potential risk factors for susceptibility

We tested for risk factors associated with nonsusceptibility to oxyimino-β-lactams, mediated by the production of β-lactamases, in *Enterobacteriaceae* isolates in the period 2004-2008 (Table 3; only factors identified as significant are shown: *P*≤0.05). Higher nonsusceptibility rates were consistently associated (for at least four of the six classes of antibiotics tested and MDR) with an age of ≥65 years, nosocomial infection and production of CTX-M-type enzymes. A protective association was found for other age groups, the acquisition of infections due to non-ESBL-producing isolates, and community-acquired infections. The internal medicine ward was a risk factor for nonsusceptibility to oxyimino-β-lactams and surgery for nonsusceptibility to trimethoprim-sulfamethoxazole. *Enterobacteriaceae* isolates collected from urine were significantly associated with nonsusceptibility to fluoroquinolones but not to the other drugs tested.

3.6. Susceptibility of transformants

As 90.1% (154/171) of isolates showed diminished susceptibility to oxyimino-cephalosporins due to CTX-M production, we performed conjugation assays to investigate further the resistances conferred by each CTX-M-type. All CTX-M-type determinants tested were found to be carried on conjugative plasmids. In general, the transformants had antibiotic resistance profiles similar to those of their parental clinical isolates (Table 4). All transformants and respective clinical isolates were resistant to the penicillins tested and to cefotaxime. Only transformants producing CTX-M enzymes of the CTX-M-9-group (CTX-M-9 and CTX-M-14) were susceptible to ceftazidime and cefepime. The *E. coli* transformant harboring CTX-M-14 (with a pI of 8.1), from a clinical isolate resistant to cefoxitin, ciprofloxacin, gentamicin and trimethoprim (with pIs of 5.4 and 8.1), was susceptible to these antibiotics.

Table 3 - Odds ratios (OR) and 95% confidence intervals (CI) from the analysis of risk factors for β-lactamase-mediated nonsusceptibility to oxyimino-β-lactams in *Enterobacteriaceae* isolates, 2004–2008[*]

Antimicrobial agent (no. of nonsusceptible isolates)	Risk Factor	OR[†]	95% CI	*P*-value
Cefotaxime (171)	Age Group (≤18)	0.11 (P)	0.02-0.47	0.013
	Age Group (≥65)	6.98	2.45-19.89	<0.001
	Genotypic Group (non-ESBL)	0.01 (P)	0-0.03	<0.001
	Ward (Internal Medicine)	13.2	1.74-100.5	0.001
	Year of Isolation (2008)	0.36 (P)	0.14-0.88	0.041
Ceftriaxone (174)	Age Group (≥65)	3.18	1.19-8.48	0.034
	Genotypic Group (non-ESBL)	0.005 (P)	0.001-0.028	<0.001
	Ward (Internal Medicine)	5.07	1.13-22.65	0.028
Aztreonam (170)	Age Group (19-40)	0.27 (P)	0.09-0.8	0.048
	Age Group (≥65)	3.62	1.45-9.04	0.009
	Genotypic Group (ESBL CTX-M)	52.98	14.38-195.2	<0.001
	Genotypic Group (non-ESBL)	0.01 (P)	0.002-0.03	<0.001
	Ward (Internal Medicine)	13.96	1.84-106	0.001
Gentamicin and/or Amikacin (129)	Age Group (19-40)	0.33 (P)	0.13-0.82	0.030
	Age Group (≥65)	2.37	1.28-4.39	0.009
	Genotypic Group (ESBL CTX-M)	3.96	1.91-8.23	<0.001
	Genotypic Group (non-ESBL)	0.1 (P)	0.03-0.27	<0.001
	Community-Acquired	0.48 (P)	0.26-0.89	0.029
	Nosocomial	2.1	1.14-3.86	0.024
Ciprofloxacin and/or Norfloxacin (147)	Age Group (≤18)	0.11 (P)	0.02-0.55	0.010
	Age Group (≥65)	7.78	3.78-16.02	<0.001
	Genotypic Group (ESBL CTX-M)	8.79	4.04-19.15	<0.001
	Genotypic Group (non-ESBL)	0.13 (P)	0.05-0.33	<0.001
	Nosocomial	2.83	1.46-5.48	0.003
	Source (Urine)	2.65	1.37-5.13	0.006
Trimethoprim-sulfamethoxazole (118)	Age Group (19-40)	0.35 (P)	0.14-0.88	0.042
	Community-Acquired	0.47 (P)	0.26-0.85	0.019
	Nosocomial	2.22	1.23-4.01	0.012
	Ward (Surgery)	4.91	1.08-22.27	0.037
MDR (134)	Age Group (19-40)	0.26 (P)	0.1-0.66	0.008
	Age Group (≥65)	2.89	1.55-5.38	0.001
	Genotypic Group (ESBL CTX-M)	5.27	2.49-11.13	<0.001
	Genotypic Group (non-ESBL)	0.07 (P)	0.02-0.22	<0.001
	Community-Acquired	0.48 (P)	0.26-0.89	0.029
	Nosocomial	2.31	1.26-4.26	0.011

[*] Differences in antimicrobial resistance were simultaneously tested for association with age categories, β-lactamase genotypic group, gender, site of infection, source, ward, and year of specimen. Only significant associations are presented: *P*-values ≤0.05 and confidence limits excluding null values (0, 1, or [n]).

[†] (P) indicates an OR-value for a protective or negative association; otherwise values are for a positive association.

Table 4 - MICs of antibiotics for representative CTX-M-producing *E. coli* isolates, transformants* and the recipient†

E. coli strain	MIC (mg/L) of antibiotic‡														
	AMX	**AMC**	**TIC**	**CF**	**CAZ**	**CCAZ**	**CTX**	**CCTX**	**CFE**	**FOX**	**IMP**	**MER**	**CIP**	**GEN**	**TMP**
E. coli K12 C600	≤4	≤4	≤4	4	≤1	≤1	≤1	≤1	≤1	2	≤0.5	≤0.5	≤0.5	0.25	0.25
INSRA7733 (CTX-M-1+TEM-1)	>4,096	64	4,096	>1,024	4	≤1	>1,024	≤1	1,024	8	≤0.5	≤0.5	≤0.5	2	>128
EcK12 C600 (CTX-M-1+TEM-1)	>4,096	32	4,096	>1,024	8	≤1	>1,024	≤1	512	8	≤0.5	≤0.5	≤0.5	2	>128
INSRA6490 (CTX-M-3+TEM-1+SHV-1)	>4,096	128	4,096	>1,024	16	≤1	>1,024	≤1	>1,024	8	≤0.5	≤0.5	≤0.5	2	>128
EcK12 C600 (CTX-M-3)	>4,096	128	4,096	>1,024	8	≤1	>1,024	≤1	1,024	4	≤0.5	≤0.5	≤0.5	0.5	>128
INSRA7604 (CTM-M-9)	4,096	16	4,096	>1,024	≤1	≤1	512	≤1	16	8	≤0.5	≤0.5	32	1	>128
EcK12 C600 (CTX-M-9)	2048	8	4,096	1,024	≤1	≤1	512	≤1	8	≤1	≤0.5	≤0.5	≤0.5	0.5	>128
INSRA5776 (CTX-M-14+TEM-1)	>4,096	32	4,096	>1,024	8	≤1	1,024	≤1	32	64	≤0.5	≤0.5	>512	64	>128
EcK12 C600 (CTX-M-14)	4,096	32	4,096	>1,024	2	≤1	512	≤1	8	4	≤0.5	≤0.5	≤0.5	1	0.25
INSRA7199 (CTX-M-15)	4,096	32	4,096	>1,024	16	≤1	1,024	≤1	256	4	≤0.5	≤0.5	≤0.5	2	0.25
EcK12 C600 (CTX-M-15)	>4,096	8	4,096	>1,024	32	≤1	>1,024	≤1	512	4	≤0.5	≤0.5	≤0.5	1	≤0.125
INSRA7751 (CTX-M-32+TEM-1)	>4,096	16	4,096	>1,024	64	≤1	>1,024	≤1	1,024	4	≤0.5	≤0.5	≤0.5	4	>128
EcK12 C600 (CTX-M-32+TEM-1)	>4,096	32	4,096	>1,024	64	≤1	>1,024	≤1	512	2	≤0.5	≤0.5	≤0.5	0.5	>128

* EcK12 C600 harboring CTX-M-1 plus TEM-1, CTX-M-3, CTX-M-9, CTX-M-14, CTX-M-15, CTX-M-32 plus TEM-1 are the transformants of the clinical *E. coli* isolates INSRA7733 (CTX-M-1+TEM-1), INSRA6490 (CTX-M-3+TEM-1+SHV-1), INSRA7604 (CTM-M-9), INSRA5776 (CTX-M-14+TEM-1), INSRA7199 (CTX-M-15), and INSRA7751 (CTX-M-32+TEM-1), respectively.

† *E. coli* K12 C600 was the recipient.

‡ AMX, Amoxicillin; AMC, Amoxicillin plus clavulanic acid; TIC, Ticarcillin; CF, Cephalothin; CAZ, Ceftazidime; CCAZ, Ceftazidime plus clavulanic acid; CTX, Cefotaxime; CCTX, Cefotaxime plus clavulanic acid; CFE, Cefepime; FOX, Cefoxitine; IMP, Imipenem; MER, Meropenem; CIP, Ciprofloxacin; GEN, Gentamicin; TMP, Trimethoprim-sulfamethoxazole.

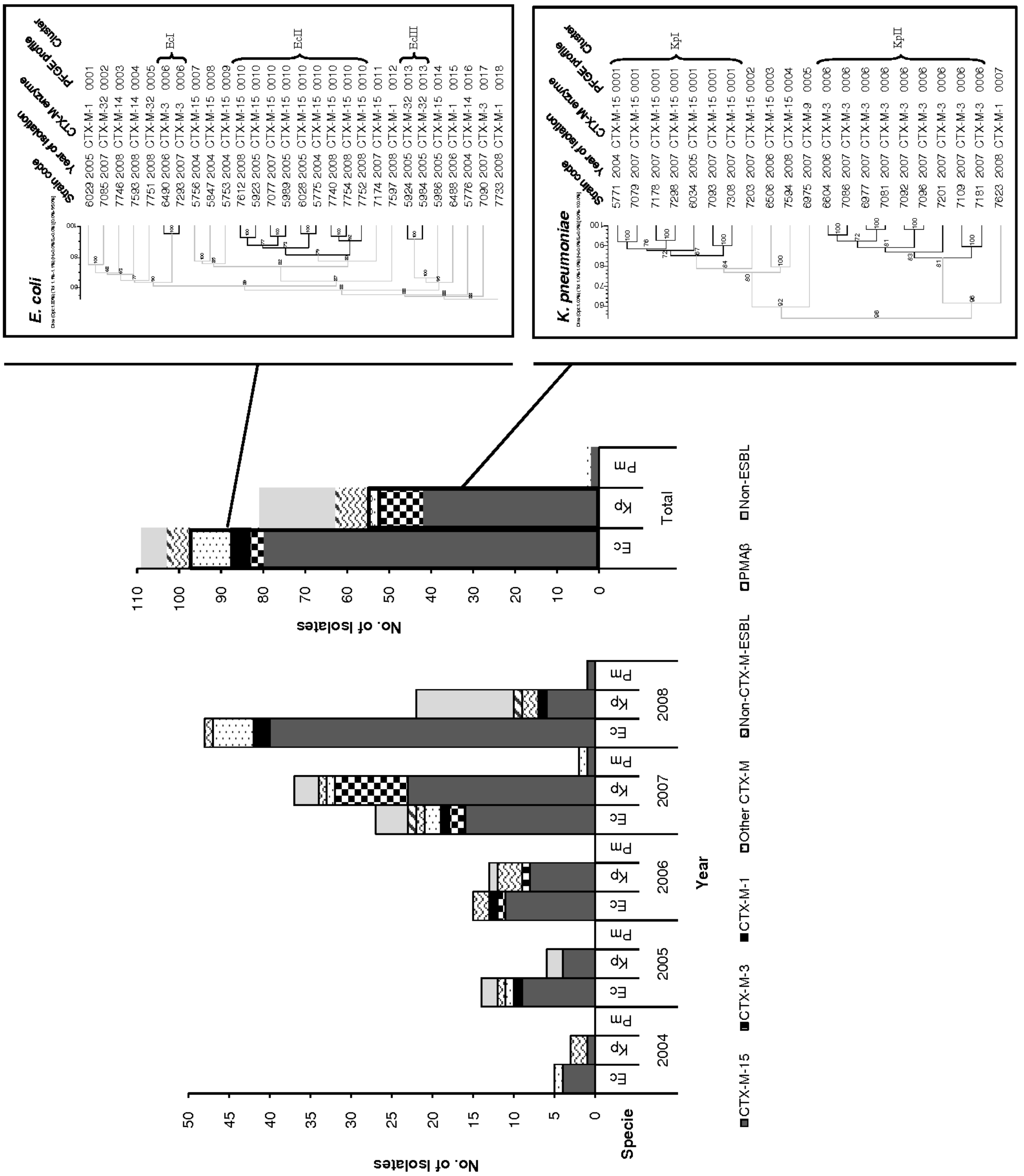
3.7. Distribution and clonality of CTX-M-producing clinical isolates

None of the clinical isolates collected in 1999 produced ESBL (Table 1). The number of CTX-M-producing isolates increased from six of eight in 2004 to 55 of 58 isolates in 2008 (Fig. 1). More *K. pneumoniae* isolates expressing due to the presence of *bla*$_{\text{CTX-M-3}}$ (n=9) and *bla*$_{\text{CTX-M-15}}$ (n=23) genes were isolated in 2007 than in the other years. Indeed, PFGE analysis (Table 1 and Fig. 1) revealed the presence of two clusters: KpI producing CTX-M-15 and KpII producing CTX-M-3. The other *K. pneumoniae* isolates were, genetically unrelated. PFGE analysis of the *E. coli* isolates identified 13 PFGE profiles, three corresponding to clones grouped into three different clusters (EcI to EcIII). Isolates carrying the *bla*$_{\text{CTX-M-15}}$ gene were identified throughout the 5 years of the study, whereas *E. coli* CTX-M-1-producers (n=6) only appeared in 2005 and the following years, and *K. pneumoniae* CTX-M-1-producers in 2008 (Fig. 1). CTX-M-3-producing isolates (n=13), both *K. pneumoniae* and *E. coli*, were found during 2006 and 2007, and were the second most common after CTX-M-15 producers. Two CTX-M-9-producing isolates were found: one *K. pneumoniae* in 2007, and one *E. coli* in 2008. CTX-M-14 (n=4) and CTX-M-32 (n=6) enzymes were also identified sporadically in *E. coli* and *P. mirabilis* isolates (Table 1).

4. Discussion

This report documents the emergence of *bla*$_{\text{ESBL}}$ genes in clinical isolates from a tertiary-level Portuguese Hospital, between two periods (1999 and 2004-2008). In the first period, β-lactamases were all non-ESBL, and included TEM-1, SHV-1 and CMY-2-types. During the second period, there was a significant increase in the number of ESBLs found, from 8 in 2004 to 58 in 2008. Although ESBLs from SHV, TEM and GES families are important, because they confer β-lactamase-mediated resistance to oxyimino-β-lactams on *Enterobacteriaceae*, the ability of CTX-M enzymes to spread between different pathogens has led to it becoming the most significant problem [5, 7, 21]. This study reinforces this view; in 2008, CTX-M-type enzymes accounted for 94.8% (55/58) of the ESBL-positive isolates. Indeed, 154 (90.1%) of the 171 isolates expressing nonsusceptibility to cefotaxime carried a *bla*$_{\text{CTX-M}}$ gene.

Fig. 1. - Emergence of 193 isolates expressing β-lactamase-mediated resistance to oxyimino-cephalosporins during period 2004 to 2008, and genetic relatedness of 21 *K. pneumoniae* and 28 *E. coli* isolates by PFGE. *K. pneumoniae* isolates with profile types Kp0001 and Kp0006, and *E. coli* isolates with profiles Ec0006, Ec0010 and Ec0013 were defined as forming clusters KpI, KpIII, EcI, EcII and EcIII, respectively.



Both CTX-M-15 and CTX-M-32 β-lactamases have the amino acid substitution Asp240Gly, responsible for resistance to ceftazidime [22, 23]. Consequently, a large proportion of our isolates were nonsusceptible to this extended-spectrum cephalosporin (95.5%). The widespread *bla*$_{\text{CTX-M-15}}$ gene was the *bla*$_{\text{CTX-M}}$ variant (124/154, 80.5%) most frequently found in our hospital, consistent with previous studies in Portugal and worldwide [7, 14, 15, 21, 24]. Although *bla*$_{\text{CTX-M-14}}$ and *bla*$_{\text{CTX-M-9}}$ genes are relatively rare in Portugal, they have been frequently isolated in Spain [21]; the genes for CTX-M-1 and CTX-M-32, are also infrequent in Portugal, but prevalent in most other European countries [4, 7, 14, 15, 21]. We believe that this is the first time that *bla*$_{\text{CTX-M-3}}$, encoding an enzyme initially reported in 1995 in *Citrobacter freundii* and *E. coli* isolates from Praski Hospital in Poland [25], has been described in Portugal, despite sporadic cases in Spain [26]. The *bla*$_{\text{CTX-M-3}}$ gene was found in 13 of our isolates (10 *K. pneumoniae* and 3 *E. coli*), and was thus the second most common CTX-M variant encountered in this hospital.

The *bla*$_{\text{CTX-M-15}}$ gene differs from *bla*$_{\text{CTX-M-3}}$ by only a single nucleotide substitution. However, one of these two *bla* genes is not necessarily the immediate progenitor of the other: the spacer region upstream of the start codon of the open reading frame differs between the two genes [27]. Thus, CTX-M-15 may not have emerged directly from CTX-M-3 in this hospital.

The selection pressure caused by increasingly inappropriate use of antibiotics over the years might be in the origin of resistance to oxyimino-β-lactams only in non-ESBL-group from the second period of the study, including the six isolates where no β-lactamases were found. This phenotype may be due to modifications in outer membrane proteins [28, 29], hyperprodution of SHV-1 or TEM-1 enzymes as a consequence of to high gene copy number [30], or a point mutation in the promoter sequence [27], compensating for the low specific activity of the β-lactamase [32]. Also, it has been reported that a larger inoculum in vivo increases the success of infections of such isolates [1, 31].

Another cause of resistance to oxyimino-β-lactams is the production of PMAβs, a resistance mechanism also identified in our set of isolates. Isolates producing these enzymes are generally less frequent than those producing ESBLs, but they are of increasing significance worldwide [33, 34]. Thus, as reported in Taiwan [35], the coexistence of ESBL and AmpC β-lactamases, namely CTX-M-3 and CMY-2, not only limits treatment options, but also complicates routine phenotypic detection of ESBLs, causing a growing problem for clinical microbiology laboratories [36, 37]. In our isolates, PMAβ was always associated with cefoxitin resistance, but this phenotype is not necessarily due to the production of PMAβ: 11.5% (1999) and 12.5% (2004-2008) of the non-ESBL group were resistant to cefoxitin. Indeed, the AmpC resistance phenotype can also result from overexpression of the chromosomal *ampC* gene, alterations in the permeability of the cell to cefoxitin, alterations in *ampC* gene or a combination of all these factors [34].

We demonstrated that β-lactamase-mediated nonsusceptibility to oxyimino-β-lactams in *Enterobacteriaceae* isolates from this hospital were consistently associated with an age of ≥65 years old, nosocomial infection and production of CTX-M-type enzymes (Table 3). Long-term hospitalizations leading to nosocomial infections and exposure to antibiotics have also been described by others as risk factors for the acquisition of ESBL-producing isolates [38, 39]. We also found that nonsusceptibility to oxyimino-β-lactams, aminoglycosides, and quinolones was associated with CTX-M-ESBL isolates, and this may be related to the dissemination of a single plasmid or to other mobile genetic elements [5, 24, 40].

K. pneumoniae ESBL-producing isolates have been described as major causes of hospital-acquired infections [1]. However, during recent years, *E. coli* producing ESBLs, mainly CTX-M enzymes, have become a major pathogen in the hospital environment [21]. In our hospital, *K. pneumoniae* were the predominant ESBL-producing isolates in 2007, but not in the previous years and in 2008; this coincided to the high frequency of CTX-M-3 in 2007, mostly in *K. pneumoniae* rather than in *E. coli*. This pattern suggests a micro-epidemic clone emerged in this hospital in 2006, in both pathogens, and disappeared in 2008, revealing the first description of CTX-M-3 in Portugal. Other outbreaks associated with Gram-negative isolates producing CTX-M-3 have been described all over the world [41, 42, 43, 44, 45, 46].

The genetic relatedness analysis of CTX-M-variant-producing *E. coli* and *K. pneumoniae* isolates corroborated these findings. The persistent *E. coli* CTX-M-15-producer clone (cluster EcII), also reported in other hospitals [14], was widespread across diverse hospital wards and over several years. *K. pneumoniae*-producing CTX-M-15 (cluster KpI) and CTX-M-3 (cluster KpII) were mostly isolated in internal medicine, and included both nosocomial and community-acquired isolates (Table 1). Overall, we observed that 38.3% (59/154) of the CTX-M-producing isolates were implicated in community-acquired infections, showing that CTX-M-3 and CTX-M-15 epidemic clones crossed the hospital-community barrier, contributing to the dissemination of β-lactamase-mediated resistance to oxyimino-β-lactams. This phenomenon is making these enzymes a threat worldwide [5, 47, 48, 49, 50].

In conclusion, we report the substantial emergence of ESBL β-lactamases, mainly CTX-M, in diverse services in a Portuguese hospital in 2004-2008, in contrast with their absence in 1999. A local and transient epidemic clone, *K. pneumoniae* producing CTX-M-3, was identified, and was isolated mostly from the internal medicine ward. This is novel for Portugal, where the main ESBLs have been CTX-M-15 enzymes [14, 15]. Given the spread of both CTX-M-3 and CTX-M-15 β-lactamases in this hospital and the recent demonstration that CTX-M-3-variants (such as CTX-M-15) confer greater resistance to extended-spectrum cephalosporins [51], monitoring isolates for the presence and evolution of bla_{ESBL}, and particularly $bla_{\mathrm{CTX\text{-}M}}$, is now imperative.

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Chapter 1. β-lactamases: antibiotic resistance impact, dissemination and co-resistance

Paper II. Diversity of plasmid-encoded AmpC β-lactamases among 2570 clinical isolates of *Enterobacteriaceae* lacking inducible chromosomal *ampC* gene from Portuguese Hospitals and the characterization of novel CMY-46 and CMY-50 enzymes

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V. Manageiro – designed study, carried out laboratory experiments, analyzed data, interpreted results and co-wrote the manuscript
D. Jones-Dias – carried out laboratory experiments and co-wrote the manuscript
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All authors have contributed to, seen and approved the manuscript.

ABSTRACT

Of the total 2570 unduplicated *Enterobacteriaceae* strains collected in 27 Portuguese hospitals, 6.3% (163) were selected as putative plasmid-mediated AmpC β-lactamases (PMAβ)-producers based on the exhibition of cefoxitin resistance and presence of synergy. Of the 163/2570, 71 (2.8%) were actually producers of such β-lactamases and were prevalent in urinary tract infections (69.3%), from patients admitted in the medicine ward (28.2%) with ≥ 65 years old (57.1%). Antimicrobial susceptibility revealed that 42.3% of the PMAβ-producers were multidrug-resistant.

Molecular characterization led to the detection of a great diversity of PMAβ-encoding genes, including *bla*$_{\text{DHA-1}}$, *bla*$_{\text{CMY-2}}$, *bla*$_{\text{CMY-39}}$, *bla*$_{\text{MIR-1}}$, *bla*$_{\text{MIR-3,}}$ and *bla*$_{\text{FOX-5}}$ genes, being noteworthy that this constitutes the first report of CMY-39, MIR-1, MIR-3 and FOX-5 in the country. Among the PMAβ producers, we detected two novel CMY-type β-lactamases that were designated CMY-46 and CMY-50. Their genetic characterization revealed a *Citrobacter freundii* chromosome-type structure, encompassing a *blc-sugE-bla*$_{\text{CMY-2-type}}$*-ampR* platform in both isolates. We have also noticed a remarkable co-expression of PMAβ with extended-spectrum β-lactamases (50.7%) and plasmid-mediated quinolones resistant determinants (78.9%), which included two *qnrC* genes.

Detection of genetic mobile elements revealed that 91.5% of PMAβ-producing isolates harbored class 1 integrons and 46.0% of the DHA-1-producing isolates bear IS*26-bla*$_{\text{DHA-1}}$ structures; IS*Ecp1* (23.5%%) and IS*903* (17.6%) were found up and downstream of *bla*$_{\text{CTX-M-15}}$ genes, respectively. Genetic-relatedness analysis of 69 isolates showed that, although CMY-producing isolates were generally unrelated, the DHA-producers could be clustered in some local epidemic clones.

We provide evidence that the identification PMAβ-producers is epidemiologically and clinically relevant since treatment options are truthfully limited.

INTRODUCTION

AmpC β-lactamases are a major group of clinically important enzymes, along with Class A β-lactamases (31, 64). These enzymes belong to class C according to the Ambler classification and to group 1 following the Bush-Jacoby functional classification (2, 10). AmpC enzymes hydrolyze oxyimino-cephalosporins, 7-α-methoxy-cephalosporins (at low levels when not induced) and monobactams, and are not inhibited by β-lactamase inhibitors such as clavulanic acid (68). AmpC-producing isolates are susceptible to carbapenems.

These enzymes are typically encoded on the chromosome of several Gram-negative bacteria, such as *Citrobacter freundii*, *Serratia marcescens, Enterobacter cloacae and Pseudomonas aeruginosa,* where its expression is usually inducible. In organisms such as *Escherichia coli* and *Shigella* spp, AmpC is also present, but is not inducible (8, 26).

The acquisition of genes coding for AmpC enzymes by transmissible plasmids has enabled their appearance in bacteria lacking or poorly expressing the chromosomal *amp*C gene, such as *E. coli, Klebsiella pneumoniae, Salmonella* spp and *Proteus mirabilis* (30, 74). Consequently, these important clinical pathogens are supplied with new and additional resistance phenotypes, once the expression of such a plasmid-mediated AmpC β-lactamase (PMAβ) is identical to that of a derepressed chromosomally-encoded AmpC enzyme (30, 74). First described in 1989 (7), PMAβs derive from chromosomal *ampC* genes (namely those of *Hafnia alvei*, *Morganella morganii*, *C. freundii*, *E. cloacae*, *Aeromonas hydrophila*, *Aeromonas caviae* and *Enterobacter asburiae*) falling into six phylogenetic groups (30, 56). Plasmids carrying *ampC* genes often carry multiple other resistance genes, including those coding for aminoglycoside and/or quinolone resistance, as well as β-lactamase encoding genes (69). Indeed, PMAβ genes can become incorporated into other different transferable elements such as transposons or integrons (13).

Infections caused by pathogenic PMAβ-producing Gram negative bacteria have increased worldwide, the majority being described as originating with *K. pneumoniae* and *E. coli* strains (30, 74). The identification of isolates containing AmpC enzymes is epidemiologically and clinically relevant due to the limitations of treatment options (52).

With this study we purpose to investigate the presence of PMAβ in clinical *Enterobacteriaceae* isolates lacking inducible chromosomal *ampC* genes, collected at different Portuguese hospitals and at different periods. We report here the occurrence of different molecular types of plasmid-encoded AmpC β-lactamases and the first description of two new CMY-2-types (designated CMY-46 and CMY-50). Finally, since plasmid-mediated quinolone resistance (PMQR) determinants, namely *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS* and *aac(6')-Ib-cr*, are commonly described in isolates co-producing extended-spectrum β-lactamases (ESBL) and PMAβ, this association was also investigated.

(The results of this study were presented in part at the 20th European Congress of Clinical Microbiology and Infectious diseases, 2010, Vienna, Austria, and at the European Scientific Conference on Applied Infectious Disease Epidemiology, 2010, Lisbon, Portugal).

MATERIAL AND METHODS

Bacterial Isolate Collection. A total of 2570 clinical non-duplicate isolates of various species of *Enterobacteriaceae* lacking inducible chromosomal *ampC* genes (i.e. 1 *Citrobacter amalonaticus*, 1824 *E. coli*, 1 *Klebsiella ornithinolytica*, 81 *Klebsiella oxytoca*, 501 *K. pneumoniae*, 3 *Klebsiella* spp, 1 *Pantoea agglomerans*, 151 *P. mirabilis*, 2 *Proteus penneri*, 3 *Proteus vulgaris* and 2 *Providencia rettgeri*) were collected, in the scope of a nation laboratory-based surveillance system; 1664 were collected consecutively between January and June 1999 (1205 *E. coli*, 56 *K. oxytoca*, 265 *K. pneumoniae*, 137 *P. mirabilis*, 1 *P. penneri*), and 906 between March 2004 and August 2009, with participant laboratories sending only isolates identified as ESBL producers using different identification systems (ATB G-5, VITEK 1, VITEK 2, and Phoenix) (i.e. 1 *Citrobacter amalonaticus*, 619 *E. coli*, 1 *Klebsiella ornithinolytica*, 25 *Klebsiella oxytoca*, 236 *K. pneumoniae*, 3 *Klebsiella* spp, 1 *Pantoea agglomerans*, 14 *P. mirabilis*, 1 *P. penneri*, 3 *P. vulgaris* and 2 *P. rettgeri*). All isolates were collected from patients in 27 Portuguese hospitals and other healthcare settings.

Antimicrobial susceptibility tests. Screening of antimicrobial susceptibility of the 2570 clinical isolates was performed using the disk diffusion method according to the guidelines of the Antibiogram Committee of the French Society for Microbiology (CA-SFM) (15). In total, twenty-one commercial disks of different antibiotics (Bio-Rad, Hercules, CA), belonging to families such as β-lactams, aminoglycosides or quinolones, were distributed (distance 30mm from edge to edge) in Mueller Hinton Agar (Difco Laboratories, Detroit, Mich) plates. The following antibiotics were tested: amoxicillin (25µg), amoxicillin plus clavulanic acid (20+10µg), ticarcillin (75µg), piperacillin (75µg), piperacillin plus tazobactam (75+10µg), cephalothin (30µg), cefuroxime (30µg), cefotaxime (30µg), ceftriaxone (30µg) ceftazidime (30µg), aztreonam (30µg), cefepime (30µg), cefoxitine (30µg), imipenem (10µg), meropenem (10µg), ciprofloxacin (5µg), norfloxacin (5µg), gentamicin (15µg), amikacin (30µg) and trimethoprim-sulfamethoxazole (1.25+23.75µg). After incubation of the plates at 37°C for 18h, inhibition zone diameters were measured for each antibiotic and the presence of synergy and / or antagonism was examined.

Isolates were considered multidrug-resistant if they had reduced susceptibility to three or more structurally unrelated antibiotics.

For each isolate producing new enzymes and its respective transformant, minimal inhibitory concentrations (MICs) were determined by a microdilution method, using Mueller-Hinton agar (Difco Laboratories, Detroit, Mich) and an inoculum of 10^4 CFU per spot. Results were interpreted according to the guidelines of the CA-SFM (10); MICs of amoxicillin, ticarcillin, cephalothin, cefuroxime, ceftazidime, cefotaxime, cefepime, cefoxitin, imipenem, meropenem, ciprofloxacin, gentamicin and trimethoprim were all tested independently, but also in combination with 2 µg/ml of clavulanic acid for amoxicillin, cefotaxime and ceftazidime.

Screening tests for PMAβ, ESBL and carbapenemases. In order to simultaneously detect and discriminate the production of several β-lactamases, such as ESBL, PMAβ and carbapenemases, the double disk synergy test (DDST) and the combined disk test (CDT) were applied by using disks of amoxicillin plus clavulanic acid placed adjacent to cefotaxime, ceftazidime, cefpodoxime and imipenem, at a distance of 20 mm from each other; a cloxacillin disk (500µg) (Rosco Diagnostica, Denmark) was placed adjacent to ceftazidime and cefoxitin; a boronic acid disk (250µg) (Rosco Diagnostica, Denmark) was placed adjacent to cefotaxime plus clavulanic acid, cefotaxime and imipenem; an imipenem disk plus EDTA (750µg) was placed adjacent to imipenem. Clinical isolates with decreased susceptibility to cefoxitin (inhibition zone diameter <18 mm), showing a keyhole or ghost zone (synergism) between boronic acid and cefotaxime plus clavulanic acid and/or showing synergism between cloxacillin and ceftazidime (and/or cefoxitin), even with susceptibility to cefoxitin, were considered presumptively PMAβ producers. Antagonism between cefoxitin, clavulanic acid and/or imipenem and extended-spectrum cephalosporins was indicative of isolates producing plasmidic inducible AmpC β-lactamases (Fig. 1).

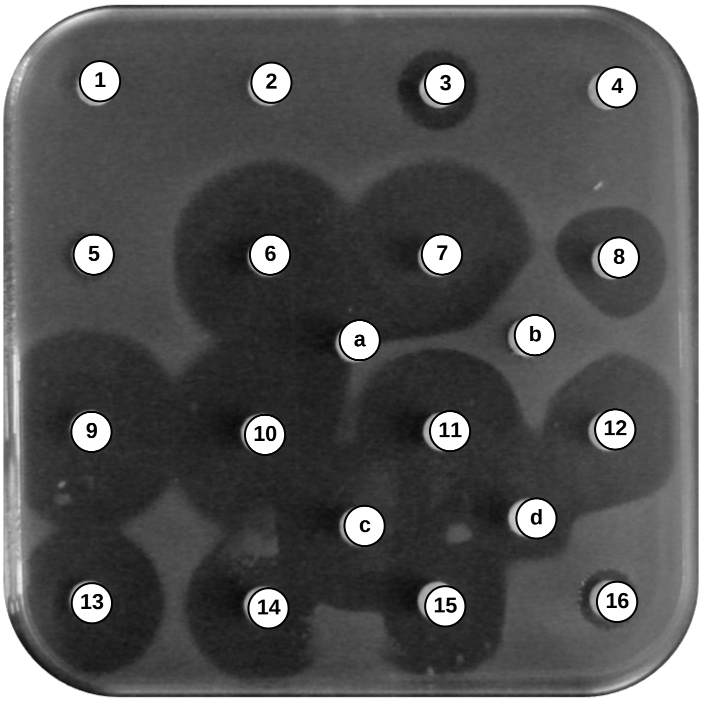


Fig.1. Disk diffusion synergy test using cloxacilin, boronic acid and EDTA as enzyme inhibitors for phenotypic detection of β-lactamases for the transformant Ec DH5α (pBK-CMY-46). Synergism between cloxacillin plus ceftazidime and cefoxitin (striped arrows), and antagonism between cefotaxime and imipinem, as well as between clavulanic acid and extended-spectrum cephalosporins (white arrow), indicated the presence of an inducible AmpC β-lactamase (the CMY-46). The following antibiotics were tested: 1, amoxicillin (25µg); 2, ticarcillin (75µg); 3, piperacillin (75µg); 4, cephalothin (30µg); 5, amoxicillin plus clavulanic acid (20+10µg); 6, cefotaxime plus clavulanic acid (30+10µg); 7, cefotaxime (30µg); 8, cefpodoxime (30µg); 9, cefepime (30µg); 10, aztreonam (30µg); 11, imipenem (10µg); 12, ceftazidime (30µg); 13, piperacillin plus tazobactam (75+10µg); 14, Cefixime (10µg); 15, cefuroxime (30µg); 16, cefoxitine (30µg); a, boronic acid disk (250µg); b, amoxicillin plus clavulanic acid (20+10µg); c, imipenem disk plus EDTA (750µg); d, cloxacillin disk (500µg).

Molecular characterization of PMAβ-encoding genes. After DNA extractions, performed as previously described (43), the presence of acquired *ampC* genes was investigated in 163 isolates, selected from the phenotypic screening as putative PMAβ producers, by using a modified multiplex PCR (56). All PCR amplifications were performed in the DNA Engine DyadTM (MJResearch, Waltham, MA, USA) and purified with ExoSAP IT (USB Corporation, Cleveland, Ohio, USA). PCR products of internal or full plasmid-encoded *ampC* genes were sequenced directly on both strands, with internal or external primers, respectively, as shown in Table 1, using the automatic sequencer ABI3100 (Applied Biosystems, Warrington, UK). DNA containing PMAβ-encoding genes was used as control.

Detection of ESBL and PMQR-encoding genes. Molecular identification of ESBL-(*bla*$_{\text{TEM}}$, *bla*$_{\text{SHV}}$, *bla*$_{\text{CTX-M}}$, *bla*$_{\text{OXA-1-type}}$ and *bla*$_{\text{GES}}$) and PMQR- (*qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *aac(6')-Ib-cr* and *qepA*) encoding genes was performed for the 71 isolates found to be PMAβ-positive, as previously described (14; 44; 54; 67; 76; 77; 80). Purification and sequencing of PCR products were carried out as referred for PMAβ genes. DNA containing ESBL- and PMQR-encoding genes was used as control.

Genetic environment characterization. The presence of class 1 integrons was determined in all PMAβ-producing isolates through PCR amplification of the integrase-specific *intI1* gene, by using specific primers and the conditions previously described (39) (Table 1). Linkage with sequences previously reported to be associated with *bla*$_{\text{DHA-1}}$, *bla*$_{\text{CMY-type}}$ and *bla*$_{\text{CTX-M}}$ genes, such as IS*Ecp1*, IS*903*, or IS*26* elements, was also investigated for the 71 PMAβ producers (20). PCR-mapping of the genetic environment of *bla*$_{\text{CMY-46}}$ and *bla*$_{\text{CMY-50}}$ was performed using primers for known antibiotic resistance and integron genes, as well as for genes encoding resistance to mercury. The PCR primers used in amplification and sequencing are listed in Table 1. Sequence alignments and generation of resistance cassette contigs were performed using *Bionumerics* version 3.5 (Applied Maths, Sint-Martens-Latem, Belgium); gene identity was confirmed at the NCBI website (http://www.ncbi.nlm.nih.gov/).

Table 1. Primers used for PCR amplification and sequencing of PMAβ and PMQR genes and for

PCR mapping of blaCMY-46 and blaCMY-50

Gene (s)	*Forward Primer Sequence (5’ → 3’)*	*Reverse Primer Sequence (5’ → 3’)*	**PCR product (bp) / Method**[a]	*Reference or source*
bla$_{\text{MOX,}}$ *bla*$_{\text{CMY-G1}}$	GCTGCTCAAGGAGCACAGGAT	CACATTGACATAGGTGTGGTGC	520 / M-PCR	56
bla$_{\text{LAT-1,}}$ *bla*$_{\text{CMY-G2}}$	TGGCCAGAACTGACAGGCAAA	TTTCTCCTGAACGTGGCTGGC	462 / M-PCR	56
bla$_{\text{CMY-G2}}$	TTACGGAACTGATTTCATG	TCGTCAGTTATTGCAGC	1169 / PCR + Seq.	This study
bla$_{\text{DHA}}$	AACTTTCACAGGTGTGCTGGGT	CCGTACGCATACTGGCTTTGC	405 / M-PCR	56
bla$_{\text{DHA}}$	CTGATGAAAAAATCGTTATC	ATTCCAGTGCACTCAAAATA	1122 / PCR + Seq.	82
bla$_{\text{ACC}}$	AACAGCCTCAGCAGCCGGTTA	TTCGCCGCAATCATCCCTAGC	346 / M-PCR	56
bla$_{\text{MIR}}$	CAGTTCTGCATTCGCCGCAC	CACCTTGTTATCACTGCCTCCGAC	889 / M-PCR	This study
bla$_{\text{MIR}}$	AAATCCCTAAGCTGTGCCCTGCTG	TTACTGCAGCGCGTCGAGGATA	1137 / PCR + Seq.	This study
bla$_{\text{FOX}}$	GGACTCATCGCCAGTATTCCAACC	AACTTCAGCAGATCCGCCGAAC	286 / M-PCR	This study
bla$_{\text{FOX}}$	TACTGACGCTGGGTAGCCT	TCACTCGGCCAACTGACTC	1124 / PCR + Seq.	This study
aac(6')-Ib-cr	TTGCGATGCTCTATGAGTGGCTA	CTCGAATGCCTGGCGTGTTT	482 / PCR + Seq.	54
qnrA	GGGTATGGATATTATTGATAAAG	CTAATCCGGCAGCACTATTA	661 / PCR	76
qnrB	CCTGAGCGGCACTGAATTTAT	GTTTGCTGCTCGCCAGTCGA	409 / PCR + Seq.	80
qnrC	GGGTTGTACATTTATTGAATC	TCCACTTTACGAGGTTCT	447 / PCR + Seq.	77
qnrD	CGAGATCAATTTACGGGGAATA	AACAAGCTGAAGCGCCTG	582 / PCR	14
qnrS	ACGACATTCGTCAACTGCAA	TAAATTGGCACCCTGTAGGC	417 / PCR + Seq.	66
intI1	TCTCGGGTAACATCAAGG	AGGAGATCCGAAGACCTC	243 / PCR + Seq.	39
5´CS-3´CS	GGCATCCAAGCAGCAAG	AAGCAGACTTGACCTGA	Variable / PCR + Seq	40
sul1	ATGGTGACGGTGTTCGG	CTAGGCATGATCTAACCCTCG	840 / PCR + Seq.	This study
qacE 1	AAGTAATCGCAACATCCG	AAAGGCAGCAATTATGAG	250 / PCR + Seq.	5
orf513	GCCAGGTCTTGAGTATCGTC	CATGTAATTGAGTCAGCGTATC	363 / PCR + Seq.	This study
fdrB - fdrD	CTCAGTTGACCACCACGAAC	GAATGCCAATAGCCGTTACGAC	920 / PCR + Seq.	This study
fdrB - ampR	CTCAGTTGACCACCACGAAC	CACCAGTCAGAATGTTCACGCA	1140 / PCR + Seq.	This study
ampR- bla$_{CMY\text{-}G2}$	TGCGTGAACATTCTGACTGGTG	TTTCTCCTGAACGTGGCTGGC	1660 / PCR + Seq.	This study
bla$_{CMY\text{-}G2}$*- sugE*	TGGCCAGAACTGACAGGCAAA	ATGTCCTGGATCGTTTTATTA	1751 / PCR + Seq.	This study
merA- urf2	TTCCCCTACCTGACGATGG	TGTTGCAGGCAGGAATAGC	1214/ PCR + Seq.	This study
merR - merA	TCTTTCTCCCCTTGCAGCG	CACCTTGTCGAACAGCCCA	Variable / PCR + Seq.	This study
merA	CGTCCAATCTGCCATAGTG	GTAGGGGAACAACTGGTCG	Seq.	This study
merD	CCTTCGAGGCGGGTATC	CCGATACCCGCCTCGAAG	Seq.	This study

[a] Method used for screening and/or identification of genes: M-PCR, Multiplex-PCR; Seq, sequencing; PCR + Seq, PCR and sequencing.

Gene transfer experiments. Amplicons comprising the *bla*$_{\text{CMY-46}}$ and *bla*$_{\text{CMY-50}}$ genes (1169bp) were cloned into the plasmid pBK-CMV, downstream of its inducible lacZ promoter, and transformed into electrocompetent *E. coli* DH5α Δ*ampC* cells using standard electroporation techniques with a Gene Pulser II apparatus, according to the manufacturer's instructions (Bio-Rad, Hercules, CA), as previously described (46). Recombinant bacteria were plated on LB agar plates containing 5 µg/ml of cefoxitin. Antimicrobial susceptibility of transformants and PCR detection and DNA sequencing of PMAβ-encoding genes were performed as described above for clinical isolates.

PFGE typing. Genetic relatedness of sixty-nine PMAβ-producing isolates (35 *E. coli*, 27 *K. pneumoniae* and 7 *K. oxytoca*) was determined using pulsed-field gel electrophoresis (PFGE), as previously described (45). Cluster analysis was performed with the *Bionumerics* version 3.5 software (Applied Maths, Sint-Martens-Latem, Belgium), using the unweighted pair group method (UPGMA) for clustering, with the arithmetic averages based on the Dice similarity index. Isolates with a dice band-based similarity coefficient value of >80% were considered to belong to the same cluster.

Analytical isoelectrofocusing (IEF). New β-lactamases were characterized by IEF of ultrasonicated bacterial extracts, as previously described (12). They were analyzed in polyacrylamide gels containing pharmalyte in the pH range 3.0 to 10.0 (GE Health-care, Waukesha, WI). *E. coli* SolRl90 (AmpC, pI 9.2) was used as control strain.

Nucleotide sequence accession number. The nucleotide sequence data of new PMAβ-encoding genes appears in the GenBank nucleotide sequence database under the following accession numbers: FN556186 for $bla_{\text{CMY-46}}$ and FN645444 for $bla_{\text{CMY-50}}$.

Statistical analysis. OpenEpi software, version 2.3 (www.OpenEpi.com), was used for statistical analysis. The chi-square test or Fisher's exact test was used when appropriate. Two-sided *P* values of <0.05 were considered to be statistically significant. Correlations between antimicrobial nonsusceptibility rates were assessed using the Spearman correlation coefficient.

RESULTS

Clinical PMAβ producer isolates. Among the 2570 isolates tested, 6.3% (108 *E. coli*, 41 *K. pneumoniae*, 11 *K. oxytoca*, 2 *P. mirabilis* and 1 *P. agglomerans*) assembled the inclusion conditions for PMAβ and were selected for further analysis. Most presumptively PMAβ-producing isolates were isolated from urine

(69.3%), and some were isolated from blood (9.2%), exsudates (6.7%), catheter (3.1%) or other sources (9.2%); In 2.5% of the cases, the sample type was unknown; 44.8% of isolates derived from patients hospitalized with nosocomial-acquired diseases, while 31.9% were identified as community acquired, according to the CDC definitions for nosocomial infections (23); 23.3% derived from unknown origins. An important number of isolates were obtained from patients admitted into the internal medicine service (28.2%), followed by outpatients (emergency room or ambulatory) (15.3%) and patients admitted in surgery (8.0%), observation (6.7%), hematology/oncology (4.3%) and pneumonology (4.3%). Ninety-three (57.1%) isolates were obtained from patients ≥ 65 years old, 34 (20.9%) were isolated from patients with 18 to 64 years old and 11 (6.7%) were isolated from patients ≤18 years old. The ages of 25 patients (15.3%) were unknown. Within the 163 collected isolates, 80 (49.1%) were from female patients, 79 (48.5%) were from male patients and in the remaining 4 (2.4%) gender was not determined.

Phenotypic and genotypic characterization of PMAβ-producing isolates. The determination of antimicrobial susceptibility of the 2570 isolates allowed the identification of 163 presumptively PMAβ-producing isolates (157 nonsusceptible to cefoxitin and 6 selected by synergy tests), for which genotypic screening by PCR and sequence analysis were performed, to detect and identify PMAβ producers, revealing that 71 out of the 163 (43.6%) contained one or more PMAβ genes.

All 71 PMAβ-producing isolates were found to be nonsusceptible to amino- and ureidopenicillins, first generation cephalosporins and cephamycins (cefoxitin), as well as to the amoxicillin plus clavulanic acid combination (Table 2). The rates of nonsusceptibility to the other β-lactam antibiotics that were screened were as follows: cefuroxime, 98.6%; ceftazidime, 97.2%; ceftazidime plus clavulanic acid, 91.5%; cefotaxime, 90.1%; aztreonam, 84.5%; piperacillin plus tazobactam, 62.0%; ceftriaxone, 57.7% and cefepime, 29.6%. All isolates were susceptible to imipenem, although 1 *K. pneumoniae* isolate had intermediate susceptibility to meropenem, but no carbapenemase was detected by IEF.

Table 2. Antibiotic susceptibility[a] of 71 PMAβ-producer Enterobacteriaceae isolates (ESBL and non-ESBL groups), among 163 presumptive PMAβ-producing isolates.

Antimicrobial agent	**PMAβ positive**						**PMAβ negative (n=92)**	
	Non-ESBL group (n=41)		**ESBL group (n=30)**		**Total (n=71)**			
	R (%)	**IR (%)**	**R (%)**	**IR (%)**	**R (%)**	**IR (%)**	**R (%)**	**IR (%)**
Amoxicillin	100.0	100.0	100.0	100.0	100.0	100.0	98.9	100.0
Amoxicillin plus Clavulanic acid	97.6	100.0	90.0	100.0	94.4	100.0	73.9	93.5
Ticarcillin	97.6	100.0	100.0	100.0	98.6	100.0	94.6	95.7
Piperacillin	90.2	100.0	96.7	100.0	93.0	100.0	83.7	95.7
Piperacillin plus Tazobactam	43.9	58.5	40.0	66.7	42.3	62.0	28.3	37.0
Cephalothin	97.6	100.0	100.0	100.0	98.6	100.0	90.2	98.9
Cefuroxime	95.1	97.6	100.0	100.0	97.2	98.6	88.0	88.0
Cefepime	0.0	34.1	3.3	23.3	1.4	29.6	29.3	35.9
Cefoxitine	95.1	100.0	86.7	100.0	91.5	100.0	57.6	93.5
Ceftazidime	78.0	95.1	96.7	100.0	85.9	97.2	51.1	68.5
Ceftazidime plus Clavulanic acid	39.0	90.2	26.7	93.3	33.8	91.5	14.7	25.8
Cefotaxime	87.8	87.8	90.0	93.3	88.7	90.1	51.1	65.2
Ceftriaxone	34.1	34.1	90.0	90.0	57.7	57.7	39.1	43.5
Aztreonam	24.4	78.0	86.7	93.3	50.7	84.5	37.0	71.7
Imipenem	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Meropenem	0.0	0.0	0.0	3.3	0.0	1.4	0.0	0.0
Ciprofloxacin	70.7	73.2	86.7	90.0	77.5	80.3	58.7	63.0
Norfloxacin	65.9	73.2	86.7	86.7	74.6	78.9	56.5	60.9
Gentamicin	36.6	36.6	80.0	80.0	54.9	54.9	22.8	31.5
Amikacin	0.0	7.3	6.7	20.0	2.8	12.7	10.9	16.3
Trimethoprim-sulfamethoxazole	73.2	73.2	66.7	66.7	70.4	70.4	55.4	55.4

[a] Susceptibility scored according to French guidelines (15). R, resistant; IR, nonsusceptible.

The 71 PMAβ-producing isolates were distributed into two groups, according to their genetic characterization: ESBL producers (n=30) and non-ESBL producers (n=41). The results presented in Table 2 allow us to determine that ceftriaxone and aztreonam were positively associated with the production of ESBLs among resistant PMAβ isolates (*P* <0.001). With respect to nonsusceptibility, only ceftriaxone had a statistically significant positive association (*P* <0.001). The DDST and CDT indicated that 98.6% of isolates presented synergy between cloxacillin and cefoxitin (and/or ceftazidime) and 77.5% showed a keyhole or ghost zone between boronic acid and cefotaxime plus clavulanic acid, alone or in combination with cloxacillin. Only 66.2% of PMAβ-producing isolates demonstrated antagonism between cefoxitin, clavulanic acid and/or imipinem and extended-spectrum cephalosporins, including not only isolates expressing genes from the *bla*$_{\mathrm{DHA}}$ family, known for being inducible, but also isolates expressing the PMAβ genes encoding CMY-39 and both the new CMY-46 and CMY-50. Only 33.8% (24/71) of PMAβ-producing isolates showed synergy between clavulanic acid and extended-spectrum cephalosporins, suggesting ESBL co-production. No ESBL genes were detected in three isolates identified as SHV-1 and TEM-1-producers, and presenting synergy between extended-spectrum cephalosporins and clavulanic acid, using phenotypic methods, which makes them false ESBL positives. Moreover, 9/30 isolates in which ESBL genes were identified by molecular screening, were false negatives for ESBL phenotype.

Overall, 54.9% of the PMAβ-producing isolates were nonsusceptible to at least one aminoglycoside, 80.3% to quinolones and 70.4% to trimethoprim-sulfamethoxazole. A total of 30/71 (42.3%) isolates (14 *E. coli*, 12 *K. pneumoniae*, 3 *K. oxytoca* and 1 *P. agglomerans*) were multidrug resistant, 19 of which were ESBL-producers and 29 of which PMQR-producers (*P* = 0.003 and 0.002, respectively).

Frequency of PMAβ-producing isolates. The occurrence rate of PMAβ-producing isolates was higher in *K. pneumoniae* (5.4%, 27/501) than in other species, namely *E. coli* (1.9%, 35/1824) (Table 3), and also higher in the second period of the study (7.1%, 64/906) relative to 1999 (0.4%, 7/1664). Overall, PCR multiplex allowed the detection of 78 PMAβ genes: 50/78 (64.1%) were positive for the DHA group; 21/78 (26.9%) corresponded to the CIT group; 6/78 (7.7%) were representative of EBC; and 1/78 (1.3%) belonged to the FOX group. Sequencing analysis confirmed the presence of 50 *bla*$_{\mathrm{DHA\text{-}1}}$, 17 *bla*$_{\mathrm{CMY\text{-}2}}$, 2 *bla*$_{\mathrm{CMY\text{-}39}}$, 5 *bla*$_{\mathrm{MIR\text{-}1}}$, 1 *bla*$_{\mathrm{MIR\text{-}3,}}$ and 1 *bla*$_{\mathrm{FOX\text{-}5}}$ genes. The sequences of two CMY-type encoding genes differed from *bla*$_{\mathrm{CMY\text{-}2}}$ by several amino acid substitutions (Table 4) and were designated CMY-46 and CMY-50. Both enzymes were identified in *E. coli* isolates collected during the period of 1999, from urine samples: CMY-46 is expressed in an isolate obtained from a patient in the ward and CMY-50 from a patient in pediatrics (77 years and 7 months old, respectively). From all isolates, four *E. coli* and one *P. mirabilis* isolates co-produced more than one PMAβ. No PMAβ gene was detected in *C. amalonaticus, K. ornithinolytica*, *Klebsiella spp, P. penneri*, *P. vulgaris* and *P. rettgeri* isolates.

Table 3– Distribution of ESBL and PMQR determinants among 71 PMAβ producing isolates, identified in 163 putative producers.

Isolates[a] (Total/ putative PMAβ)	PMAβ plus ESBL[b] positive								
	by year:		No of PMQR determinants:						
	1999	2004-2009	*qnrB1*	*qnrB2*	*qnrB4*	*qnrC*	*qnrS1*	*aac(6')-Ib-cr*	Total[c]
E. coli (1824/108)				2	13	2	1	16	35
	CMY-2	CMY-2						1	6
		CMY-2, CTX-M-15						3	5
	CMY-39			1					2
	CMY-46			1					1
	CMY-50								1
		MIR-1						1	1
		MIR-3							1
		CMY-2, MIR-1						1	2
	DHA-1	DHA-1			2		1	2	3
		DHA-1, CTX-M-15			2	1		2	3
		DHA-1, CTX-M-15, SHV-12			5			3	5
		DHA-1, SHV-12			3			2	3
		DHA-1, CMY-2							1
		DHA-1, CMY-2, MIR-1, SHV-12			1	1		1	1
K. oxytoca (81/11)					6				7
		DHA-1			1				2
		DHA-1, SHV-12			4				4
		DHA-1, SHV-31			1				1
K.pneumoniae (501/41)			1		25		4	17	27
		CMY-2, CTX-M-15	1						1
		DHA-1			19		4	15	20
		DHA-1, CTX-M-15			3			1	3
		DHA-1, SHV-12			3			1	3
P.agglomerans (1/1)					1				1
		DHA-1, CTX-M-14, SHV-12			1				1
P. mirabilis (151/2)			1					1	1
		CMY-2, MIR-1, FOX-5						1	1

[a] Total number of studied isolates/number of producers (identified by phenotype).

[b] ESBL if any

[c] Total number of PMAβ detected by PCR multiplex and characterized by sequencing.

Table 4- Comparison of amino acid substitutions of CMY PMAβs firstly encountered in this study.

PMAβ	Amino acid at position no.[a]																				pI	Accession Number
	32	35	49	102	105	124	126	133	143	164	190	193	194	198	208	236	242	253	261	348		
CMY-2	V	Q	A	Q	R	D	R	H	T	K	T	Q	N	D	P	A	H	A	R	V	9.0	X91840
CMY-46			T		S		T	R	A			K			A	V	R				9.2	FN556186
CMY-50	I	E		R	S	E	T	R		Q			S	N				E	C	A	9.2	FN645444

[a] Numbering according to Ambler (2)

ESBL and PMQR mechanisms plus PMAβ. Among the 71 PMAβ-producing isolates, 18 (25.4%) had one gene of the *bla*$_{\text{CTX-M}}$ family, 17 of which were *bla*$_{\text{CTX-M-15}}$ and 1 *bla*$_{\text{CTX-M-14.}}$ No PMAβ-producing isolates were positive for the *bla*$_{\text{GES}}$ gene, but sequencing analysis confirmed the presence of *bla*$_{\text{TEM-1}}$ (n=33), *bla*$_{\text{TEM-39}}$ (or *bla*$_{\text{IRT-10}}$) (n=1), *bla*$_{\text{TEM-40}}$ (or *bla*$_{\text{IRT-11}}$) (n=1), *bla*$_{\text{SHV-1}}$ (n=15), *bla*$_{\text{SHV-11}}$ (n=16) and other *bla*$_{\text{ESBL}}$ genes, such as *bla*$_{\text{SHV-12}}$ (n=17) and *bla*$_{\text{SHV-31}}$ (n=1); 41 isolates presented genes from the *bla*$_{\text{OXA-1-type}}$ group (Table 3). The ubiquitous chromosomal *amp*C gene was detected in all *E. coli* isolates.

A total of 56/71 (78.9%) PMAβ-producing isolates were positive for PMQR determinants (Table 3). Among the Qnr families, *qnrA* and *qnrD* genes were not detected, but the *qnrB, qnrS* and *qnrC* genes were detected in 48/71 (67.6%), 5/71 (7.0%) and 2/71 (2.8%) isolates, respectively. The QnrB determinants were most commonly detected in *K. pneumoniae* (26/48, 54.2%), followed by *E. coli* (15/48, 31.3%), *K. oxytoca* (6/48, 12.5%), and *P. agglomerans* (1/48, 2.1%) isolates. When considering all *qnr* gene determinants, *qnrB4-like* was the predominant one, mostly among *K. oxytoca* (6/6, 100%), *P. agglomerans* (1/1, 100%), *K. pneumoniae* (25/26, 96.2%) and *E. coli* (13/15, 86.7%) isolates; the *qnrB2-like* gene was only carried by *E. coli* isolates (2/15, 13.3%) while the *qnrB1-like* gene only by *K. pneumoniae* isolates (1/26, 3.8%). QnrS, encoded by the *qnrS1* allele, was detected in four *K. pneumoniae* and one *E. coli* isolate. QnrC was detected in two *E. coli* isolates. Thirty-four out of 71 isolates were positive for *aac(6')-Ib-cr* gene (47.9%), 17 of them (50.0%) encountered in *K. pneumoniae*, 16 (47.1%) in *E. coli* and 1 (2.9%) in *P. mirabilis* isolates. PMQR determinants in PMAβ-producing isolates were detected alone or in combination (Table 3).

Transfer of antibiotic resistance experiences. Transfer of β-lactam resistance was not achieved with any of the *E. coli* producing CMY-46 and CMY-50, neither in conjugation assays with *E. coli* C600 as a recipient nor in transformation assays by electroporation of total-DNA preparations into *E. coli* DH5α (data not shown). Hence, the *bla*$_{\text{CMY-46}}$ and *bla*$_{\text{CMY-50}}$ genes were cloned downstream of the inducible *lac*Z promoter in the plasmid pBK-CMV and transformed into electrocompetent *E. coli* DH5α Δ*ampC* by electroporation. Antibiotic susceptibilities of clinical isolates and respective transformants, namely EcDH5α (pBK-CMY-46) and EcDH5α (pBK-CMY-50) are show in Table 5. The clinical isolates, INSRA1169 and INSRA3413, were nonsusceptible to amoxicillin, amoxicillin plus clavulanic acid, ticarcillin, cephalothin, cefoxitin, ciprofloxacin and gentamicin. INSRA1169 was also nonsusceptible to ceftazidime and trimethoprim. In comparison to the clinical isolates, transformants EcDH5α (pBK-CMY-46) (Fig. 1) and EcDH5α (pBK-CMY-50) exhibited diminished susceptibility only to penicillins alone or in combination with clavulanic acid, cephalothin and cefoxitin.

Table 5 – MICs of antibiotics for clinical isolates producing CMY-46 and CMY-50 PMAβs and *E. coli* transformants and recipients.[a]

E. coli **strain**	**pI**	**MIC (µg/ml)[b]**															
		AMX	**AMC[c]**	**TIC**	**CF**	**CXM**	**CAZ**	**CCAZ[c]**	**CTX**	**CCTX[c]**	**FEP**	**FOX**	**IMP**	**MER**	**CIP**	**GEN**	**TMP**
EcDH5α Δ*ampC*	-	≤4	≤4	≤4	4	≤1	≤1	≤1	≤1	≤1	≤1	4	≤0.5	≤0.5	≤0.5	0.5	0.25
EcDH5α Δ*ampC* (pBK-CMV)	-	≤4	≤4	≤4	4	≤1	≤1	≤1	≤1	≤1	≤1	4	≤0.5	≤0.5	≤0.5	0.5	0.25
INSRA1169	7.4; 9.2	>4096	512	4096	1024	16	4	≤1	≤1	≤1	≤1	64	1	≤0.5	4	>128	>128
EcDH5α (pBK-CMY-46)	9.2	>4096	16	≤4	16	≤1	≤1	≤1	≤1	≤1	≤1	16	≤0.5	≤0.5	≤0.5	2	0.5
INSRA3413	9.2	32	64	64	128	4	≤1	≤1	≤1	≤1	≤1	64	1	≤0.5	2	64	0.5
EcDH5α (pBK-CMY-50)	9.2	8	16	≤4	16	≤1	≤1	≤1	≤1	≤1	≤1	16	≤0.5	≤0.5	≤0.5	1	0.5

[a] *E. coli* EcDH5α (pBK-CMY-46) and *E. coli* EcDH5α (pBK-CMY-50) were transformants of *E. coli* INSRA1169 (harboring CMY-50 and OXA-1-type enzymes and Qnr2-type determinant) and *E. coli* INSRA3413 (harboring CMY-50 enzyme), respectively; *E. coli* EcDH5α and *E. coli* EcDH5α (pBK-CMV) were the recipients.

[b] AMX, amoxicillin; AMC, amoxicillin-clavulanic acid; TIC, ticarcillin; CF, cephalothin; CXM, cefuroxime; CAZ, ceftazidime; CCAZ, ceftazidime-clavulanic acid; CTX, cefotaxime; CCTX, cefotaxime-clavulanic acid; FEP, cefepime; FOX, cefoxitin; IMP, imipenem; MER, meropenem; CIP, ciprofloxacin; GEN, gentamicin and TMP, trimethoprim.

[c] Clavulanic acid, at fixed concentration of 2µg/ml.

Characterization of the genetic environment. Overall, 65/71 (91.5%) of PMAβ-producing isolates were found positive for the integrase gene, *intI1*: 32 *E. coli*, 27 *K. pneumoniae*, 4 *K. oxytoca*, 1 *P. agglomerans* and 1 *P. mirabilis*. The IS*26* element was identified in 23 out of 50 (46.0%) DHA-1-producing isolates; in four strains (2 *E. coli* and 2 *K. pneumoniae*), the IS*Ecp1* element was found upstream of the $bla_{\text{CTX-M-15}}$ genes; three strains (1 *E. coli* and 2 *K. pneumoniae*) carried an IS*903* element downstream from its $bla_{\text{CTX-M-15}}$ gene (Fig. 2).

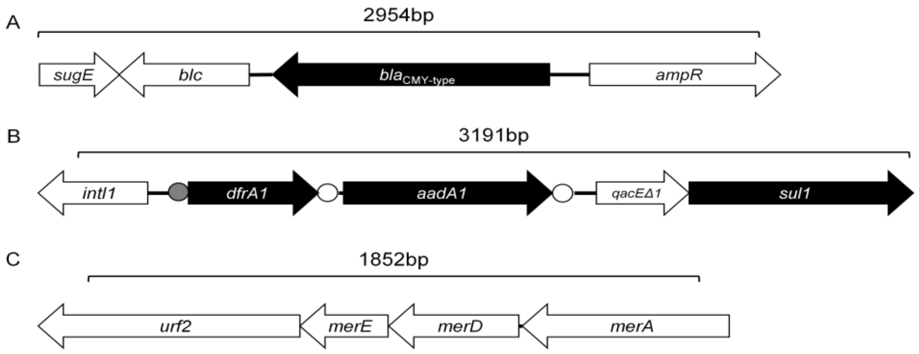


Fig.3. Schematic representation of the same three structures found within the genetic environment of $bla_{\text{CMY-46}}$ and $bla_{\text{CMY-50}}$. The directions of transcription of the corresponding genes are depicted by arrows. A: sequence including the $bla_{\text{CMY-type}}$ genes, which seems to be *C. freundii*-derived; B: class 1 integron, with *attI1* site (grey circle) and the two *attc* regions (open circles); C: truncated mercury resistance operon.

Figure 3 shows a schematic presentation of the three antimicrobial resistance regions encountered within the genetic environment of the novel CMY-encoding genes. Sequencing of the $bla_{\textbf{CMY}\text{-46}}$- and $bla_{\textbf{CMY}\text{-50}}$-carriyng region revealed the presence of the *blc* gene (encoding an outer membrane lipoprotein) and the sugE gene (encoding a small multidrug resistance protein responsible for resistance to quaternary ammonium compounds) downstream of the open reading frame (Fig. 3A). The *ampR* gene (encoding the transcriptional regulator of *ampC* expression), showing an opposite direction of transcription, was identified upstream (Fig. 3A).

By characterizing the genetic environment of $bla_{\textbf{CMY}\text{-46}}$ and $bla_{\textbf{CMY}\text{-50}}$ genes, we observed that none was located within a class 1 integron. However, the presence of two resistant gene cassettes were identified (Fig. 3B): a class 1 integron in the $bla_{\textbf{CMY}\text{-46}}$ surroundings, consisting of *intI1* (which encodes the integrase), a *dfrA1* gene (encoding trimethoprim resistance), an *aacA1* gene cassette (responsible for aminoglycoside resistance), *qacE* Δ *1* (which encodes the quaternary ammonium compound resistance protein), and *sul1* (encoding sulfamethoxazole resistance).

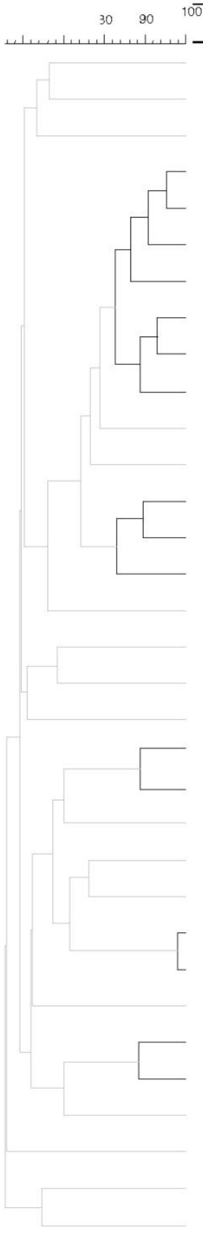
The subsequent use of primers specific to known resistance-encoding genes generated a PCR-amplicon that, upon sequencing, was found to represent a truncated mercury resistance operon (Fig. 3C).

Clonal relationships and geographic distribution of PMAβ-producing isolates. Molecular typing of *E. coli* isolates identified 22 PFGE profile types (Fig. 2A): 17 included genetically unrelated clones (0001 to 0003, 0005, 0006, 0008 to 0011, 0013 to 0015, 0017 and 0019 to 0022) and the remaining five, defined as clusters I to V, included either related or closely related (with >80% similarity) or undistinguishable (with 100% homology) clones. The largest cluster (I, including profile type 0004) consisted of 7 isolates (20.0%) producing CMY-2, but mainly DHA-1 and co-expressing CTX-M-15 and SHV-12 plus TEM-1 and/or OXA-1-type, with QnrB4-type alone or in association with Aac(6')-Ib-cr; only in isolates producing CMY-2 was there not co-expression of a PMQR determinant. Isolates from cluster I were all but one (E) isolated in two hospitals (A and B), from three different regions, in 2008 and 2009. From the 35 *E. coli* isolates, two were non-typable by PFGE.

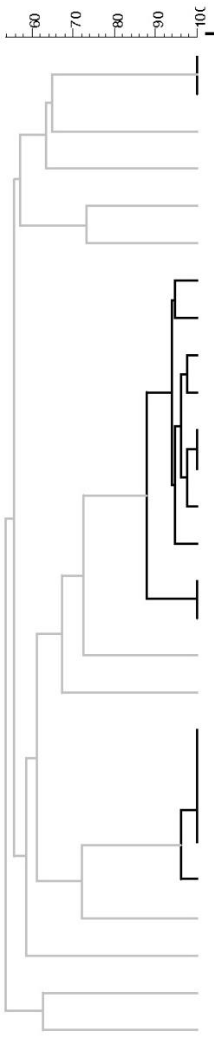
Regarding *K. pneumoniae*, dendrogram analysis identified 13 PFGE patterns (Fig. 2B), from which 10 were clones genetically unrelated, and three (profile types 0001, 0006 and 0009) grouped into clusters I to III, respectively. Among 27 isolates, 10 (37.0 %) were identified with profile type 0006 (Cluster II), which included clones co-producing DHA-1 (10/10), OXA-1-type (10/10), SHV-11 (9/10), TEM-1 (9/10), SHV-1 (1/10) and CTX-M-15 (1/10). All isolates expressed also QnrB4-type and Aac(6')-Ib-cr, and 3 of them co-expressed QnrS1. The clones were obtained from the same hospital (B), with all but one isolate collected in 2004.

PFGE grouped five of the seven *K. oxytoca* isolates into one cluster (I, with >90% similarity), while the remaining two isolates exhibited unique genotypes (Fig. 2C); all expressed QnrB4-type and four co-expressed SHV-12 enzymes. In this cluster, three isolates were collected in 2008, in one hospital in the South region, and the remaining were collected in 2005 and 2009, in a North region hospital.

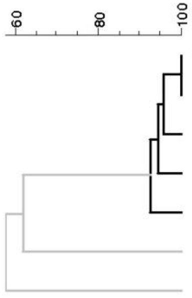
A



PFGE Profiles	Hospital Code (Region)[a]	Year	PMAβ	Other, β-Lactamases	PMQR determinants	Insertion Sequences[d]	Nonsusceptibility Profile [b, c]
Ec 0001	F (LVT)	1999	CMY-46	OXA	QnrB2-type		CXM CAZ AZT CIP NOR GEN SXT
Ec 0002	E (LVT)	2005	DHA-1	SHV-12, TEM-1	QnrB4-type, Aac(6')-Ib-cr	IS26 (d/DHA-1)	CXM CAZ CCAZ CTX CRO AZT CIP NOR GEN AN SXT
Ec 0003	J (LVT)	1999	CMY-2	-	-		TPZ CXM CAZ CCAZ CTX CRO AZT
Ec 0004 (Cluster I)	E (LVT)	2008	CMY-2	CTX-M-15, TEM-1, OXA	-		TPZ CXM CAZ CCAZ CTX CRO AZT CIP NOR AN SXT
	A (Sth)	2008	DHA-1	CTX-M-15, SHV-12, SHV-1, OXA	QnrB4-type	IS26 (d/DHA-1)	TPZ CXM CAZ CTX CRO AZT CIP NOR AN SXT
	B (Nth)	2008	DHA-1	CTX-M-15, SHV-12, SHV-1, TEM-1, OXA	QnrB4-type	IS26 (d/DHA-1)	TPZ CXM CAZ CCAZ CTX CRO AZT CIP NOR GEN AN SXT
	A (Sth)	2008	DHA-1	CTX-M-15, SHV-12, TEM-1, OXA	QnrB4-type, Aac(6')-Ib-cr	IS26 (d/DHA-1), IS903 (d/CTX-M)	TPZ CXM CAZ CCAZ CTX CRO AZT CIP NOR GEN SXT
	A (Sth)	2008	DHA-1	CTX-M-15, SHV-12, TEM-1, OXA	QnrB4-type, Aac(6')-Ib-cr		TPZ CXM CAZ CTX CRO AZT CIP NOR GEN SXT
	B (Nth)	2009	CMY-2	CTX-M-15, OXA	-		TPZ CXM CAZ CCAZ CTX CRO AZT CIP NOR GEN AN
	B (Nth)	2009	DHA-1	CTX-M-15, TEM-1, OXA	QnrB4-type, Aac(6')-Ib-cr, QnrC	IS26 (d/DHA-1) ISEcp1 (u/CTX-M)	TPZ CXM CAZ CCAZ CTX CRO AZT CIP NOR GEN
Ec 0005	B (Nth)	2008	DHA-1	TEM-1, TEM-40 (IRT-11), OXA	QnrB4-type, Aac(6')-Ib-cr	IS26 (d/DHA-1)	TPZ CXM CAZ CCAZ CTX CIP NOR GEN AN
Ec 0006	B (Nth)	2008	DHA-1	CTX-M-15, OXA	QnrB4-type		CXM CAZ CCAZ CTX CRO AZT CIP NOR GEN SXT
Ec 0007 (Cluster II)	B (Nth)	2005	DHA-1	CTX-M-15, TEM-1, OXA	Aac(6')-Ib-cr	IS26 (d/DHA-1)	TPZ CXM FEP CAZ CCAZ CTX CRO AZT
	B (Nth)	2008	CMY-2	CTX-M-15, OXA	Aac(6')-Ib-cr	ISEcp1 (u/CTX-M)	TPZ CXM CAZ CCAZ CTX CRO AZT CIP NOR GEN SXT
	A (Sth)	2008	DHA-1	CTX-M-15, SHV-12, TEM-1, OXA	QnrB4-type, Aac(6')-Ib-cr	IS26 (d/DHA-1), ISEcp1 (u/CTX-M)	TPZ CXM CAZ CCAZ CTX CRO AZT CIP NOR GEN SXT
Ec 0008	A (Sth)	2008	CMY-2	CTX-M-15, OXA	Aac(6')-Ib-cr		TPZ CXM CAZ CCAZ CTX CRO AZT CIP NOR GEN
Ec 0009	G (Nth)	1999	CMY-2	TEM-1	-		TPZ CXM CAZ CCAZ CTX CRO AZT SXT
Ec 0010	L (LVT)	2005	MIR-3	OXA	-		TPZ CXM CAZ CCAZ CTX CRO AZT
Ec 0011	F (LVT)	2007	CMY-2	TEM-1	-		CXM CAZ CCAZ CTX AZT CIP NOR SXT
Ec 0012 (Cluster III)	B (Nth)	2005	DHA-1, CMY-2	TEM-1	-	IS26 (d/DHA-1)	CXM CAZ CCAZ CTX AZT CIP NOR SXT
	B (Nth)	2005	DHA-1	TEM-1, TEM-39 (IRT-10), OXA	QnrB4-type, Aac(6')-Ib-cr, QnrS1	IS26 (d/DHA-1)	CXM FEP CAZ CTX CRO AZT CIP NOR AN SXT
Ec 0013	K (Nth)	1999	DHA-1	TEM-1	-		TPZ CXM CAZ CCAZ CTX AZT CIP NOR SXT
Ec 0014	C (LVT)	1999	CMY-39	SHV-1	QnrB2-type		CXM CAZ CCAZ CTX CRO AZT
Ec 0015	H (LVT)	2009	CMY-2, MIR-1	TEM-1	-		TPZ CXM CAZ CCAZ CTX CRO AZT CIP NOR GEN
Ec 0016 (Cluster IV)	B (Nth)	2008	DHA-1	SHV-12, TEM-1	QnrB4-type	IS26 (d/DHA-1)	CXM CAZ CCAZ CTX CRO AZT CIP NOR GEN SXT
	B (Nth)	2009	DHA-1, CMY-2, MIR-1	SHV-12, TEM-1, OXA	QnrB4-type, Aac(6')-Ib-cr, QnrC	IS26 (d/DHA-1)	TPZ CXM CAZ CCAZ CTX CRO AZT CIP NOR GEN AN SXT
Ec 0017	D (Cn)	1999	CMY-39	SHV-1	-		SXT
Ec 0018 (Cluster V)	E (LVT)	2005	MIR-1	TEM-1	Aac(6')-Ib-cr		CXM CAZ CCAZ CTX AZT SXT
	E (LVT)	2005	CMY-2	TEM-1	Aac(6')-Ib-cr		CXM CAZ CCAZ CTX AZT SXT
Ec 0019	M (Nth)	2005	CMY-2	-	-		CXM CAZ CCAZ CTX CRO AZT GEN SXT
Ec 0020	K (Nth)	1999	CMY-50	-	-		[e]
Ec 0021	B (Nth)	2005	DHA-1	SHV-12, OXA	QnrB4-type, Aac(6')-Ib-cr		TPZ CXM FEP CAZ CCAZ CTX CRO AZT CIP NOR GEN AN SXT
Ec 0022	F (LVT)	2008	CMY-2	CTX-M-15	Aac(6')-Ib-cr		CXM CAZ CCAZ CTX CRO AZT GEN



PFGE Profiles	Hospital Code (Region)^a	Year	PMAβ	Other, β-Lactamases	PMQR, determinants	Insertion Sequences^d	Nonsusceptibility Profile $^{b, c}$
Kp 0001 **(Cluster I)**	B (Nth)	2004	DHA-1	SHV-11, OXA	QnrB4-type, Aac(6')-Ib-cr	IS26 (d/DHA-1)	TPZ CXM FEP CAZ CCAZ CTX CIP NOR
	B (Nth)	2004	DHA-1	SHV-11, OXA	QnrB4-type, Aac(6')-Ib-cr,		CXM FEP CAZ CCAZ CTX CRO AZT CIP NOR
Kp 0002	B (Nth)	2005	DHA-1	SHV-12, TEM-1, OXA	QnrB4-type		CXM CAZ CCAZ CTX CRO AZT CIP GEN AN SXT
Kp 0003	I (LVT)	2009	CMY-2	CTX-M-15, SHV-1, OXA	QnrB1-type	ISEcp1 (u/CTX-M)	TPZ CXM CAZ CCAZ CTX CRO AZT MER CIP NOR GEN SXT
Kp 0004	B (Nth)	2008	DHA-1	SHV-1, OXA	QnrB4-type, Aac(6')-Ib-cr	IS26 (d/DHA-1)	CXM CAZ CCAZ CTX CRO AZT CIP NOR
Kp 0005	B (Nth)	2009	DHA-1	SHV-12, SHV-11, OXA	QnrB4-type, Aac(6')-Ib-cr	IS26 (d/DHA-1)	TPZ CXM FEP CAZ CCAZ CTX CRO AZT CIP NOR
Kp 0006 **(Cluster II)**	B (Nth)	2004	DHA-1	SHV-11, TEM-1, OXA	QnrB4-type, Aac(6')-Ib-cr		TPZ CXM FEP CAZ CCAZ CTX CIP NOR GEN SXT
	B (Nth)	2004	DHA-1	SHV-11, TEM-1, OXA	QnrB4-type, Aac(6')-Ib-cr		TPZ CXM FEP CAZ CCAZ CTX AZT CIP NOR GEN SXT
	B (Nth)	2004	DHA-1	SHV-11, TEM-1, OXA	QnrB4-type, Aac(6')-Ib-cr		TPZ CXM FEP CAZ CCAZ CTX AZT CIP NOR GEN SXT
	B (Nth)	2004	DHA-1	SHV-11, TEM-1, OXA	QnrB4-type, Aac(6')-Ib-cr, QnrS1		TPZ CXM FEP CAZ CCAZ CTX CRO AZT CIP NOR GEN SXT
	B (Nth)	2004	DHA-1	SHV-11, TEM-1, OXA	QnrB4-type, Aac(6')-Ib-cr, QnrS1	IS26 (d/DHA-1)	TPZ CXM FEP CAZ CCAZ CTX AZT CIP NOR GEN SXT
	B (Nth)	2004	DHA-1	SHV-11, TEM-1, OXA	QnrB4-type, Aac(6')-Ib-cr	IS26 (d/DHA-1)	TPZ CXM FEP CAZ CCAZ CTX AZT CIP NOR GEN SXT
	B (Nth)	2004	DHA-1	SHV-11, TEM-1, OXA	QnrB4-type, Aac(6')-Ib-cr, QnrS1		TPZ CXM FEP CAZ CCAZ CTX CRO CIP NOR GEN SXT
	B (Nth)	2004	DHA-1	SHV-11, OXA	QnrB4-type, Aac(6')-Ib-cr		TPZ CXM FEP CAZ CCAZ CTX CIP NOR SXT
	B (Nth)	2005	DHA-1	SHV-11, TEM-1, OXA	QnrB4-type, Aac(6')-Ib-cr		TPZ CXM FEP CAZ CCAZ CTX CIP NOR GEN SXT
	B (Nth)	2004	DHA-1	CTX-M-15, SHV-1, TEM-1, OXA	QnrB4-type, Aac(6')-Ib-cr		TPZ CXM FEP CAZ CCAZ CTX CIP NOR GEN SXT
Kp 0007	F (LVT)	2008	DHA-1	SHV-1, OXA	QnrB4-type, Aac(6')-Ib-cr		CXM CAZ CCAZ AZT CIP NOR SXT
Kp 0008	B (Nth)	2008	DHA-1	CTX-M-15, SHV-1, OXA	QnrB4-type	ISEcp1 (u/CTX-M), IS903 (d/CTX-M)	TPZ CXM CAZ CCAZ AZT CIP NOR
Kp 0009 **(Cluster III)**	B (Nth)	2008	DHA-1	SHV-1, OXA	QnrB4-type		TPZ CXM CAZ CCAZ CTX AZT CIP NOR SXT
	B (Nth)	2008	DHA-1	SHV-1, OXA	QnrB4-type		TPZ CXM CAZ CCAZ CTX AZT CIP NOR SXT
	B (Nth)	2008	DHA-1	CTX-M-15, SHV-1, TEM-1, OXA	QnrB4-type	IS903 (d/CTX-M)	TPZ CXM CAZ CCAZ CIP NOR SXT
	B (Nth)	2008	DHA-1	SHV-11, OXA	-		TPZ CXM CAZ CCAZ AZT CIP NOR SXT
	B (Nth)	2008	DHA-1	SHV-11, OXA	QnrB4-type		TPZ CXM CAZ CCAZ CIP NOR SXT
Kp 0010	B (Nth)	2008	DHA-1	SHV-1, OXA	QnrB4-type, Aac(6')-Ib-cr		TPZ CXM CAZ CCAZ CTX AZT CIP NOR SXT
Kp 0011	B (Nth)	2004	DHA-1	SHV-11, TEM-1, OXA	QnrB4-type, Aac(6')-Ib-cr, QnrS1		TPZ CXM FEP CAZ CCAZ CTX AZT CIP NOR GEN SXT
Kp 0012	H (LVT)	2006	DHA-1	SHV-12, SHV-11	QnrB4-type	IS26 (d/DHA-1)	CXM FEP CAZ CCAZ CTX CRO AZT GEN
Kp 0013	B (Nth)	2008	DHA-1	SHV-1, OXA	QnrB4-type		TPZ CXM CAZ CCAZ CTX AZT CIP NOR GEN



PFGE Profiles	Hospital Code (Region)[a]	Year	PMAβ	Other β-Lactamases	PMQR determinants	Insertion Sequences[d]	Nonsusceptibility Profile [b, c]
Ko 0001 (Cluster I)	M (Nth)	2005	DHA-1	SHV-31	QnrB4-type	IS26 (d/DHA-1)	CXM CAZ CCAZ CTX CRO AZT CIP NOR GEN SXT
	A (Sth)	2008	DHA-1	SHV-12 OXA	QnrB4-type	IS26 (d/DHA-1)	TPZ CXM FEP CAZ CCAZ CTX CRO AZT CIP NOR GEN SXT
	A (Sth)	2008	DHA-1	SHV-12, SHV-1, OXA	QnrB4-type	IS26 (d/DHA-1)	CXM CAZ CCAZ CTX CRO AZT CIP NOR GEN
	A (Sth)	2008	DHA-1	SHV-12	QnrB4-type	IS26 (d/DHA-1)	CXM CAZ CCAZ CTX CRO AZT CIP NOR GEN
	M (Nth)	2009	DHA-1	SHV-12	QnrB4-type	IS26 (d/DHA-1)	CXM CAZ CCAZ CTX CRO AZT CIP NOR GEN SXT
Ko 0002	F (LVT)	2006	DHA-1	OXA	QnrB4-type		TPZ CXM CAZ CCAZ CTX AZT CIP NOR SXT
Ko 0003	M (Nth)	2005	DHA-1	TEM-1	-		CXM CAZ CCAZ CTX AZT GEN AN SXT

[a] LVT: Lisbon and Tagus Valley; Nth: North; Sth: South; Centre (Cn).

[b] TPZ, piperacillin-tazobactam; CXM, cefuroxime; FEP, cefepime; CAZ, ceftazidime; CCAZ, ceftazidime-clavulanic acid; CTX, cefotaxime; CRO, ceftriaxone; AZT, aztreonam; IMP, imipinem; MER, meropenem; CIP, ciprofloxacin; GEN, gentamicin; AN, amikacin; SXT, trimethoprim plus sulfamethoxazole.

[c] All isolates were nonsusceptible to amoxicillin, amoxicillin-clavulanic acid, ticarcillin, piperacillin, cephalothin, and cefoxitin.

[d] Enzymes in parenthesis were found upstream (u/) or downstream (d/) of insertion sequences.

[e] Resistant only to β-lactam antibiotics from [c].

-, negative

Discussion

PMAβ-producing *Enterobacteriaceae* isolates are increasingly being found worldwide (30). This study documents for the first time the occurrence and dissemination of these enzymes in Portuguese hospitals and healthcare institutions, as well as their association with ESBL production and PMQR determinants.

In our study, the prevalence of PMAβs in *Enterobacteriaceae* lacking an inducible chromosomal *ampC* gene was 2.8% of 2570 clinical isolates (9.9% were obtained in 1999 and 90.1% in the second period; 2004-2009). This value is higher than what was found by Mulvey *et al* (0.09%) or Pitou *et al* (0.16%), although Hopkins *et al* (24%) and Potz *et al* (16.9%) described higher values (27, 48, 58, 61).

The rate of occurrence of PMAβs was higher in *Klebsiella* spp. isolates (27/501, 5.4%) than in *E. coli* isolates (35/1824, 1.9%), with a predominance of the DHA-1 β-lactamase (50/71; 70.4%), followed by CMY-2 (21/71; 29.6%), as was observed previously in China, South Korea, the United Kingdom and the United States (1, 19, 38, 79). However, other studies in the United States and Taiwan demonstrated a prevalence of CMY-2 in *E. coli* isolates (25, 81). Isolates producing PMAβs from the ACC family, whose phenotype includes susceptibility to cefoxitin and non-inducibility (6, 24), were not detected, despite the known dissemination of *bla*$_{\text{ACC-1}}$ in the Mediterranean region (57). PMAβs from the CMY-1-group were also not found in our study.

PMAβs usually confer resistance to penicillins, oxyimino-β-cephalosporins (at low levels), cephamycins and aztreonam, but remain susceptible to cefepime, cefpirome and carbapenems (30, 68). Our work suggests that reduced susceptibility to cefoxitin in *Enterobacteriaceae* may be an indicator of AmpC activity, but this should be confirmed using other tests, namely multiplex PCR and the synergy disk diffusion test with cloxacillin and/or boronic acid, due to the co-expression with other β-lactamases, namely ESBLs, which makes it increasingly difficult to identify PMAβs solely on the basis of the phenotype (32, 70, 83). For the purposes of this study, clinical isolates were considered as presumptive PMAβ producers if they demonstrated resistance or decreased susceptibility to cefoxitin, and/or showed synergism between boronic acid and ceftazidime plus clavulanic acid and/or between cloxacillin and ceftazidime (and/or cefoxitin). All 71 PMAβ-producers were nonsusceptible to cefoxitin, although only 1.4%, 22.5%, and 76.1% showed synergy with boronic acid, cloxacillin or both, respectively. Fifty-six per cent (92/163) of presumptive PMAβ-producer isolates were negative for PMAβ genes using molecular screening methods, suggesting that their selection using phenotypic methods could have been influenced by the production of other β-lactamases; indeed, 50 of these 92 isolates, when screened by multiplex PCR for the presence of other *bla* genes, were TEM-, SHV- or OXA-1-type producers (data not shown). Over-expression of chromosomal *ampC* gene or decreased outer membrane permeability may also explain cefoxitin nonsusceptibility (reviewed in 30).

In this study, the percentages of antimicrobial resistance to penicillins are very similar for ESBL and non-ESBL producers, although resistances to ceftriaxone and monobactam were statistically associated with the production of ESBLs. Regarding non-β-lactam antibiotics, only amikacin seems to have some effect on PMAβ-producers (12.7% of nonsusceptibility). Carbapenems may be used to treat infections caused by these isolates (51, 83), but resistance to these antibiotics may arise due to outer membrane porin loss or efflux pump activation (9, 34, 37); this was probably the case with the only *K. pneumoniae* isolate that was nonsusceptible to meropenem, since we did not find any enzyme capable of confering this phenotype (data not shown). Fluoroquinolones did not seem to be a good therapeutic option (80.3% and 78.9% of nonsusceptibility to ciprofloxacin and norfloxacin, respectively), as previously reported by others (36, 78).

The *qnr* and *aac(6')-Ib-cr* genes are frequently found among *Enterobacteriaceae* producing PMAβ and/or ESBL (53, 55, 59, 75). This study shows the association between QnrB4-type and DHA-1 determinants (*P*<0.0001), especially in *K. pneumoniae* isolates; indeed, all DHA-1-producing *K. pneumoniae* isolates evaluated in this study were positive for QnrB4-type. The association of DHA-1 and SHV-12 was also significant (*P*=0.0015) among 34.0% (17/50) of the co-producing isolates. The study also demonstrates that most DHA-1 producers also co-expressed the QnrB4-type determinant, and only in the second period of the study (after 2004), suggesting their dissemination in plasmids. Regarding other Qnr determinants, QnrB1 was only detected in one *K. pneumoniae* isolate, and QnrB2, already previously described in Portugal among clinical and veterinary isolates (21, 60), was found in two *E. coli* isolates collected in 1999, one of them expressing the new CMY-46 enzyme and the other expressing CMY-39. Only five DHA-1-producing isolates were found to produce QnrS1, neither of them co-expressing an ESBL (Fig. 2). Indeed, as reported by Poirel et al (59), the *qnrS1* gene was mostly identified in non-ESBL-positive isolates; different results were obtained in Algeria by Iabadene *et al* (29) and in France by Crémet *et al* (16). Contrasting with recent studies in the Iberian Peninsula (21, 35), where QnrA was detected, no isolate from this study showed neither this nor the QnrD variant. QnrC was encountered in two *E. coli* strains. To our knowledge, this is the first report of these enzymes outside of China (77). Interestingly, two *qnrB4*- and one *qnrB2*-positive isolates were found in norfloxacin and/or ciprofloxacin susceptible isolates, demonstrating the difficulty of detecting this resistance mechanism using phenotypic methods. Although *qnr* genes confer only low-level resistance on their own, when found in the presence of other mechanisms they enhance the selection of mutants with increased levels of fluoroquinolone resistance from the population (65, 66). In this work, only 6 DHA-1-producer isolates (5 *E. coli* and 1 *K. pneumoniae*) harbored the $bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1}}$, $bla_{\text{OXA-1}}$ and *aac(6')-Ib-cr* genes in combination (17, 18, 71), and all but one were resistant to quinolones.

PFGE was used to evaluate the clonality of the PMAβ-producing isolates. We showed that *E. coli* and *K. pneumoniae* DHA-1-producers were prevalent in hospital B throughout the different years of the study (with an epidemic local clone of *K. pneumoniae* in 2004) consistently co-producing DHA-1, SHV-11, TEM-1, OXA-1-type, QnrB4-type and Aac(6')-Ib-cr, excepting for two isolates. A different epidemic local clone was also identified in 2008 for both *E. coli* and *K. pneumoniae* isolates. However, there was little evidence of clones spreading between hospitals and/or other health institutions. Regarding CMY-producers, PFGE analysis revealed that the majority of isolates were genetically unrelated and distributed among the different health institutions, with a higher prevalence in *E. coli* isolates collected during the period of 1999. Overall, we demonstrated heterogeneity of profiles among PMAβ-producers, with some epidemic local clones, as in other countries (49, 79).

The two new CMY-2-type PMAβs found in this study, the CMY-46 and CMY-50 enaymes, had 9 and 13 amino acid substitutions, respectively (Table 4). Two of these mutations (Q193K plus P208A for CMY-46 and N194S plus D198N for

CMY-50) are in the Ω loop (between amino acids 178 and 226), which interacts by hydrogen bonding with helix H-2, close to the active Ser64. Substitutions in this region have been previously linked to the extension of the hydrolysis spectrum (42, 50, 62). However, both isolates and transformants producing CMY-46 and CMY-50 were nonsusceptible only to amoxicillin, amoxicillin plus clavulanic acid, cephalothin and cefoxitin, leading us to assume that these enzymes are not extended-spectrum AmpC cephalosporinases (Table 4), a group of β-lactamases recently reported in *E. coli* isolates (50). It is noteworthy that not all PMAβ-producing isolates meet the criteria for resistance to cephamycins and oxyimino-cephalosporins (30, 57).

Inducible PMAβs have been previously reported in *C. freundii* (CMY-13), *M. morganii* (DHA-1 and DHA-2) and *Enterobacter* (ACT-1) (4, 22, 63). Therefore, the presence of an intact *ampC-ampR* cassette in both new *bla*$_{\text{CMY}}$ genetic regions implied that the production of CMY-46 and CMY-50 is inducible (41, 47). This *ampC-ampR* cassette was identical to the sequence flanking the *bla*$_{\text{ampC}}$ gene in the *C. freundii* chromosome (41, 72). The *bla*$_{\textbf{CMY}\text{-46}}$-*ampR* region sequence was 96% identical to that of *C. freundii* (accession no. AY125469), while the homology of *bla*$_{\textbf{CMY}\text{-50}}$-*ampR* region was 99%. The deduced amino acid sequence of the AmpR open reading frame was 98% (AmpR$_{\text{CMY-46}}$) to 100% (AmpR$_{\text{CMY-50}}$) identical to the known *C. freundii* AmpR- protein. The *frdD*, *frdC*, and *frdB* genes, comprising part of the fumarate operon that is adjacent to *ampC-ampR* in the *C. freundii* chromosome, were not identified in the surroundings of these two new PMAβ-encoding genes.

The detection of a truncated mercury resistance operon in the surroundings of the new *bla*$_{\text{CMY}}$ genes (Fig. 2C), previously reported as belonging to a “*kan* region” that also includes a kanamycin resistance gene (11), is of great concern, since mercury resistance may help to promote antibiotic resistance through indirect selection (3). Furthermore, the presence of a *sul1*-type class 1 integron, containing the *dfrA1* and *aadA2* gene cassettes, was detected and may explain the observed resistance to trimethoprim and/or aminoglycosides.

In this study, 91.5% of PMAβ-producing isolates were positive for the *intI*1 gene (10.8% in 1999 and 89.2% in 2004-2009), which suggests the presence of class 1 integrons, most of them also carrying insertion sequences. We also demonstrated an association between DHA-1 and IS*26*, among all insertion sequences identified in PMAβ-producing isolates. All isolates displayed the same genetic organization, an IS26 element downstream of *bla*$_{\text{DHA-1}}$. This linkage of *bla*$_{\text{DHA-1,}}$ and also of *qnrB4* genes, on IS26 or similar structures has been described mainly in *K. pneumoniae* (28, 33, 73). Our results reinforce the important role that mobile genetic elements play in the dissemination of PMAβ mechanisms.

In conclusion, the present data suggests a nationwide dissemination of PMAβ resistance mechanisms (Fig. 3), mostly encoded by multidrug-resistant clones (74.6%), namely encoding resistance to β-lactams (100.0%), fluoroquinolones (96.2%), amynoglycosides (75.5%), and trimethoprim-sulphamethoxazole (86.8%). Thus, the high association between the PMAβ genes and those encoding for PMQR (both the *qnr* and *aac(6`)-Ib-cr* genes) and/or ESBLs is of high concern in clinical practices, since the therapeutic options for the treatment of infections caused by these resistant isolates are very limited.

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Chapter 1. β-lactamases: antibiotic resistance impact, dissemination and co-resistance

Paper III. Carbapenem-hydrolyzing class D β-lactamase-producing Acinetobacter baumannii isolated in community and health care facilities in Portugal

This chapter was submitted as:

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Contributions of the authors for the following manuscript:

V. Manageiro – designed study, carried out laboratory experiments, analyzed data, interpreted results and co-wrote the manuscript
D. Jones-Dias – carried out laboratory experiments, co-drafted and revised the manuscript
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M. Caniça – designed study, interpreted results, co-wrote the manuscript and supervised work
All authors have contributed to, seen and approved the manuscript.

Summary

Of the 127 *Acinetobacter baumannii* multidrug-resistant isolates recovered in Portugal, 98.4% carried *bla*$_{\text{OXA-66}}$, 0.8% *bla*$_{\text{OXA-104}}$, 0.8% *bla*$_{\text{OXA-71}}$ genes; 77.2% carried *bla*$_{\text{OXA-23}}$, 18.1% *bla*$_{\text{OXA-24}}$, 27.6% *bla*$_{\text{TEM-1}}$, 2.4% *bla*$_{\text{CTX-M-15-type}}$, 0.8% *bla*$_{\text{TEM-110}}$ genes. We firstly reported ST187 in Portugal, new ST188, and epidemic ST118 (OXA-23- or OXA-24-producer). ST118 disseminated since 2009, in all but one of nine hospitals, suggests importation from elsewhere. ST98 and ST92, which co-existed before 2009, seemed to have been replaced by ST118. The identification of a pandrug-resistant ST118 and carbapenem-resistant ST92, ST98 and ST118 isolates, both in community and health care facilities, demonstrates the menace of *A. baumannii*-associated infections.

Keywords: *Acinetobacter baumannii,* pandrug-resistance, CHDL.

Introduction

Acinetobacter baumannii is a ubiquitous pathogen capable of colonization or causing both community and healthcare-associated infections.[1] This pathogenic bacterium is naturally resistant to aminopenicillins, first and second-generation cephalosporins, ertapenem, fosfomycin, trimethoprim and nitrofuranes. Extensive use of antimicrobial chemotherapy to treat bacterial infections, particularly carbapenems, has contributed to the emergence of carbapenem-hydrolyzing class D β-lactamases (CHDL), and further expression of multidrug-resistant *A. baumannii* (MDR-Ab) strains. These enzymes include the acquired OXA-23, OXA-24/40 and OXA-58, as well as intrinsic OXA-51.[2] These strains reported worldwide are the cause of several nosocomial outbreaks.[1]

We report an evaluation of the carbapenem resistance of OXA-type producing *A. baumannii* from various geographic regions of Portugal and in a worldwide context, and their correlation with the genetic relatedness of this pathogen. This will allow to understand how this pathogen menace healthcare-associated infections.

Methods

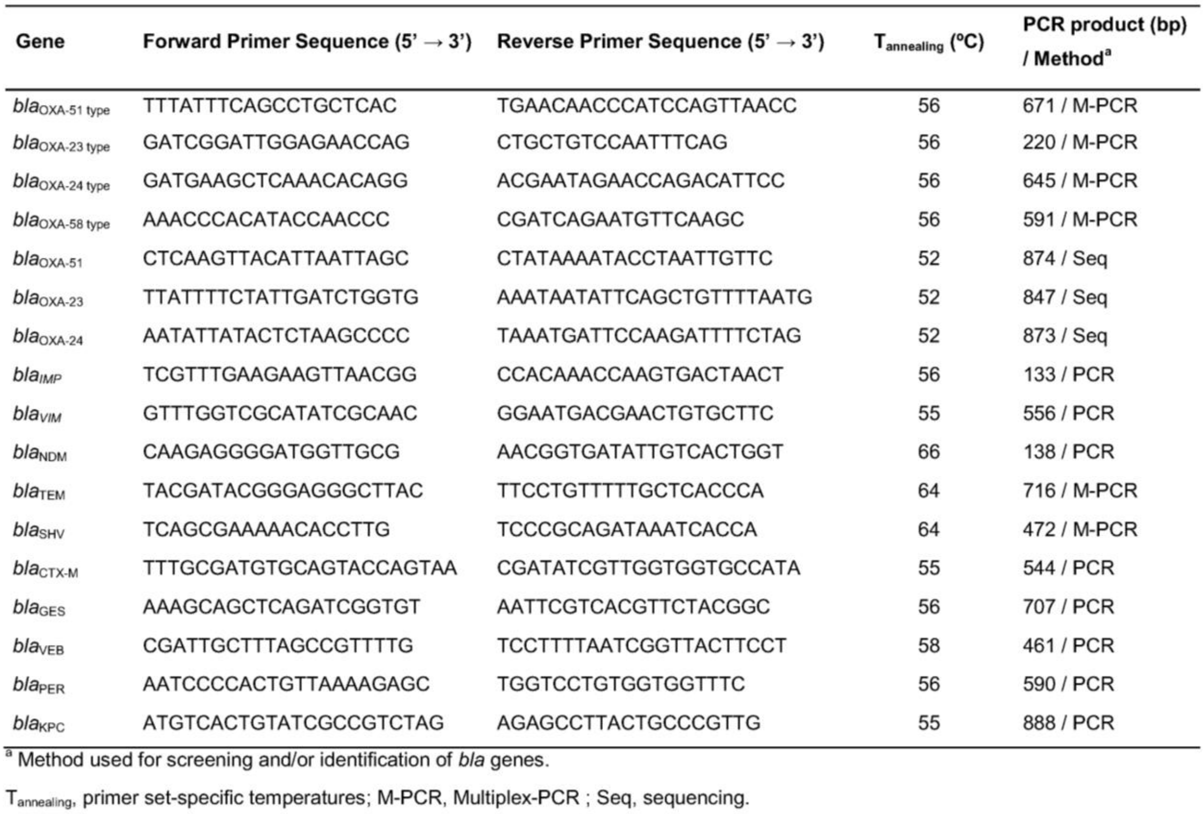
Bacterial strain collection. The study included 127 clinical *A. baumannii* isolates. A total of 116 non repetitive isolates were collected consecutively, between April 2009 and April 2010, in nine Portuguese hospitals (each with between 100 and 743 beds) participating in the Antibiotic Resistance Surveillance Program in Portugal (ARSIP), in four different geographic regions; the isolates were sent to National Institute of Health (NIH), in Lisbon. Additionally, 11 isolates collected in between 2005 and 2008, mainly in hospital D, were included for comparison and analysis of genetic evolution. All but one of the *A. baumannii* strains were identified in the participating hospitals as carbapenem non-susceptible isolates.

Antimicrobial susceptibility testing. A disk diffusion susceptibility test (DDST) was used to analyze the antimicrobial susceptibility of clinical isolates; the results were interpreted according to the clinical breakpoints of the French Society for Microbiology (CA-SFM, http://www.sfm-microbiologie.org/). In parallel, a group of 25 isolates representative of each pulsed-field gel electrophoresis (PFGE) profile and hospital (Figure 1), subjected to multilocus sequence typing (MLST), was also tested for susceptibility carbapenems by a broth microdilution method and to tigecycline and colistin by using E-test strips (bioMérieux, Marcy l'Etoile, France). Results were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints (http://www.eucast.org/); BSAC breakpoints (http://www.bsac.org.uk/) were used for tigecycline. Isolates were scored as MDR-Ab when non-susceptible to at least one agent in each of three or more structurally unrelated antibiotics, excluding natural resistances, and were considered as pandrug-resistant (PDR) when non-susceptible to all seven antimicrobial categories tested.

PCR amplification and gene sequencing. PCR and sequencing with specific primers were used to test for and identify genes encoding CHDLs ($bla_{\text{OXA-23}}$, $bla_{\text{OXA-24}}$, $bla_{\text{OXA-51}}$ and $bla_{\text{OXA-58}}$), class B metallo-β-lactamases (MBLs; bla_{IMP}, bla_{VIM} and bla_{NDM}) and class A *β*-lactamases (bla_{TEM}, bla_{SHV}, $bla_{\text{CTX-M}}$, bla_{GES}, bla_{VEB}, bla_{PER} and bla_{KPC}) (Table 1). PCR conditions comprised a first cycle of 7 min of denaturation at 94°C, followed by 30 cycles of denaturation at 94°C for 30 s, annealing for 1 min at specific temperatures (Table 1), and extension at 72°C for

1 min per kb product. Cycling was followed by a final extension at 72°C for 10 min. Species identification for all *A. baumannii*, was confirmed in the NIH, also by PCR amplification of the *bla*$_{\text{OXA-51-like}}$ gene, as previously described.[3]

Table 1 - Primers, synthesized for this study, used for PCR amplification and sequencing of *bla* genes.



Gene	Forward Primer Sequence (5' → 3')	Reverse Primer Sequence (5' → 3')	$T_{\text{annealing}}$ (ºC)	PCR product (bp) / Method[a]
bla$_{\text{OXA-51 type}}$	TTTATTTCAGCCTGCTCAC	TGAACAACCCATCCAGTTAACC	56	671 / M-PCR
bla$_{\text{OXA-23 type}}$	GATCGGATTGGAGAACCAG	CTGCTGTCCAATTTCAG	56	220 / M-PCR
bla$_{\text{OXA-24 type}}$	GATGAAGCTCAAACACAGG	ACGAATAGAACCAGACATTCC	56	645 / M-PCR
bla$_{\text{OXA-58 type}}$	AAACCCACATACCAACCC	CGATCAGAATGTTCAAGC	56	591 / M-PCR
bla$_{\text{OXA-51}}$	CTCAAGTTACATTAATTAGC	CTATAAAATACCTAATTGTTC	52	874 / Seq
bla$_{\text{OXA-23}}$	TTATTTTCTATTGATCTGGTG	AAATAATATTCAGCTGTTTTAATG	52	847 / Seq
bla$_{\text{OXA-24}}$	AATATTATACTCTAAGCCCC	TAAATGATTCCAAGATTTTCTAG	52	873 / Seq
bla$_{IMP}$	TCGTTTGAAGAAGTTAACGG	CCACAAACCAAGTGACTAACT	56	133 / PCR
bla$_{\text{VIM}}$	GTTTGGTCGCATATCGCAAC	GGAATGACGAACTGTGCTTC	55	556 / PCR
bla$_{\text{NDM}}$	CAAGAGGGGATGGTTGCG	AACGGTGATATTGTCACTGGT	66	138 / PCR
bla$_{\text{TEM}}$	TACGATACGGGAGGGCTTAC	TTCCTGTTTTTGCTCACCCA	64	716 / M-PCR
bla$_{\text{SHV}}$	TCAGCGAAAAACACCTTG	TCCCGCAGATAAATCACCA	64	472 / M-PCR
bla$_{\text{CTX-M}}$	TTTGCGATGTGCAGTACCAGTAA	CGATATCGTTGGTGGTGCCATA	55	544 / PCR
bla$_{\text{GES}}$	AAAGCAGCTCAGATCGGTGT	AATTCGTCACGTTCTACGGC	56	707 / PCR
bla$_{\text{VEB}}$	CGATTGCTTTAGCCGTTTTG	TCCTTTTAATCGGTTACTTCCT	58	461 / PCR
bla$_{\text{PER}}$	AATCCCCACTGTTAAAAGAGC	TGGTCCTGTGGTGGTTTC	56	590 / PCR
bla$_{\text{KPC}}$	ATGTCACTGTATCGCCGTCTAG	AGAGCCTTACTGCCCGTTG	55	888 / PCR

[a] Method used for screening and/or identification of *bla* genes.

$T_{\text{annealing}}$, primer set-specific temperatures; M-PCR, Multiplex-PCR ; Seq, sequencing.

PFGE. The genetic diversity of 83 isolates representative of the different hospitals and years was investigated through a modified PFGE protocol,[4] with *Apa*I used to digest the genomic DNA of *A. baumannii*. Genomic analysis was performed as previously.[4]

MLST. In order to compare single nucleotide polymorphisms with the whole genome profile provided by PFGE, MLST was conducted on a set of 25 representative isolates of each PFGE profile and hospital, as previously described.[5] The resulting sequences were assigned to sequence types (STs) using the tools on the *A. baumannii* MLST webpage (http://pubmlst.org/abaumannii/).

Nucleotide sequence accession number. The sequence of *bla*$_{OXA-104}$ β-lactamase gene was submitted to the EMBL Nucleotide Sequence Database under the accession no. FR667694.

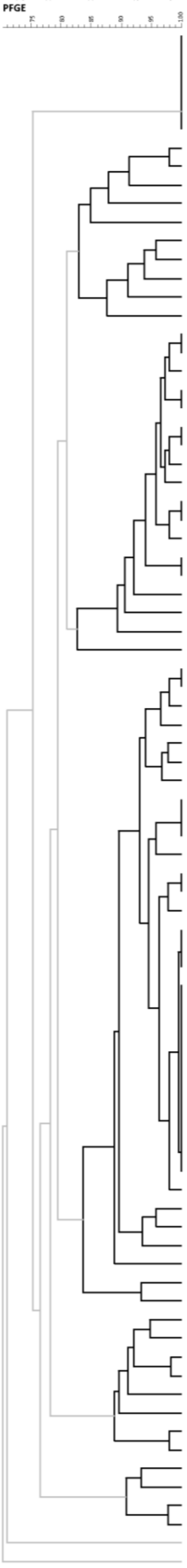
Results and Discussion

Overall, bacterial isolates were mainly from urine (40.2%), sputum (22.0%) and blood (11.0%), from male patients (58.3%) over 65 years old (68.5%), and from patients hospitalized in internal medicine services (44.1%), intensive care units (15.0%) and surgery (10.2%). Seventeen isolates were identified as the cause of community-acquired infections; the majority of isolates were from elderly (≥65 years old) patients, admitted at the emergency room. We have no information about previous hospitalizations.

DDST indicated that all isolates were MDR; however, seven were susceptible to imipenem, one was susceptible to both imipenem and meropenem (INSRA 10187) and all of them were susceptible to colistin and non-susceptible to tigecycline (Figure 1).

Contrary to DDST, two other isolates showed susceptibility to both carbapenems (INSRA10182, INSRA10183), by microdilution. Regarding colistin, one isolate was non-susceptible (INSRA9855) by E-test, which is of great concern. DDST and E-test against tigecycline was not concordant for four (16%) isolates: by E-test, three isolates were intermediate (MIC of 2 mg/L) and one was susceptible (MIC of 1 mg/L). Susceptibility testing to tigecycline must be carefully interpreted, as this drug might be an important therapeutic option in infections caused by MDR-Ab.[1]

Fig. 1. PFGE dendroGram of 83 *A. baumannii* isolates. Strains with PFGE profile types from 0001 to 0006 were defined as forming clusters I to VI, respectively (indicated by vertical curly brackets on the right). Strain code, hospital code, regions and ward, biological product, year of isolation, susceptibility (screened by disk diffusion test and quantified by MIC), β-lactamases identified, MLST sequence types and PFGE profile are also shown. TEM, CTX-M and CHDLs are presented as black circles. CA, Community-Acquired; ICU, Intensive Care Unit; IM, Internal Medicine; NF, Nephrology; OP, Ophthalmology; ORL, Otorhinolaryngology; OT, Orthopaedy; OW, Observation ward; PN, Pneumology; SUR, Surgery; SURV, Surgery Vascular; Unk, Unknown; af, Ascitic fluid; ba, Bronchial aspirate; bl, Blood; bs, Bronchial Secretions; ct, Catheter; db, Duodenal Biopsy; pe, Purulent Exudate; sp, Sputum; ta, Tracheal aspirate ; ur, Urine; w, Wound; I, imipenem; M, meropenem; T, tigecycline; C, colistin.



Strain code	Hospital code	Hospital Regions	Hospital Ward	Biological Product	Year	IMP/MER Screen	Antibiotic Susceptibility Profile MICs (mg/L)				β-lactamases detected							MLST	PFGE profile	PFGE Cluster
							I	M	T	C	CTX-M	TEM	OXA-66	OXA-71	OXA-104	OXA-23	OXA-24			
10180	D	LVT	OW	bl	2005	S/I							◆						0001	I
10181	D	LVT	ICU	ur	2005	S/I							◆						0001	
10182	D	LVT	ICU	bs	2005	S/I	≤0.5	1	8	0.5			◆					ST98	0001	
10183	D	LVT	ICU	ct	2006	S/I	≤0.5	1	8	0.5			◆					ST98	0001	
10184	D	LVT	ICU	ct	2006	S/I							◆						0001	
10185	D	LVT	NF	db	2006	S/I							◆						0001	
9770	F	LVT	CA	bl	2009	R/R							◆				◆		0002	II
10189	D	LVT	ORL	ur	2008	R/R							◆				◆		0002	
9317	F	LVT	IM	ur	2009	R/R	32	256	16	0.5			◆				◆	ST98	0002	
10186	D	LVT	ICU	ur	2006	R/R	128	512	8	0.5			◆				◆	ST98	0002	
6095	F	LVT	IM	ur	2005	R/R	128	256	16	0.25		◆	◆				◆	ST98	0002	
10075	F	LVT	SUR	ur	2010	R/R							◆				◆		0002	
10085	F	LVT	IM	ur	2010	R/R							◆				◆		0002	
9086	H	Ctr	IM	ur	2009	R/R	64	128	2	0.25			◆				◆	ST98	0002	
10054	H	Ctr	CA	ur	2010	R/R							◆				◆		0002	
9744	E	LVT	IM	ba	2009	R/R	64	128	4	0.5			◆				◆	ST98	0002	
8660	A	Nth	IM	w	2009	R/R	32	32	16	0.5		◆	◆			◆		ST92	0003	III
10042	A	Nth	NU	sp	2010	R/R						◆	◆			◆			0003	
9551	A	Nth	SUR	bl	2009	R/R						◆	◆			◆			0003	
8661	A	Nth	IM	sp	2009	R/R	32	64	32	0.25		◆	◆			◆	◆	ST92	0003	
9192	B	Nth	CA	sp	2009	R/R	32	128	8	0.5		◆	◆			◆		ST92	0003	
10028	A	Nth	IM	sp	2009	R/R							◆			◆			0003	
10034	A	Nth	PN	bl	2010	R/R						◆	◆			◆			0003	
9389	A	Nth	IM	sp	2009	R/R						◆	◆			◆			0003	
8659	A	Nth	CA	ur	2009	R/R	32	32	8	0.25		◆	◆			◆		ST92	0003	
9060	C	Nth	SUR	pe	2009	R/R	32	64	8	0.5			◆			◆		ST92	0003	
9309	B	Nth	SURV	bl	2009	R/R						◆	◆			◆			0003	
9456	B	Nth	SURV	bl	2009	R/R						◆	◆			◆			0003	
8818	A	Nth	ICU	bs	2009	R/I	8	4	128	0.5		◆	◆			◆		ST92	0003	
8956	A	Nth	IM	sp	2009	R/R						◆	◆			◆			0003	
9857	H	Ctr	ICU	pe	2009	R/R	32	32	16	0.5		◆	◆			◆		ST92	0003	
9085	H	Ctr	IM	ur	2009	R/R						◆	◆			◆			0003	
9123	A	Nth	IM	bs	2009	R/R							◆			◆			0003	
10188	D	LVT	ICU	af	2007	R/R	16	32	8	0.5		◆	◆			◆		ST92	0003	
8882	F	LVT	IM	ur	2009	R/R							◆			◆			0004	IV
9497	C	Nth	IM	ur	2009	R/R							◆			◆			0004	
9038	C	Nth	SUR	bl	2009	R/R							◆			◆			0004	
9767	F	LVT	CA	ur	2009	R/R	32	32	2	0.5			◆			◆		ST118	0004	
9688	C	Nth	IM	sp	2009	R/R	32	32	2	0.25			◆			◆		ST118	0004	
9811	C	Nth	CA	ur	2010	R/R							◆			◆			0004	
10121	C	Nth	CA	ur	2010	R/R							◆			◆			0004	
9858	H	Ctr	IM	sp	2009	R/R							◆			◆			0004	
9862	H	Ctr	OT	sp	2010	R/R							◆			◆			0004	
10050	H	Ctr	CA	ur	2010	R/R							◆			◆			0004	
9552	I	LVT	PN	ur	2009	R/R							◆			◆			0004	
9313	F	LVT	IM	bl	2009	R/R							◆			◆			0004	
9318	F	LVT	OT	ur	2009	R/R							◆			◆			0004	
9269	I	LVT	PN	bl	2009	R/R							◆			◆			0004	
10192	D	LVT	ICU	ct	2009	R/R							◆			◆			0004	
10195	D	LVT	IM	ur	2010	R/R							◆			◆			0004	
10194	D	LVT	IM	sp	2010	R/R							◆			◆			0004	
8973	I	LVT	CA	ur	2009	R/R							◆			◆			0004	
9675	F	LVT	SUR	ur	2009	R/R							◆			◆			0004	
10190	D	LVT	ICU	ur	2009	R/R							◆			◆			0004	
10191	D	LVT	ICU	ct	2009	R/R					◆		◆			◆			0004	
10193	D	LVT	ICU	ct	2010	R/R							◆			◆			0004	
10197	D	LVT	ICU	ct	2010	R/R							◆			◆			0004	
10198	D	LVT	SUR	pe	2010	R/R	32	32	4	0.5			◆			◆		ST118	0004	
10199	D	LVT	ICU	ct	2010	R/R							◆			◆			0004	
10201	D	LVT	ICU	bl	2010	R/R							◆			◆			0004	
10202	D	LVT	IM	ur	2010	R/R							◆			◆			0004	
10203	D	LVT	OP	ct	2010	R/R							◆			◆			0004	
8846	I	LVT	PN	bl	2009	R/R	32	128	2	0.5			◆			◆		ST118	0004	
9165	C	Nth	IM	sp	2009	R/R							◆			◆			0004	
9328	F	LVT	SUR	pe	2009	R/R							◆			◆			0004	
10124	C	Nth	SUR	ur	2010	R/R							◆			◆			0004	
9395	I	LVT	PN	bs	2009	R/R	32	32	2	0.5			◆			◆		ST118	0004	
8970	H	Ctr	SUR	pe	2009	R/R	32	128	16	0.5			◆			◆		ST118	0004	
9095	H	Ctr	SUR	ur	2009	R/R							◆			◆			0004	
9855	G	ARM	Unk	ur	2009	R/R	128	64	16	4			◆				◆	ST118	0005	V
9856	G	ARM	Unk	ur	2009	R/R							◆				◆		0005	
9851	G	ARM	Unk	bl	2009	R/R							◆				◆		0005	
9853	G	ARM	Unk	ur	2009	R/R							◆				◆		0005	
9849	G	ARM	Unk	bl	2009	R/R							◆				◆		0005	
9832	G	ARM	Unk	pe	2010	R/R							◆				◆		0005	
9852	G	ARM	Unk	ur	2009	R/R							◆				◆		0005	
9854	G	ARM	Unk	ta	2009	R/R							◆				◆		0005	
9735	E	LVT	CA	ur	2009	R/R							◆			◆			0006	VI
9747	E	LVT	IM	sp	2009	R/R							◆			◆			0006	
9750	E	LVT	IM	ur	2009	R/R	64	128	4	0.25			◆			◆		ST118	0006	
9754	E	LVT	IM	ur	2009	R/R							◆			◆			0006	
8662	A	Nth	IM	ur	2009	R/R	64	128	1	0.5					◆		◆	ST188	0007	
10187	D	LVT	OW	ur	2006	S/S	≤0.5	1	8	0.5				◆				ST187	0008	

Overall, we identified that the 127 isolates carried the *bla*$_{OXA-66}$ (98.4%), *bla*$_{OXA-71}$ (0.8%) or *bla*$_{OXA-104}$ (0.8%) CHDL genes, which belong to *bla*$_{OXA-51}$ group; 77.2% of the isolates carried *bla*$_{OXA-23}$, 18.1% *bla*$_{OXA-24}$, 27.6% *bla*$_{TEM-1}$, 2.4% *bla*$_{CTX-M-15-type}$, and 0.8% *bla*$_{TEM-110}$ genes; none carried OXA-58 or MBL-encoding genes.

PFGE analysis identified eight profiles: 81 MDR-Ab related (>80% similarity) or indistinguishable (100% homology) clones were grouped into six different clusters (I-VI) (Figure 1). PFGE clusters correlated with CHDL production and non-susceptibility to carbapenems, and were associated with five distinct sequence types (STs) (Figure 1): ST92 (allelic profile, 1-3-3-2-2-7-3), ST98 (allelic profile, 1-12-3-2-2-3-3), ST118 (allelic profile, 1-3-3-2-2-3-3), ST187 (allelic profile, 1-1-1-1-1-9-6) and the novel ST188 (allelic profile, 31-33-67-40-16-58-7). The association of studying CHDL-encoding genes with the genotype analysis increased the discrimination of clones circulating in Portugal and in other countries; in this context, we show that there are three major STs contributing to the high prevalence of carbapenem resistance in *A. baumannii*: ST92 (OXA-23-producer, mostly associated with TEM expression), ST118 (OXA-23- or OXA-24-producer) and ST98 (OXA-24-producer).

We also demonstrated by PFGE analysis that these three STs have a common ancestor, which is consistent with the fact they belong to the same clonal complex.[6,7] Interestingly, all carbapenem-susceptible MDR-Ab isolates lacking CHDLs other than OXA-66 belonged to the ST98 and were isolated between 2005 and 2006; to our knowledge this is the first description of carbapenem-susceptible MDR-Ab isolates from ST98 (http://pubmlst.org/abaumannii/). These isolates were indistinguishable by PFGE, suggesting that they existed in Portugal before the acquisition of the *bla*$_{OXA-24}$ gene. In our study, ST98 and ST92 both single-locus variant of ST118, were simultaneously present before 2009, as also reported by others.[8] However, since 2009 they seemed to have been replaced by the epidemic ST118, suggesting importation and spread of this ST from elsewhere. In fact, ST118 was previously identified in Italy, France, UK and Norway (http://pubmlst.org/abaumannii/).[6,9] Another possibility is that this lineage may have emerged from close STs spread in the continental Portuguese regions since ST118 differ at only 2 loci, *gyrB* and *gpi*, in respect to ST98 and ST92, respectively. Furthermore, the geographic distance between the Madeira Island and the continental Portuguese regions may hypothesize that ST118 has evolved through the acquisition of different resistance genes, namely *bla*$_{\text{OXA-24}}$ and *bla*$_{\text{OXA-23}}$. OXA-23 producer clones, all belonging to ST118 and ST92 (the last also described in several continents),[6,9] have successfully spread among all hospitals in our study (Figure 1), suggesting clonal expansion; ST118 was identified in all but one hospital and ST92 in all but two. Indeed, ST118 seems to be replacing ST98 described so far as endemic in Portugal.[8] Worryingly, Portugal may have favorable features that contribute to the rapid adaptation of ST118 to hospital environments, being five isolates from a military hospital; indeed, the phenotype of INSRA9855, with ST118, makes it a step closer to a PDR *A. baumannii* isolate. Clones from each cluster I (ST98), V and VI (ST118) were only detected in a single hospital (D, G and E, respectively), suggesting three different epidemic local clones.

ST187 identified in 2006, in a carbapenem-susceptible OXA-71-producing *A. baumannii* isolate, was also recently described in France, Germany and Spain.[6] The *bla*$_{\text{OXA-104}}$ gene, identified in 2009, in a ST188 MDR-Ab, also with *bla*$_{\text{OXA-24}}$ gene, carries 182 synonymous mutations comparing with the first-described;[10] the molecular evolution of *bla*$_{\text{OXA-104}}$ gene suggests an adaptation of MDR-Ab to the selective pressure imposed by the clinical use of carbapenems.

In conclusion, this study provides new data regarding the molecular epidemiology of MDR-Ab in Portugal and in an international context. Here, at our knowledge, we report the first appearance of the epidemic ST118 OXA-23-producer, in both community and Portuguese health care facilities, even in military patients, as well as one ST92 isolate co-producing OXA-23 and OXA-24. Carbapenem susceptible ST98 isolates, and MDR-Ab-producing TEM-110 or CTX-M-15-type were also stated. We also show that efficacy of colistin (one of the LAST-RESORT antibiotics for treating MDR-Ab infections) is increasingly compromised. Overall, our results emphasize the need of a concerted action to manage carbapenem use, as well as to control the worldwide clonal expansion of MDR-Ab.

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Transparency declarations

None to declare.

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Chapter 2.

Paper IV. The Lys234Arg substitution in the enzyme SHV-72 is a determinant for resistance to clavulanic acid inhibition

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Contributions of the authors for the following manuscript:

N. Mendonça – designed study, carried out laboratory experiments, analyzed the data, interpreted the results, drafted and co-wrote the manuscript

V. Manageiro – designed study, carried out laboratory experiments and co-drafted the manuscript

F. Robin –carried out laboratory experiments

M. J. Salgado – worked on patient population and clinical data

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M. Caniça – designed study, interpreted results, co-wrote the manuscript and supervised work

R. Bonnet – designed study, interpreted results, co-wrote the manuscript and supervised work

All authors have contributed to, seen and approved the manuscript.

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The Lys234Arg Substitution in the Enzyme SHV-72 Is a Determinant for Resistance to Clavulanic Acid Inhibition▽

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The new β-lactamase SHV-72 was isolated from clinical *Klebsiella pneumoniae* INSRA1229, which exhibited the unusual association of resistance to the amoxicillin-clavulanic acid combination (MIC, 64 μg/ml) and susceptibility to cephalosporins, aztreonam, and imipenem. SHV-72 (pI 7.6) harbored the three amino acid substitutions Ile8Phe, Ala146Val, and Lys234Arg. SHV-72 had high catalytic efficiency against penicillins (k_{cat}/K_m, 35 to 287 $\mu M^{-1} \cdot s^{-1}$) and no activity against oxyimino β-lactams. The concentration of clavulanic acid necessary to inhibit the enzyme activity by 50% was 10-fold higher for SHV-72 than for SHV-1. Molecular-dynamics simulation suggested that the Lys234Arg substitution in SHV-72 stabilized an atypical conformation of the Ser130 side chain, which moved the Oγ atom of Ser130 around 3.5 Å away from the key Oγ atom of the reactive serine (Ser70). This movement may therefore decrease the susceptibility to clavulanic acid by preventing cross-linking between Ser130 and Ser70.

The most common resistance mechanism in bacteria against β-lactam antibiotics is the production of β-lactamases (EC 3.5.2.6), which hydrolyze and inactivate β-lactams. β-Lactamases are divided into four major classes (A to D) on the basis of their primary sequence (1). While class B is composed of metalloenzymes that necessitate the presence of zinc cations for activity, classes A, C, and D are serine hydrolases (1, 7). Class A enzymes comprise several enzyme families, including the clinically relevant enzymes TEM and SHV (26).

TEM and SHV enzymes initially had preferential activity against penicillins, as in the case of enzymes SHV-1 and TEM-1. Oxyimino β-lactams that are resistant to their hydrolytic activity and β-lactam inhibitors, such as clavulanic acid and tazobactam, have been developed to get around the activities of these enzymes (6, 9, 26). Nevertheless, the presence of point mutations in TEM and SHV enzymes has expanded the substrate spectrum to include oxyimino β-lactams and/or has conferred resistance to the inhibitors (3, 6, 9, 26).

More than 28 inhibitor-resistant TEM enzymes have been detected. They harbor amino acid substitutions at positions 69, 130, 165, 182, 244, 275, and/or 276 that confer resistance to inhibitors (9, 15). Only three natural inhibitor-resistant SHVs (IRS) have been reported (http://www.lahey.org/studies). It has been proposed that substitutions at positions 69, 130, and 187 are involved in their resistance to inhibitors (10, 14, 31). IRS enzymes have also been constructed in vitro by site saturation mutagenesis at position 244 (37).

In this study, we performed a phenotypic, molecular, and biochemical characterization of the new IRS-type β-lactamase SHV-72 from a clinical *K. pneumoniae* strain and investigated by molecular-dynamics simulations (MDSs) the role of the Lys234Arg substitution in its resistance to clavulanic acid.

MATERIALS AND METHODS

Bacterial strains and plasmid. *Klebsiella pneumoniae* INSRA1229 was isolated from sputum of an 80-year-old male in an internal medicine service of a general hospital in Lisbon in 1999. *Escherichia coli* DH5α Δ*ampC* and plasmid pBK-CMV (Stratagene, Amsterdam, The Netherlands) were used for the cloning experiments (Table 1) (13). bla_{SHV-1}-encoding *E. coli* C600 was used as a control strain for PCR.

Susceptibility testing. MICs were determined by the agar dilution method according to the recommendations of the French Society of Microbiology (8). Strains were tested against cefotaxime (Sanofi Aventis), ceftriaxone and trimethoprim (Roche Pharmaceuticals), aztreonam and cefepime (Bristol-Myers Squibb), amoxicillin, cefuroxime, ceftazidime, clavulanic acid, and ticarcillin (GlaxoSmithKline), cefoxitin (Labesfal), amdinocillin (Leo Pharma), cephalothin (Sigma), cefoperazone (Pfizer), piperacillin and tazobactam (Wyeth Pharmaceuticals), imipenem (Merck Sharp & Dohme, Lda), ciprofloxacin (Bayer HealthCare), gentamicin (Schering-Plough), and penicillin (Laboratórios Atral). MICs of β-lactam antibiotics were determined alone and combined at a fixed concentration of clavulanic acid (2 μg/ml) (amoxicillin, ceftriaxone, cefotaxime, ceftazidime, and aztreonam) or tazobactam (4 μg/ml) (piperacillin).

Isoelectric focusing. Cell extracts were obtained by ultrasonic treatment, and isoelectric focusing was performed with polyacrylamide gels containing ampholines with a pH range of 3.5 to 9.5, as previously described (4), with IRT-2 (pI 5.2), TEM-1 (5.4), TEM-2 (pI 5.6), OXA-1 (pI 7.4), SHV-1 (pI 7.6), CTX-M-15 (pI 8.9), and AmpC (pI 9.2) as standards.

Amplification, sequencing, and cloning of β-lactamase genes. The β-lactamase-encoding genes were detected and sequenced, as described elsewhere (28). The complete SHV-encoding open reading frame of the bla_{SHV-1} and bla_{SHV-72} genes was amplified with the specific primers SHVsf and SHVsr (28). The bla_{SHV-1} and bla_{SHV-72} genes were cloned as follows: proofreading Isis DNA polymerase (Qbiogene, Irvine, CA) was used for bla_{SHV-72}, and proofreading iProofTM high-fidelity DNA polymerase (Bio-Rad Laboratories Inc., Hercules, CA) was used for bla_{SHV-1}. PCR products were ligated in the SmaI site of the plasmid pBK-CMV, and the recombinant plasmids were electroporated in *E. coli* DH5α Δ*ampC*. The transformants harboring the recombinant SHV-encoding plasmids (pBK-SHV-72 and pBK-SHV-1) were selected on Mueller-Hinton agar

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or description	Source or reference
Strains		
K. pneumoniae	Clinical strain harboring the natural plasmid pINSRA1229	This study
E. coli C600	Strain harboring the natural plasmid pINSRASHV-1	This study
E. coli DH5α	*supE44 ΔlacU169 (ϕ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1 ΔampC*	13
Plasmids		
pINSRASHV-1	Plasmid from *E. coli* C600 containing $bla_{\text{SHV-1}}$ gene; resistance phenotype: amoxicillin	This study
pINSRA1229	Natural plasmid from *K. pneumoniae* INSRA1229 containing $bla_{\text{SHV-72}}$ gene; resistance phenotype: IRS	This study
pBK-SHV-72	Recombinant plasmid containing 891-bp fragment with $bla_{\text{SHV-72}}$ gene; resistance phenotype: IRS, kanamycin	This study
pBK-SHV-1	Recombinant plasmid containing 891-bp fragment with $bla_{\text{SHV-1}}$ gene; resistance phenotype: amoxicillin, kanamycin	This study
pBK-CMV	Phagemid vector; resistance phenotype: kanamycin	Stratagene

supplemented with 30 μg/ml kanamycin and 16 μg/ml ticarcillin. The sequence and the orientation of the inserted open reading frames were determined from PCR experiments, which were performed with different combinations of the primers pBK-CMV1′ (5′-CTAGTGGATCCAAAGAATTCAAAAAGC-3′), pBK-CMV2′ (5′-AATTGGGTACACTTACCTGGTACCC-3′), SHVsf, and SHVsr.

β-Lactamase preparation. The SHV-producing clones were grown for 18 h at 37°C in 6 liters of Luria-Bertani broth complemented with yeast extract, 30 μg/ml of kanamycin, and 16 μg/ml of ticarcillin. After centrifugation, bacterial pellets were suspended with MES-NaOH (20 mM) (pH 6) and disrupted by ultrasonic treatment as previously described (5). The extract was then clarified by centrifugation and treated with DNase I (Roche Applied Science, Meylan, France). Purification was carried out by ion-exchange chromatography with an SP Sepharose column or a HiPrep 16/10 SP HF column (Amersham Pharmacia Biotech) and gel filtration chromatography with a Superose 12 or HiPrep 16/60 Sephacryl S-100 HR column (Amersham Pharmacia Biotech), using a fast-protein liquid chromatography system as previously described (4). The protein concentration was estimated by using the BCA protein assay kit (Pierce, Rockford, IL). The purity of enzymes was estimated by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis as previously described (4).

Determination of β-lactamase kinetic constants. The Michaelis constant (K_m) and catalytic activity (k_{cat}) of the SHV-1 and SHV-72 enzymes were obtained with purified extracts by a computerized microacidimetric method, using a 702 SM Titrino pHstat apparatus (Metrohm, Herisau, Switzerland) (20). These kinetic parameters were determined by analysis of the complete hydrolysis time courses, and the kinetic progress curves were fitted by nonlinear least-squares regression (20). The concentrations of the β-lactamase inhibitors (clavulanate and tazobactam) necessary to inhibit the enzyme activity by 50% (IC_{50}s) were determined as described elsewhere (4), using 200 μM ticarcillin as the reporter substrate. The kinetic constants were determined three times for each substrate tested.

MDS. The model of the mutant enzyme (SHV-72) was constructed on the basis of the SHV-1 crystal structure (19). The SHV-72 and SHV-1 enzymes were solvated with water in a periodic cubic box that was large enough to contain the system and 1 nm of solvent on all sides. Version 1.8.2 of the VMD software package was used to manipulate the two systems (16). The GROMACS software package, version 3.2 (24), and the geometric and charge parameters of the OPLSAA (optimized potentials for liquid simulations in all-atom) force field (18) were used to carry out all energy minimizations and MDSs. TIP3P parameters were used for the water molecules (17). The particle-mesh Ewald method was used to treat long-range electrostatics (12). All covalent bond lengths were constrained by the SHAKE algorithm (32) with a relative tolerance of 10^{-4}. The systems were equilibrated as reported previously (27), and MDSs of 400 ps were then made with a time step of 1.5 fs and coordinates collected every 0.0015 ps. The velocities of all atoms were generated from a Maxwellian distribution. The temperature was kept constant at 300 K, while the pressure was kept constant by the weak coupling constant of 1 bar using Berendsen's algorithms (25).

Nucleotide sequence accession number. The new bla_{SHV} nucleotide sequence was submitted to the EMBL nucleotide sequence database as $bla_{\text{SHV-72}}$ with accession number AM176547.

RESULTS

Phenotypic characterization. The clinical *K. pneumoniae* INSRA1229 strain exhibited resistance to amoxicillin (2,048 μg/ml), ticarcillin (512 μg/ml), and piperacillin (32 μg/ml) (Table 2). The strain was susceptible to cephalosporins, monobactams, imipenem, ciprofloxacin, gentamicin, and trimethoprim. *K. pneumoniae* INSRA1229 was also resistant to amoxicillin

TABLE 2. MICs of β-lactam antibiotics for the clinical *K. pneumoniae* strain INSRA1229, SHV-72- and SHV-1-producing transformants, and the recipient *E. coli* DH5α Δ*ampC*

Antimicrobial drug	MIC (μg/ml) for strain			
	E. coli DH5α Δ*ampC*	*E. coli* DH5α-SHV-1[a]	*E. coli* DH5α-URA1229[b]	*K. pneumoniae* INSRA1229
Amoxicillin	8	2,048	512	2,048
Amoxicillin + CLA[c]	8	8	64	64
Ticarcillin	4	1,024	8	512
Piperacillin	2	64	4	32
Piperacillin + TAZ[d]	1	2	2	2
Amdinocillin	0.125	0.5	0.25	0.25
Cephalothin	8	16	4	8
Cefuroxime	4	4	8	2
Cefoperazone	≤0.25	4	≤0.25	2
Ceftriaxone	0.03	0.03	0.03	0.03
Ceftriaxone+ CLA	0.06	0.03	0.03	≤0.015
Cefotaxime	0.06	0.06	0.06	0.125
Cefotaxime + CLA	0.06	0.06	0.06	0.06
Ceftazidime	0.25	0.5	0.25	0.06
Ceftazidime + CLA	0.125	0.25	0.25	0.06
Aztreonam	0.06	0.125	0.125	≤0.015
Aztreonam + CLA	0.06	0.125	0.125	≤0.015
Cefepime	0.03	0.03	0.03	0.03
Cefoxitin	4	8	8	4
Imipenem	0.25	0.25	0.125	0.125
Ciprofloxacin	≤0.125	≤0.125	≤0.125	≤0.125
Gentamicin	≤0.125	0.25	0.25	0.5
Trimethoprim	≤0.125	≤0.125	≤0.125	0.5

[a] *E. coli* DH5α-SHV-1 was the transformant producing SHV-1.

[b] SHV-72-producing *E. coli* DH5α-URA1229 was the transformant corresponding to *K. pneumoniae* INSRA1229.

[c] CLA, clavulanic acid at a fixed concentration of 2 μg/ml.

[d] TAZ, tazobactam at a fixed concentration of 4 μg/ml.

TABLE 3. Kinetic parameters of SHV-72 and SHV-1

Antibiotic	SHV-1			SHV-72		
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)
Penicillin G	23 ± 0.42	1,937 ± 82	84.2 ± 2.0	19 ± 0.92	5,438 ± 176	286.2 ± 23.5
Amoxicillin	31 ± 1.29	1,044 ± 10	33.7 ± 1.1	26 ± 6.97	5,876 ± 79	226.0 ± 66.4
Ticarcillin	11 ± 3.40	220 ± 49	20.0 ± 1.7	36 ± 3.45	1,279 ± 24	35.5 ± 2.8
Piperacillin	24 ± 0.53	1,490 ± 96	62.1 ± 2.7	43 ± 5.45	1,918 ± 33	44.6 ± 6.5
Cephalothin	40 ± 1.46	128 ± 33	3.2 ± 0.8	141 ± 12.5	38 ± 7	0.3 ± 0.1
Ceftazidime	ND[a]	<0.1	ND	ND	<0.1	ND
Cefotaxime	ND	<0.1	ND	ND	<0.1	ND

[a] ND, not determinable.

combined with clavulanic acid (64 μg/ml). The MIC of piperacillin-tazobactam was much lower than that for the amoxicillin-clavulanic acid combination (Table 2).

Molecular characterization of *bla*$_{SHV-72}$ gene. The *bla*$_{SHV}$ gene of INSRA1229 was amplified and sequenced. The sequence showed the nonsynonymous nucleotide mutations A10T, C425T, and A689G, compared to the sequence of *bla*$_{SHV-1}$. According to Ambler numbering (1), these mutations lead to the two previously described amino acid substitutions Ile8Phe and Ala146Val and to the new substitution Lys234Arg. The corresponding enzyme was designated SHV-72, and its gene was cloned downstream of the *lac*Z promoter of the plasmid pBK-CMV, as well as *bla*$_{SHV-1}$. The SHV-72-producing clone, designated *E. coli* DH5α-URA1229, exhibited a β-lactam resistance phenotype similar to that of the clinical strain (Table 2).

Biochemical properties of β-lactamase SHV-72. The clinical strain and the corresponding clone produced only β-lactamases of pI 7.6, which is compatible with the amino acid sequence of SHV-72. SHV-72 and SHV-1 were purified from the *E. coli* clones by ion exchange and gel filtration. The rate of purity was estimated to be ≥96% for SHV-72 and ≥95% for SHV-1 on a sodium dodecyl sulfate-polyacrylamide gel as a band of 28 kDa, which corresponded to the molecular mass deduced from the amino acid sequence (data not shown).

The kinetic parameters of SHV-72 were determined for seven β-lactams and compared with those of SHV-1 (Table 3). k_{cat} values of SHV-72 against penicillins were higher than those of SHV-1, and K_m values were comparable or slightly higher (19 to 43 μM versus 11 to 31 μM). Overall, the catalytic efficiency against penicillins was slightly higher for SHV-72 (k_{cat}/K_m, 36 to 286 $\mu M^{-1} \cdot s^{-1}$) than for SHV-1 (k_{cat}/K_m, 20 to 84 $\mu M^{-1} \cdot s^{-1}$), except for piperacillin (k_{cat}/K_m, 45 $\mu M^{-1} \cdot s^{-1}$ versus 62 $\mu M^{-1} \cdot s^{-1}$). In contrast with penicillins, SHV-72 had a lower catalytic efficiency against cephalothin (11-fold) than SHV-1. Neither SHV-1 nor SHV-72 exhibited catalytic activity against oxyimino β-lactams.

The IC_{50}s for SHV-1 and SHV-72 are as follows: for clavulanic acid, the IC_{50} was 0.17 μM for SHV-1 and 1.72 μM for SHV-72; for tazobactam, the IC_{50} was 0.11 μM for SHV-1 and 0.08 μM for SHV-72. The IC_{50} of clavulanic acid was 10-fold higher for SHV-72 than for SHV-1, and the IC_{50}s of tazobactam were similar for the two enzymes. Tazobactam was 22-fold more active than clavulanic acid against the SHV-72 enzyme.

MDS. The enzyme SHV-72 was modeled from the crystallographic structure of SHV-1 (19). The behaviors of SHV-72 and SHV-1 were compared during MDSs of 400 ps at a temperature of 300 K. The MDS for each model were checked for stability by monitoring several overall properties, such as the radius of gyration, secondary structure, root mean squared deviation (RMSD) from the initial structure, and the kinetic and potential energies (data not shown). These parameters were found to stabilize after about 100 ps, and hence, data from 300 ps were used for all subsequent analyses. The radius of gyration and the RMSDs of Cα atoms were similar to those for the crystallographic structure of SHV-1 (Table 4). The secondary structure was also preserved during the simulation (Table 4). The relatively small deviations were further evidence of the inherent stability of the model and indicated that the dynamic structures of the models remained in the realm of the crystal SHV-1 geometry during the course of the simulation. The largest fluctuations were localized in loops connecting the secondary structure elements, as is usual in MDSs of proteins (data not shown). The introduction of the Ala146Val

TABLE 4. Summary of statistical data for 300-ps MDSs

Enzyme and use	Radius of gyration (Å)[a]	Cα RMSD (Å)	Secondary structure analysis (%)			
			Helix	β-sheet	Coil	Turn
Molecular modeling						
SHV-72	1.81 ± 0.01	0.88 ± 0.12[b]	41.5 ± 2.7	16.7 ± 0.8	18.2 ± 0.8	14.6 ± 1.8
SHV-1	1.80 ± 0.00	0.90 ± 0.16[b]	42.8 ± 2.6	17.2 ± 0.4	18.2 ± 0.6	12.7 ± 1.4
Crystal structure						
SHV-1[c]	1.79	1.20 ± 0.11[d]	44.5	17.0	17.3	13.2

[a] Average radius of gyration of nonhydrogen atoms.

[b] RMSD of α-carbon atoms with respect to the minimized starting structures.

[c] Crystal structure of β-lactamase SHV-1 (PDB identification code 1SHV) (19).

[d] RMSD of α-carbon atoms with respect to the molecular dynamics structures.

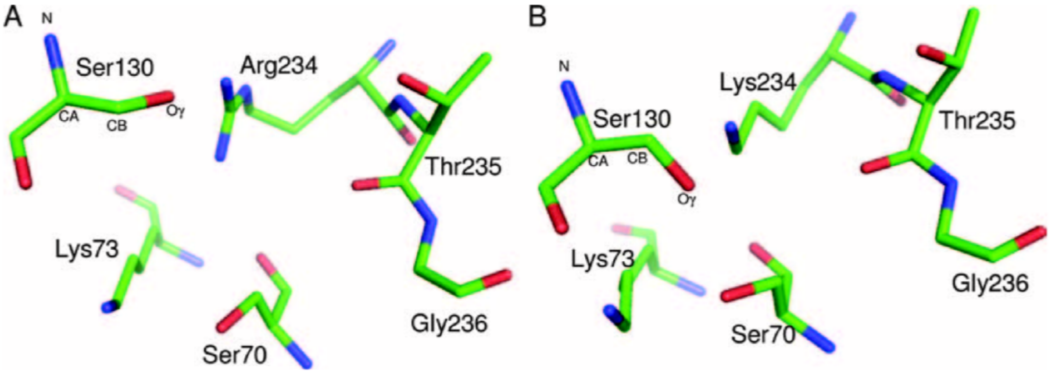

FIG. 1. The two conformations of the Ser130 side chain. (A) SHV-72 exhibiting a Ser130 χ1 angle value of −145°. (B) SHV-1 exhibiting a Ser130 χ1 angle value of −60°. χ1 is the angle between the plane containing the atoms N, CA, and CB and the plane containing the atoms CA, CB, and Oγ.

and Lys234Arg substitutions caused no overall or large-scale deviation of the dynamic properties.

Position 146, which is located at the surface of the protein and at a distance from the catalytic site (distance between Cα of positions 70 and 140, 16.7 Å), did not modify the positioning of surrounding residues. Residue Arg234 is located in the catalytic site and adopted the conformation observed in the crystallographic structure of the class A β-lactamase PSE-4 (Fig. 1A) (24). Overall, the architectures of the active site were identical for the two enzymes SHV-72 and SHV-1. All residues of the active site except residue Ser130 had similar positioning in SHV-1 and SHV-72.

The behaviors of the Ser130 side chain were different in the two enzymes. In the initial set of MDSs, the χ1 angle of Ser130 was around −145° in the starting models (Fig. 1A and 2A and C), as observed in the crystal structures of class A enzymes such as the TEM-, SHV- and CTX-M-type enzymes (11, 19, 29). After 130 ps of MDS, the Ser130 χ1 angle of SHV-72 increased to −61 ± 11° and thereafter remained stable until the end of the simulation (Fig. 2A). In SHV-1, the χ1 angle kept the value of −145 ± 12° during almost all of the simulation (Fig. 2C).

In a second set of MDSs, the χ1 angle of Ser130 was set at −61° in the starting models by manual modeling (Fig. 1B and 2B and D). For SHV-72, the χ1 angle value of Ser130 was −67 ± 10° and stable (Fig. 2B). In contrast, the Ser130 χ1 angle of SHV-1 decreased after 160 ps from −58 ± 10° to −153 ± 20° and remained stable until the end of the MDS (Fig. 2D). The

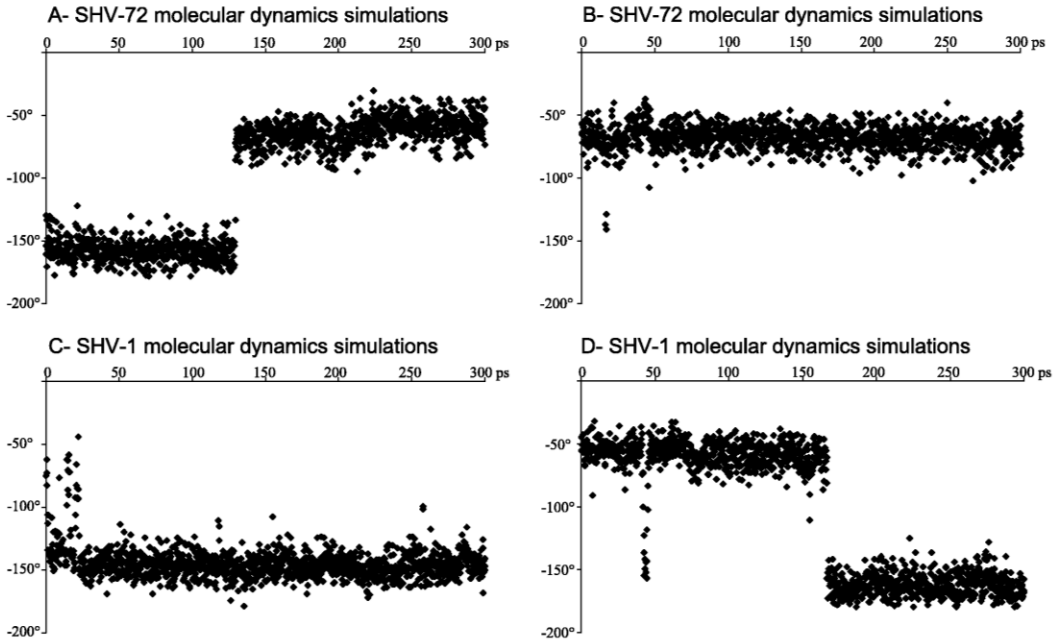

FIG. 2. χ1 angle of residue Ser130 during 300-ps MDSs. (A) SHV-72 with a starting χ1 angle of −148°. (B) SHV-72 with a starting χ1 angle of −60°. (C) SHV-1 with a starting χ1 angle of −148°. (D) SHV-1 with a starting χ1 angle of −60°.

Lys234Arg substitution thus stabilized an atypical conformation of the Ser130 side chain. This conformation was characterized by an $\chi1$ angle between −50° and −77°, which moved the Oγ atom of Ser130 around 3.5 Å away from the key Oγ atom of the reactive serine (Ser70).

DISCUSSION

The study of β-lactamases that are resistant to inhibitors is of great importance owing to the restricted number of drugs capable of eluding bacterial resistance. In this work we have characterized a new enzyme, SHV-72, from a *K. pneumoniae* strain isolated in a Portuguese hospital, that exhibits unusual resistance to amoxicillin and the amoxicillin-clavulanic acid combination. SHV-72 harbored three substitutions: Ile8Phe in the leader peptide, Ala146Val at a distance from the catalytic pocket (distance between Cα atoms 146 and 70, ≈17 Å), and the Lys234Arg substitution in the catalytic pocket. To our knowledge, this last substitution is observed for the first time in a natural SHV/TEM-type β-lactamase (23). The resulting new enzyme induced a resistance phenotype compatible with that of an inhibitor-resistant penicillinase. No effect was observed on the imipenem MIC, despite the presence of the Ala146Val substitution (30). The kinetic constant confirmed a high catalytic efficiency against penicillins, no significant activity against oxyimino β-lactams, and a decreased susceptibility to clavulanate in comparison with results for SHV-1.

Only the three SHV-type enzymes SHV-10, SHV-26, and SVH-49 have been previously described as resistant to inhibitors. In contrast to SHV-72, these enzymes have an increase in K_m values and/or a decrease in k_{cat} values against penicillins (10, 14, 31). In SHV-10 and SHV-49, the amino acid substitutions Ser130Gly and Met69Ile reduced activity against β-lactam substrates (14, 31). The introduction of Lys234Arg in TEM-1 by site-directed-mutagenesis experiments induce a 10-fold decrease of the affinity against penicillins (23). The mutations observed in SHV-72 did not significantly affect K_m values and did not decrease catalytic activities against penicillins.

Among IRSs, SHV-10 is the most resistant (with an IC_{50} 41-fold higher than that for SHV-1) to inhibitors and SHV-26 the least (with an IC_{50} threefold higher than that for SHV-1) (10, 30). SHV-72, like SHV-49 (14), was 10-fold more resistant to clavulanic acid than SHV-1. The Ser130Gly substitution in SHV-10 also induces resistance to inhibitors in TEM-, OXY- and CTX-M-type enzymes (2, 22, 31, 33). The mechanism of inhibition by clavulanic acid is based on the formation of a covalent cross-link between Oγ atoms of Ser70 and Ser130 by residual atoms of the inhibitor. In enzymes harboring Gly130, this residue, which is deprived of the side chain, prevents cross-linking with position 70 (34–36). In SHV-49, the Met69Ile substitution is responsible for resistance to inhibitors (14). By analogy with inhibitor-resistant TEMs (38), substitution at position 69 may decrease the susceptibility to inhibitors because of the modification of Ser130 side-chain positioning. In SHV-72, the Lys234Arg substitution is probably responsible for resistance to inhibitors owing to its location in the catalytic pocket and in the vicinity of residue 130.

To understand the role of Arg234 in resistance to clavulanic acid, SHV-72 was modeled from the SHV-1 crystal structure (19) and analyzed during MDSs. SHV-72 and SHV-1 models exhibited different behaviors only in the local region of residue 234. In Lys234-harboring class A β-lactamases, the conformation of the Ser130 side chain is such that its $\chi1$ values are in the range of −120.5° to −163.5°, as for SHV-1 (−140°). In SHV-72 MDSs, an alternative conformation of the Ser 130 side chain ($\chi1 \approx -64°$) appeared because of a hydrogen bond with Arg234, as previously observed in the crystal structure of the Arg234-harboring enzyme PSE-4 (24). This alternative conformation is probably stabilized because of the restricted ability of Arg234 to move and establish a hydrogen bond, since hydrogen bonds are favorable only in the plane of the rigid arginine guanidium group. This conformation is probably involved in the weak susceptibility to inhibitors of SHV-72.

The change in the $\chi1$ angle by around −64° moved the Ser130 Oγ atom away from the reactive Ser70 Oγ atom. This movement of the Oγ atom of Ser130, which is the ultimate covalent attachment point for the inhibitors, may therefore prevent the cross-link. Such a resistance mechanism has been previously proposed for TEM-32, for which the Met69Ile substitution induces, by another mechanism, the same movement of the Ser130 side chain ($\chi1$ angle, −64°) (38). The effects of the Met69Ile, Ser130Gly, and Lys234Arg substitutions therefore share a common logic for inhibitor resistance.

The Oγ atom of Ser130 plays a role in the hydrolysis of β-lactams (21). In SHV-72, this role could presumably be disrupted. However, the enzyme did not lose its catalytic efficiency. This behavior may be explained by the coexistence of the two Ser130 conformations and/or the replacement of the Ser130 Oγ atom by a water molecule, as observed in the crystal structures of the enzymes deprived of Ser130, such as SHV-10 and TEM-76 (35, 36).

In conclusion, we report a new SHV-type penicillinase resistant to clavulanic acid. The resistance to clavulanic acid is induced by the Lys234Arg substitution, which probably affects the positioning of the Ser130 side chain, a key element of the inhibition reaction mediated by clavulanic acid.

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Chapter 2. Class A β-lactamases: function meets structure

Paper V. Biochemical study of a new inhibitor-resistant β-lactamase, SHV-84, produced by a clinical *Escherichia coli* strain

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Biochemical Study of a New Inhibitor-Resistant β-Lactamase, SHV-84, Produced by a Clinical *Escherichia coli* Strain[▽]

Inhibitor-resistant TEM (IRT) β-lactamases derive from TEM-1 or TEM-2 enzymes by point mutations in the corresponding coding gene (http://www.lahey.org/studies). Inhibitor-resistant SHV (IRS) β-lactamases are less commonly identified than IRT enzymes, and mainly in *Klebsiella pneumoniae* strains (3, 4, 5, 8, 9). In this study, we performed a phenotypic, molecular, and biochemical characterization of a new IRS enzyme, SHV-84, produced by a clinical *Escherichia coli* strain.

E. coli INSRA4590 was isolated from a patient admitted in 1999 to Centro Hospitalar de Coimbra, Portugal. Antimicrobial susceptibility, isoelectric focusing for isoelectric point determination, identification of β-lactamase-encoding genes, and extraction and purification of a new β-lactamase were performed as previously described (1, 7). Antimicrobial susceptibility results were interpreted by using French Society of Microbiology criteria (2). *E. coli* INSRA4590 exhibited high-level resistance to the penicillins tested (except piperacillin) and to the combination of amoxicillin and clavulanic acid but was susceptible to the combination of piperacillin and tazobactam (Table 1); it was susceptible to all cephalosporins tested and to aztreonam.

The clinical strain expressed SHV-1 with a pI of 7.6 and the new SHV-84 β-lactamase with a pI of 7.4, which differed from SHV-1 by the amino acid substitution Lys234Arg. This mutation has been described in other IRS enzymes, such as SHV-56 (5) and SHV-72 (also encountered in Portugal) (8), in which it was associated with the substitution Leu35Gln and the substitutions Ile8Phe and Ala146Val, respectively. The recombinant SHV-encoding plasmid (pBK-SHV-84) and the corresponding transformant *E. coli* DH5α were obtained as previously described (8). The SHV-84-producing transformant DH5α-SHV-84 exhibited a β-lactam resistance phenotype similar to that of the clinical strain (Table 1), and the corresponding β-lactamase had a pI of 7.4.

The kinetic constants of the purified enzyme (≥99% pure; data not shown) and the concentrations of inhibitors required to inhibit enzyme activity by 50% (IC_{50}s) were determined as previously reported (6, 8) and compared with those of SHV-1. Unlike SHV-72, SHV-84 showed a lower affinity for penicillins (K_m, 64 to 101 μM) and decreased catalytic activity against these antibiotics (k_{cat}, 216 to 1,042 s^{-1}) than SHV-1 (K_m, 11 to 31 μM; k_{cat}, 220 to 1,937 s^{-1}) (Table 1). In SHV-72, mutations other than Lys234Arg might confer increased affinity of the enzyme for penicillins, as well as better catalytic activity against those antibiotics. SHV-84, like SHV-72, exhibited lower affinity for cephalothin (K_m, 169 to 141 μM) than SHV-1. No hydrolysis of extended-spectrum cephalosporins was detected. However, SHV-84 was less susceptible to clavulanic acid (IC_{50}, 2.21 μM) than SHV-1 (IC_{50}, 0.17 μM), as observed for SHV-72 (IC_{50}, 1.72 μM) (Table 1) (8). The IC_{50} of tazobactam for SHV-84 was 3.5-fold lower than that for SHV-1 (IC_{50}s, 0.03 and 0.15 μM, respectively).

In conclusion, this study underlines the importance of the Lys234Arg substitution in resistance to clavulanic acid in nature, since we demonstrate that this mutation alone is responsible for decreased susceptibility to β-lactamase inhibitors. These results corroborate those previously obtained by molecular dynamic simulation in a study using the model of the mutant SHV-72 enzyme, in which the authors suggested a change in the positioning of the Ser130 side chain induced by Arg234 (8).

Nucleotide sequence accession number. The new bla_{SHV} nucleotide sequence was submitted to the EMBL Nucleotide

TABLE 1. MICs for clinical, transformant, and recipient *E. coli* strains and kinetic parameters of SHV-84 and SHV-1 β-lactamases

Antimicrobial(s)	MIC (μg/ml) for *E. coli* strain:				Kinetic parameters of enzyme[a]					
					SHV-1[c]			SHV-84		
	DH5α	DH5α-SHV-1 (pBK-SHV-1)[b]	DH5α-SHV-84(pBK-SHV-84)[b]	INSRA4590 (SHV-84)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)
Penicillin G	ND[f]	ND	ND	ND	23 ± 0.42	1,937 ± 82	84.2 ± 2.0	64 ± 0.81	611 ± 43	9.5 ± 0.8
Amoxicillin	≤2	2,048	2,048	512	31 ± 1.29	1,044 ± 10	33.7 ± 1.1	101 ± 2.75	1,042 ± 116	10.4 ± 1.4
Amoxicillin + CLA[d]	≤2	8	>64	64	ND	ND	ND	ND	ND	ND
Ticarcillin	≤2	1,024	>4,096	>4,096	11 ± 3.40	220 ± 49	20.0 ± 1.7	83 ± 5.97	216 ± 20	2.5 ± 0.01
Piperacillin	1	64	128	64	24 ± 0.53	1,490 ± 96	62.1 ± 2.7	68 ± 2.90	406 ± 15	6.0 ± 0.03
Piperacillin + TAZ[e]	1	2	2	4	ND	ND	ND	ND	ND	ND
Cephalothin	1	16	8	8	40 ± 1.46	128 ± 33	3.2 ± 0.8	169 ± 0.31	4 ± 0.3	0.02 ± 0.002
Ceftazidime	0.06	0.5	0.25	0.25	142 ± 3.18	<0.1	NH[g]	NH	<0.1	NH
Cefotaxime	≤0.015	0.06	0.06	0.125	257 ± 20.65	<0.1	NH	NH	<0.1	NH
Aztreonam	≤0.015	0.125	0.03	0.03	ND	ND	ND	ND	ND	ND

[a] Values are means ± standard deviations.
[b] *E. coli* DH5α-SHV-1 and *E. coli* DH5α-SHV-84 were the transformants producing SHV-1 and SHV-84, respectively. The MICs for SHV-1 are from reference 8.
[c] The kinetic constants of SHV-1 are from reference 8.
[d] CLA, clavulanic acid at a fixed concentration of 2 μg/ml.
[e] TAZ, tazobactam at a fixed concentration of 4 μg/ml.
[f] ND, not determined.
[g] NH, not determinable because the hydrolysis rates were too low.

Sequence Database as $\mathit{bla}_{\text{SHV-84}}$ with accession number AM087453.

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Chapter 2. Class A β-lactamases: function meets structure

Paper VI. Characterization of the inhibitor-resistant SHV β-lactamase (SHV-107) in a clinical *Klebsiella pneumoniae* strain co-producing GES-7 enzyme

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Abstract

The clinical *K. pneumoniae* INSRA6884 strain exhibited non-susceptibility to all penicillins tested (MICs of 64 to >2048 µg/ml). The MICs of penicillins were weakly reduced by clavulanate (from 2048 to 512 µg/ml) and tazobactam restored the piperacillin susceptibility. Molecular characterization identified the genes *bla*$_{\text{GES-7}}$ and a new β-lactamase gene blaSHV-107, which encoded an enzyme that differed from SHV-1 by the amino acid substitutions Leu35Gln and Thr235Ala. The SHV-107-producing *E. coli* strain exhibited only a β-lactam resistance phenotype with respect to amoxicillin, ticarcillin and amoxicillin/clavulanate combination. The kinetic parameters of the purified SHV-107 enzyme revealed strong affinity to penicillins. However, catalytic efficiency for these antibiotics was lower for SHV-107 than for SHV-1. No hydrolysis was detected against oxyimino β-lactams. The IC_{50} for clavulanic acid was 9-fold higher for SHV-107 than for SHV-1, but tazobactam inhibitory effects did not change in comparison to the parental enzyme. Molecular dynamics simulation suggested that the substitution Thr235Ala affects the accommodation of clavulanate in the binding site and therefore its inhibitory activity.

Keywords: Antibiotic resistance, β-lactam inhibitor, β-lactamase, ESBL, inhibitor-resistant SHV.

Introduction

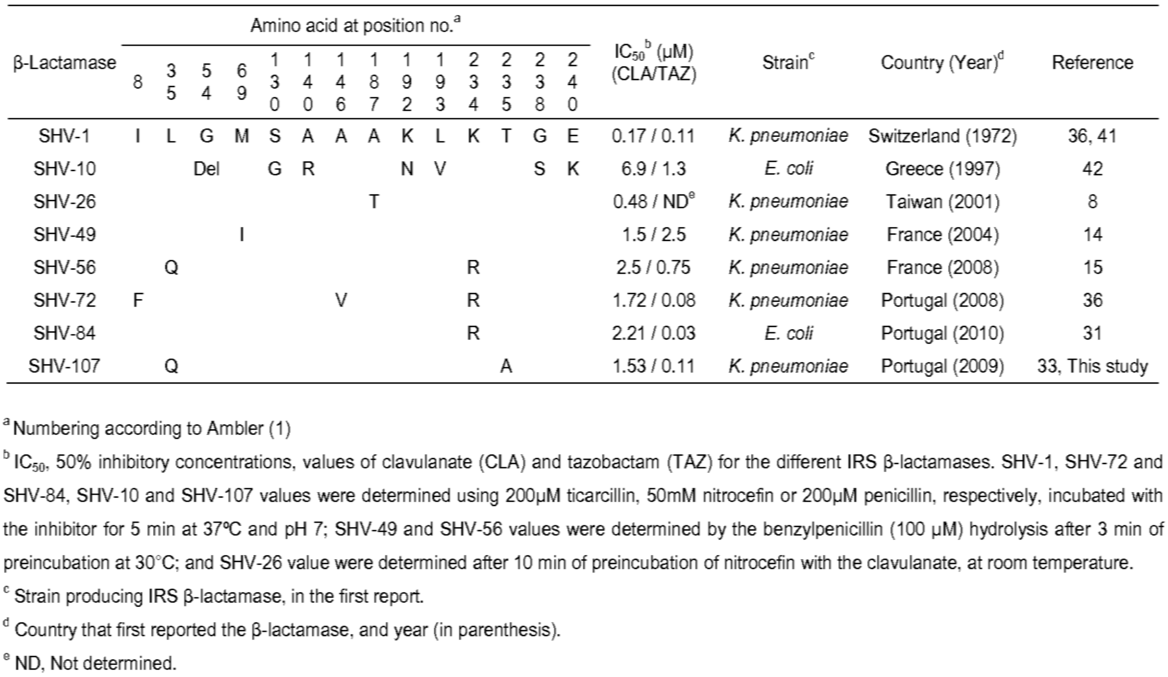
β-Lactamases are the most important mechanism of β-lactam drug resistance in Gram-negative bacteria. They hydrolyze and inactivate β-lactams and are divided into four major classes (A-D) on the basis of their sequence (1). Class B is composed of metallo-enzymes which require the presence of zinc cations for activity, classes A, C and D are hydrolases with an active site serine (1, 5). Class A enzymes comprise several enzyme families, including the clinically relevant β-lactamases TEM-1 and SHV-1 (40). These enzymes confer resistance to penicillins and to narrow spectrum cephalosporins, but are inhibited by β-lactamase inhibitors such as clavulanate, which have been developed to evade the activity of these β-lactamases (18, 43).

However, the broad spectrum β-lactamases TEM-1, TEM-2 and SHV-1 have acquired point mutations in key positions, often near the active site, that have given rise to (i) TEM/SHV-type extended-spectrum β-lactamases (ESBL), which hydrolyze oxyimino β-lactams, (ii) TEM/SHV-type inhibitor resistant β-lactamases and (iii) complex mutant TEMs (CMTs), which can associate resistance to β-lactam inhibitors and activity against oxyimino β-lactams (4, 11, 13, 29, 39). In addition, many recent reports have described the emergence of class A ESBLs belonging to other families, such as GES, VEB, PER and the worldwide disseminated CTX-M family (2, 25). The GES family, namely GES-1, has also been reported in large areas including Portugal (33).

Since 1992, about 35 inhibitor resistant TEMs (IRTs) and 11 CMTs have been found (http://www.lahey.org/Studies/). However, only six inhibitor-resistant SHVs (IRS) have been detected clinically so far, mainly in *Klebsiella pneumoniae* strains, in different countries (8, 14, 15, 31, 37, 42) (Table 1). No complex mutant SHV has been encountered, which is surprising since SHV enzymes are usually more susceptible to inactivation by clavulanate than TEM, due to differences in the enzyme active sites (4, 11). In the SHV family, naturally occurring inhibitor resistant phenotypes harbor substitutions at positions Met69 (SHV-49), Ser130 (SHV-10), Arg187 (SHV-26) and Lys234 (SHV-56, SHV-72 and SHV-84) (numbering according to Ambler classification) (1, 11) (Table 1). Most enzymes and the corresponding substitutions have been further investigated. However, the recently reported enzyme SHV-107 (AM941848) (33), which is suspected to belong to the IRS group, has not been characterized.

In this study, we report in a clinical *K. pneumoniae* strain the co-expression of the ESBL GES-7 and a new IRS-type enzyme called SHV-107, which harbors the substitution Thr235Ala. The kinetic constants of SHV-107 were determined and molecular modeling was undertaken to investigate the role of Thr235Ala in the resistance to clavulanate.

Table 1 - Comparison of amino acid substitutions, inhibitory properties and epidemiology of IRS β-lactamases.



β-Lactamase	Amino acid at position no.[a]														IC_{50}[b] (μM) (CLA/TAZ)	Strain[c]	Country (Year)[d]	Reference
	8	35	54	69	130	140	146	187	192	193	234	235	238	240				
SHV-1	I	L	G	M	S	A	A	A	K	L	K	T	G	E	0.17 / 0.11	*K. pneumoniae*	Switzerland (1972)	36, 41
SHV-10			Del		G	R			N	V			S	K	6.9 / 1.3	*E. coli*	Greece (1997)	42
SHV-26								T							0.48 / ND[e]	*K. pneumoniae*	Taiwan (2001)	8
SHV-49				I											1.5 / 2.5	*K. pneumoniae*	France (2004)	14
SHV-56		Q									R				2.5 / 0.75	*K. pneumoniae*	France (2008)	15
SHV-72	F						V				R				1.72 / 0.08	*K. pneumoniae*	Portugal (2008)	36
SHV-84											R				2.21 / 0.03	*E. coli*	Portugal (2010)	31
SHV-107		Q										A			1.53 / 0.11	*K. pneumoniae*	Portugal (2009)	33, This study

[a] Numbering according to Ambler (1)

[b] IC_{50}, 50% inhibitory concentrations, values of clavulanate (CLA) and tazobactam (TAZ) for the different IRS β-lactamases. SHV-1, SHV-72 and SHV-84, SHV-10 and SHV-107 values were determined using 200μM ticarcillin, 50mM nitrocefin or 200μM penicillin, respectively, incubated with the inhibitor for 5 min at 37ºC and pH 7; SHV-49 and SHV-56 values were determined by the benzylpenicillin (100 μM) hydrolysis after 3 min of preincubation at 30°C; and SHV-26 value were determined after 10 min of preincubation of nitrocefin with the clavulanate, at room temperature.

[c] Strain producing IRS β-lactamase, in the first report.

[d] Country that first reported the β-lactamase, and year (in parenthesis).

[e] ND, Not determined.

Materials and methods

Bacterial strains and plasmid. *K. pneumoniae* INSRA6884 was isolated from sputum of a 92 year old female, in 2006, in an internal medicine service at the Centro Hospitalar in Coimbra, Portugal (33). *Escherichia coli* DH5α ∆*ampC* and *E. coli* BL21(DE3) were used for initial cloning experiments and overexpression of the β-lactamase-encoding gene, respectively. *E. coli* C600 was used for mating-out assays. Plasmid pBK-CMV (Stratagene, Amsterdam, The Netherlands) was used for cloning experiments (9).

Antimicrobial susceptibility testing and ESBL detection. MICs were determined by the agar dilution method, in Mueller Hinton medium, according to the French Society of Microbiology (SFM) (7). MICs of β-lactam antibiotics were determined alone and combined at a fixed concentration of clavulanate (2 µg/ml) with amoxicillin, ceftriaxone, cefotaxime, ceftazidime and aztreonam, or of tazobactam (4 µg/ml) with piperacillin. We considered as an ESBL producer a strain for which any MIC of extended-spectrum cephalosporins and/or monobactam was three-fold lower in the presence of 2 µg/ml of clavulanate, as described previously (34).

Analytical isoelectric focusing (IEF). β-Lactamases were characterized by isoelectric focusing of ultrasonicated bacterial extracts, as previously described (6), with strains *E. coli* C600 (SHV-1, pI 7.6), *K. pneumoniae* INSRA5767 (TEM-24, pI 6.5 and SHV-1, pI 7.6) and *E. coli* INSRA7813 (GES-1, pI 5.8) as standards.

DNA amplification and sequencing of β-lactamase genes. β-lactamase genes bla_{SHV}, bla_{OXA}, bla_{TEM}, bla_{CTX} and bla_{GES} were detected by PCR amplification as previously described (33). PCR products were purified with ExoSAP IT (USB Corporation, Cleveland, Ohio, USA), and all amplicons were further sequenced directly on both strands using the automatic sequencer ABI3100 (Applied Biosystems, Warrington, UK).

Cloning. $bla_{\mathrm{SHV\text{-}107}}$ gene was cloned downstream of the inducible lacZ promoter in the plasmid pBK-CMV and transferred into electrocompetent *E. coli* DH5α Δ*ampC* by electroporation as previously described (35). After plasmid DNA extraction using the Wizard *Plus* Midipreps purification system (Promega, Madison, WI), the recombinant SHV-encoding plasmid (pBK-SHV-107) was transformed by electroporation into the expression host *E. coli* BL21(DE3) strain. *E. coli* transformants were selected on LB agar supplemented with 30 µg/ml of kanamycin and 50 µg/ml of amoxicillin. To confirm the presence and to determine the orientation of the inserted genes, PCR was performed as previously (35).

β-Lactamase gene transfer experiments. Direct transfer of the β-lactam resistance phenotype was performed by liquid and solid mating-out assays at 37°C using streptomycin-resistant *E. coli* K12 C600 as the recipient strain. Transconjugants were selected on Mueller-Hinton agar plates containing 25µg/ml streptomycin and 100µg/ml amoxicillin. Selected transformants were subjected to antibiotic susceptibility testing, IEF and PCR analysis.

Purification of β-lactamase. SHV-107 was produced from *E. coli* **BL21**(DE3) in LB broth, supplemented with 30µg/ml kanamycin, 16µg/ml ticarcillin and 0.1mM isopropyl-β-D-thiogalactopyranoside (Sigma Chemical Co., St. Louis, Mo.), overnight at 30ºC. The enzyme was extracted by ultrasonic treatment, and the clarified supernatant was purified as described elsewhere (34). SHV-107 total protein concentration was estimated by the BCA protein assay kit (Pierce, Rockford, IL, USA). The purity of enzyme extracts was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (3).

Determination of β-lactamase kinetic constants. The Michaelis constant (K_m) and catalytic activity (k_{cat}) of SHV-107 were determined with purified extracts by a computerized microacidimetric method, using 702 SM Titrino pHstat (Metrohm, Herisau, Swiss) (31). The concentrations of the inhibitors (clavulanate and tazobactam) required to inhibit enzyme activity by 50% (IC_{50}) were determined as previously described (3). Specific activity and IC_{50} were monitored with penicillin G (200 µM) as the reporter substrate.

The inhibitor kinetics with clavulanate were determined under steady-state conditions for SHV-1 and SHV-107 as previously described (19, 32). The enzyme activity was monitored with UV-1800 spectrophotometer (Shimadzu, Champs sur Marne, France) in PBS 20mM, pH 7,4 at 25°C using 100µM chromogenic substrate nitrocephin (Δε, 17,400 M^{-1} cm^{-1} at 482 nm) (Sigma Aldrich, France) as the reporter substrate.

The kinetic constants were determined three times for each subtract tested.

Molecular modelling. The structural model of SHV-107 was constructed from the crystal structure of SHV-1 using Chimera (30). Molecular dynamics simulations (MDSs) were performed as previously described with GROMACS software package, version 4.0 (45) using the parameters of the OPLSAA force field (26) in a periodic cubic box. The systems were equilibrated and MDS of 400 ps were then made at 300 K and at constant pressure. The radius of gyration, the secondary structure, the root mean squared deviation (RMSD) from the initial structure and the kinetic and potential energies (data not shown) were stable after about 100 ps and, hence, data from 300 ps were used for all subsequent analyses.

The AutoDock 4.0 package was used to perform molecular docking (38). Coordinates for clavulanate structure were obtained using CACTVS (http://www2.ccc.uni-erlangen.de/software/cactvs/) and the CORINA server (22). Structure optimization was calculated at the 6-31G** level using the Gamess quantum chemistry software (44). All the torsion angles in clavulanate were set free to perform flexible docking and the Gasteiger–Hückel atomic charge was assignated. Kollman united atom partial charges were assigned for the receptor, which was kept rigid during the docking study. Water molecules, ions and ligands were removed, except the catalytic water molecule. The size of the docking grid box (x, y, z) was assigned to be 40, 40, and 40 Å and it was centered at the experimentally observed position of class A β-lactamase ligands. The grid spacing inside the docking box was setup to 0.30 Å. The empirical free energy function and Lamarckian genetic algorithm were used for docking with the following settings: a maximum number of 25,000,000 energy evaluations, an initial population of 150 randomly placed individuals, a maximum number of 27,000 generations, a mutation rate of 0.02, a crossover rate of 0.80 and an elitism value (number of top individuals that automatically survive) of 1. For the local search, the Solis and Wets algorithm was used with a maximum of 300 iterations per search; 100 independent docking runs were performed for each docking experiment. Results were clustered according to the default RMSD criteria.

Results

***bla* gene analysis**. The clinical strain *K. pneumoniae* INSRA6884 was isolated in 2006 from sputum of a 92 year old female in a Portuguese hospital (Centro Hospitalar de Coimbra). We analyzed its β-lactamase content, because preliminary susceptibility tests showed an unusual phenotype to β-lactams. The screening of *bla* genes by PCR and sequencing identified the ESBL-encoding gene *bla*$_{\text{GES-7}}$ gene and the recently described gene *bla*$_{\text{SHV-107}}$ (33). Isoelectric focusing of β-lactamases revealed the production of two β-lactamases of pIs 6.9 and 7.5, respectively. The deduced amino sequence of *bla*$_{\text{GES-7}}$ presented Glu107Lys and Leu125Ala substitutions (Ambler numbering) in comparison to GES-1 sequence (1). SHV-107 enzyme presented the amino acid substitutions Leu35Gln and Thr235Ala, in comparison to SHV-1 sequence (Table 1) (33). Mating out assays provided an *E. coli* C600 transconjugant, which produced the β-lactamase of pI 6.9 and only harbored *bla*$_{\text{GES-7.}}$ It was designated *E. coli* C600 (GES-7). Cloning experiments were performed to isolate *bla*$_{\text{SHV-107}}$ in *E. coli* BL21(DE3). The corresponding strain was designated *E. coli* BL21(DE3) (pBK-SHV-107).

Phenotypic characterization. The transconjugant *E. coli* C600 (GES-7) originating from the clinical *K. pneumoniae* INSRA6884 strain only acquired resistance to amoxicillin, ticarcillin, cephalothin, cefuroxime and ceftazidime, in contrast to the clinical strain, which was resistant to all penicillins and all cephalosporins tested except cefepime and cefoxitin (Table 2). The addition of clavulanate drastically reduced the MIC value of oxyimino-cephalosporins and aztreonam in both the *E. coli* C600 (GES-7) transconjugant and the clinical strain. Like the clinical strain, *E. coli* BL21(DE3) (pBK-SHV-107) acquired resistance to amoxicillin and ticarcillin and exhibited a synergy of amoxicillin and piperacillin in combination with clavulanate (2-fold) and tazobactam (8-fold), respectively (Table 2). Both transformants and the clinical strain were susceptible for all other antibiotics tested, including imipinem, kanamycin, ciprofloxacin, nalidixic acid and trimethoprim.

Table 2 - MICs of β-lactam antibiotics for the *K. pneumoniae* clinical strain producing the SHV-107 enzyme, recipient and transformant.[a]

Antimicrobial drug	MIC (µg/ml) for strains:				
	K. pneumoniae INSRA 6884 (SHV-107 + GES-7)	*E. coli* BL21	*E. coli* BL21 (pBK-SHV-107)	*E. coli* C600	*E. coli* C600 (GES-7)
Amoxicillin	2048	≤2	256	≤2	256
Amoxicillin + CLA[b]	512	≤2	64	≤2	8
Ticarcillin	>2048	≤ 2	128	≤ 2	2048
Piperacillin	64	1	8	0.5	4
Piperacillin + TAZ[c]	4	1	1	0.5	0.5
Mecillinam	1	≤ 0.015	≤ 0.015	≤ 0.015	0.25
Cephalothin	128	1	1	4	32
Cefuroxime	64	0.5	1	2	16
Cefoperazone	8	≤ 0.25	≤ 0.25	≤ 0.25	0.5
Ceftazidime	128	0.06	0.06	0.06	32
Ceftazidime + CLA	8	0.03	0.03	≤ 0.015	0.5
Ceftriaxone	4	≤ 0.015	≤ 0.015	≤ 0.015	0.5
Ceftriaxone + CLA	0.5	≤ 0.015	≤ 0.015	≤ 0.015	≤ 0.015
Cefotaxime	4	≤ 0.015	≤ 0.015	0.03	0.5
Cefotaxime + CLA	0.25	≤ 0.015	≤ 0.015	≤ 0.015	0.03
Aztreonam	2	≤ 0.015	≤ 0.015	0.06	1
Aztreonam + CLA	0.125	≤ 0.015	≤ 0.015	0.03	0.03
Cefepime	0.25	≤ 0.015	≤ 0.015	≤ 0.015	0.06
Cefoxitin	4	2	2	2	2
Imipenem	0.25	0.25	0.25	≤ 0.06	≤ 0.06
Kanamycin	4	≤ 0.125	≤ 0.125	≤ 0.125	≤ 0.125
Nalidixic acid	4	≤ 0.003	≤ 0.003	4	4
Ciprofloxacin	0.03	≤ 0.003	≤ 0.003	0.007	0.007
Trimethoprim	0.5	≤ 0.125	≤ 0.125	≤ 0.125	≤ 0.125

[a] *E. coli* BL21 (pBK-SHV-107) and *E. coli* C600 (GES-7) were a transformant and conjugant, respectively, of *K. pneumoniae* INSRA6884 (harboring SHV-107 plus GES-7 enzyme).

[b] CLA, clavulanate at a fixed concentration of 2 µg/ml.

[c] TAZ, tazobactam at a fixed concentration of 4 µg/ml.

Enzymatic parameters of β-lactamase SHV-107. After purification by ion exchange and gel filtration, the purity was estimated to be ≥ 98% on a sodium dodecyl sulfate-polyacrylamide gel. The band of 28.8 kDa corresponds to the molecular mass deduced from the amino acid sequence (data not shown). Two-fold changes or less between the SHV-1 and SHV-107 β-lactamases were obtained for *Km* values for penicillins (11 to 31 µM and 11 to 42 µM, respectively) (Table 3). In contrast, K_m values were 4-fold higher for cephalothin or not determinable for the other cephalosporins, because the hydrolysis rate was too low. Catalytic efficiency against penicillins was 2- to 9-fold lower for

SHV-107 (k_{cat}/K_m, 2.2 to 34.4 µ$M^{-1}.s^{-1}$) than for SHV-1 (k_{cat}/K_m, 20.0 to 84.2 µ$M^{-1}.s^{-1}$) (Table 3). Amoxicillin, with a k_{cat} value of 657s^{-1}, was for the substrate hydrolyzed fastest by SHV-107.

IC_{50} results, obtained by using benzylpenicillin as the substrate, showed that clavulanate was 14-fold less active than tazobactam against the SHV-107 enzyme (Table 1). IC_{50} values for SHV-107 were 9-fold higher with clavulanate than for SHV-1 and no loss of sensitivity was shown to tazobactam in comparison to the parental enzyme. SHV-1 and SHV-107 inhibitor kinetics with clavulanate was therefore further investigated (Table 4). K_i for clavulanate was almost 9-fold higher for SHV-107 than for SHV-1 (1.82 µM versus 0.21 µM). k_{inact} and k_{cat} values were closely related for both enzymes. Consequently, the second-order rate constants, k_{inact}/K_i and k_{cat}/K_i, were 10-fold reduced for SHV-107 than for SHV-1, because of the increase in K_i.

Table 3 - Comparative kinetic parameters for SHV-1 and SHV-107 β-lactamases.

Antibiotic	Kinetic parameters of enzyme:[a]					
	SHV-1[b]			SHV-107[c]		
	K_m (µM)	k_{cat} (s^{-1})	k_{cat}/K_m (µ$M^{-1}.s^{-1}$)	K_m (µM)	k_{cat} (s^{-1})	k_{cat}/K_m (µ$M^{-1}.s^{-1}$)
Penicillin G	23 ± 0.42	1,937 ± 82	84.2 ± 2.0	11 ± 0.85	378 ± 6	34.4 ± 2.7
Amoxicillin	31 ± 1.29	1,044 ± 10	33.7 ± 1.1	42 ± 4.11	657 ± 10	15.9 ± 1.3
Ticarcillin	11 ± 3.40	220 ± 49	20.0 ± 1.7	23 ± 1.86	51 ± 7	2.2 ± 0.4
Piperacillin	24 ± 0.53	1,490 ± 96	62.1 ± 2.7	31 ± 0.39	412 ± 87	13.4± 3.0
Cephalothin	40 ± 1.46	128 ± 33	3.2 ± 0.8	179 ± 8.80	<0.1	<0.001
Ceftazidime	142 ± 3.18	<0.1	ND	ND	<0.1	ND
Cefotaxime	257 ± 20.65	<0.1	ND	ND	<0.1	ND

[a] Values are means ± standard deviations.
[b] The kinetic constants for SHV-1 are from reference 34.
[c] *E. coli* BL21 (pBK-SHV-107) was the transformant producing SHV-107.
ND, not determinable, because the hydrolysis rates were too low.

Table 4 - Clavulanate kinetic parameters of SHV-1 and SHV-107 β-lactamases

	K_i (µM)	k_{inact} (s^{-1})	k_{inact}/K_i (s^{-1}.µM^{-1})	k_{cat} (s^{-1})	k_{cat}/K_i (s^{-1}.µM^{-1})	k_{cat}/k_{inact}
SHV-1	0.21	0.050	0.23	0.94	4.48	19
SHV-107	1.82	0.042	0.02	0.75	0.41	18

Molecular Modeling. Enzyme SHV-107 was modeled from the crystallographic structure of SHV-1 (30). The behaviors of SHV-107 and SHV-1 were compared during MDSs of 300 ps at a temperature of 300 K. The radius of gyration and the RMSDs of Cα atoms were similar to those of the crystallographic structure of SHV-1 and the secondary structure was also preserved during the simulation (data not shown), indicating that the dynamic structure of the models remained in the realm of the crystal SHV-1 geometry throughout the simulation. The introduction of Thr235Ala substitution caused no overall or large scale deviation of dynamic properties. Likewise, the survey of residues and water molecules located in the vicinity of position 235 revealed no dramatic change in this area (data not shown).

To investigate further the mechanism of resistance to clavulanate, we used the minimized structures of clavulanate to carry out docking simulations with the SHV-1 crystal structure and the SHV-107 model using Autodock software package. 100 docking experiments were performed for each protein and penicillin. The positioning of clavulanate in the catalytic pocket was then compared by RMSD analysis. For SHV-1, all docking experiments provided a similar conformation and positioning of clavulanate in the catalytic pocket (RMSD value for clavulanate atoms <1Å). The oxygen atom of β-lactam carbonyl function was at a hydrogen bond distance of the Ser70 and Ala237 N atoms (2.8 to 3.0 Å), which form the oxianion hole. C3 carboxylate function of clavulanate established electrostatic interaction with Ser130 and Thr235 (2.7 to 2.9 Å).

For SHV-107, 98% of clavulanate-docking experiments provided a similar positioning of clavulanate in the SHV-107 catalytic pocket (RMSD value for clavulanate atoms <1Å). However, the positioning of clavulanate in SHV-107 was clearly different from that in SHV-1. Clavulanate was flipped (Fig. 1). Consequently, the oxygen atom of β-lactam carbonyl function did not interact with the oxianion hole. Instead, Ser70 and Ala237 N atoms and Ala237 O atom shared hydrogen bonds with the C2 substituent of clavulanate (2.8 to 3.0 Å) and the β-lactam ring was accommodated in the vicinity of position 244.

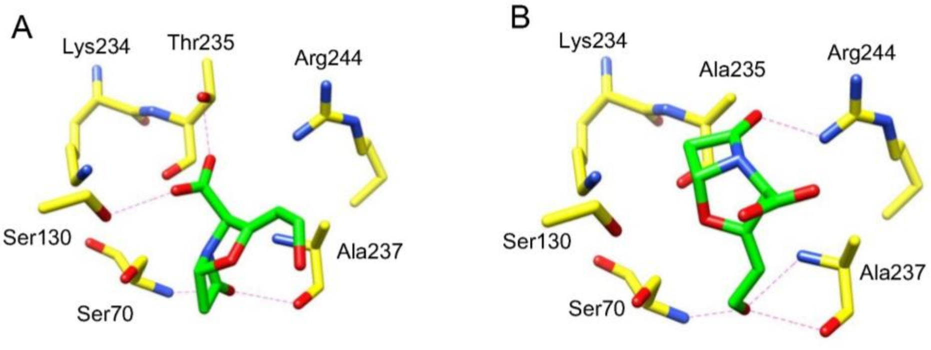


Fig. 1 - Clavulanate positioning in SHV-1 (A) and SHV-107 (B) binding site after docking experiments using Autodock. Carbon atoms are in yellow for the protein or in green for clavulanate; oxygen atoms are red and nitrogen atoms blue. Hydrogen bonds are indicated by dashed lines.

Discussion

Resistance to β-lactam antibiotics continues to increase, largely due to the emergence of various β-lactamases. Inhibitor resistant β-lactamases are one of the most important causes of resistance to β-lactam-inhibitor combinations. In this study, we characterized a clinical *K. pneumoniae* strain, isolated in a Portuguese hospital, co-expressing two different β-lactamases: the IRS enzyme SHV-107 and the ESBL GES-7.

GES-7 (formerly *bla*$_{\text{IBC-1}}$) is an ESBL class A β-lactamase that differs from GES-1 in two amino acid residues, namely Glu107Lys and Leu125Ala, which was first observed in early 2000, in Greece, in *Enterobacter cloacae* and *E. coli* strains (23, 46), and more recently in Brazil in *K. pneumoniae* (12). The corresponding coding gene *bla*$_{\text{GES-7}}$ has been observed in class 1 integrons located in a multidrug-resistant transferable plasmid of *E. cloacae* and *E. coli* clinical strains (23, 27, 46). This gene has also been found in chromosomal location and associated with the metallo-β-lactamase-encoding gene *bla*$_{\text{VIM-2}}$ (20, 21). We report for the first time *bla*$_{\text{GES-7}}$ in Portugal. Although not associated with class 1 integrons (data not shown), this gene was encoded by a conjugative plasmid isolated from a *K. pneumoniae* clinical strain.

This clinical strain also produced an IRS β-lactamase ($IC_{50} \geq 1 \mu M$), designated SHV-107. This enzyme is the seventh member of this subgroup, which is characterized by a resistance to clavulanate (Table 1). Regarding tazobactam

SHV-107 exhibited an higher inhibitory activity than clavulanic acid as it has a triazole group at the C-2 β-methyl position, which lead to the improvement of IC_{50}s (11). It combines the amino acid substitutions Leu35Gln and Thr235Ala. The first substitution Leu35Gln, frequently encountered and observed alone in the SHV-11 enzyme (35), is known to have no significant impact on kinetic constants (37). To our best knowledge, the second substitution is the first natural substitution reported at position 235 in class A enzymes (Table 1).

In class A enzymes, Thr235 residue is part of a conserved element (Lys234-Ser/Thr235-Gly236). It is situated on the β3 strand of a β-sheet in the α/β domain and forms a major wall of the catalytic cavity site (10). The hydroxyl function of Ser/Thr-235 contributes to the binding of the conserved C3/C4 carboxylate function of β-lactams (47). We observed that the substitution Thr235Ala affected SHV-107 kinetic constants against β-lactams without dramatic consequence on the resistance to penicillins. Likewise, previous enzymatic studies of a TEM-1 Ser235Ala mutant, obtained by site-directed mutagenesis, showed similar results for penicillins as TEM-1 enzyme, although the mutant had greatly reduced cephalosporinase activity (16, 24). In the SHV β-lactamase family, residue Thr235 is also critical for cephalosporinase activity, because its substitution by Ala235 in SHV-107 leads to severe impairment of the catalytic constant against cephalosporins, as observed in TEM-type enzyme (17, 24). On the other hand, Thr235Ala substitution affected susceptibility to clavulanate and consequently the susceptibility to the combination amoxicillin-clavulanate. Three IRS enzymes, SHV-56, SHV-72 and SHV-84, which presented an amino acid substitution in the vicinity of position 235, also showed decreased susceptibility to clavulanate (15, 31, 35). Indeed, molecular-dynamics simulations suggested that substitution Lys234Arg observed in this mutant, probably affects the positioning of the Ser130 side chain, a key element in the inhibition reaction mediated by clavulanate (35).

To explain the role of substitution Thr235Ala in resistance against clavulanate, we investigated the recognition of clavulanate by the SHV-107 binding site using molecular modeling tools. It emerges from this study that Thr235 favors the interaction of β-lactam carbonyl function with the oxyanion hole. This interaction is a key factor in active serine acylation by clavulanate and therefore in the inhibition process mediated by this inhibitor. Substitution Thr235Ala may therefore affect the correct orientation of clavulanate in the binding site and consequently the inhibition activity of clavulanate. This point is supported by the increase of clavulanate K_i with SHV-107.

In conclusion, our findings show that mutations at Thr235 of the SHV-1 β-lactamase contributes to decreased susceptibility to β-lactamase inhibitors. Given the increasing number of new enzymes conferring resistance to β-lactamase inhibitors, further investigation should be undertaken on IRS enzymes. This emergence may be the consequence of the widespread use of penicillins with β-lactamase inhibitors (J01CR) in different European countries, a practice that needs to be re-assessed and modified (18).

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Chapter 2. Class A β-lactamases: function meets structure

Paper VII. Biochemical characterization of SHV-55, an extended-spectrum class A β-lactamase from *Klebsiella pneumoniae*

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Biochemical Characterization of SHV-55, an Extended-Spectrum Class A β-Lactamase from *Klebsiella pneumoniae*▽

We biochemically characterized the *Klebsiella pneumoniae* extended-spectrum SHV-55 enzyme carrying the amino acid substitutions Tyr7→Phe (as in SHV-28) and Gly238→Ser and Glu240→Lys (both found in SHV-5) identified in a previous study (5). The SHV-55 extended-spectrum β-lactamase differed from SHV-5 only in the signal peptide region (1). The $bla_{\mathrm{SHV\text{-}55}}$ gene was obtained as described by Mendonça et al. (6), and transformants were selected on Luria broth agar supplemented with 30 μg of kanamycin/ml and 16 μg of amoxicillin/ml. SHV-55 was extracted and purified according to the previously described protocol (6). The Michaelis constant (K_m) and catalytic activity (k_{cat}) of purified extracts of SHV-55 were obtained by using a computerized microacidimetric method and a 702 SM Titrino pH-stat apparatus (Metrohm, Herisau, Switzerland) (3). The complete hydrolysis time courses were analyzed, and the kinetic progress curves were fitted by nonlinear least-squares regression. These kinetic parameters were determined and compared to those of the SHV-1 enzyme for 10 β-lactams (Table 1).

SHV-55 has a high affinity (K_m, 5 to 10 μM) for penicillins, similar to that of SHV-5 (1) and higher than that of SHV-1 (K_m, 11 to 31 μM). SHV-55 presented higher affinity values (K_m, 9 to 58 μM) than SHV-1 (K_m, 40 to 257 μM) for narrow-, extended-, and broad-spectrum cephalosporins and monobactams. This finding may be a consequence of the Gly238→Ser substitution present in the active sites of both SHV-55 and SHV-5, which pushes the β-strand out and away from the reactive Ser70 (2). This effect results in a slightly expanded active site that may improve binding and accommodate cephalosporins with bulky side chains (4). SHV-55 presented a higher affinity for cefotaxime than for ceftazidime (K_ms, 21 and 58 μM, respectively), as did SHV-5 (1, 7). This finding is surprising because both enzymes possess the Glu240→Lys substitution, which increases hydrolytic activity against ceftazidime (8) due to the change in the electrostatic charge of the exposed group at position 240 (2). The enzymatic activities (k_{cat}s) of SHV-55 for penicillin G and amoxicillin were 84- and 45-fold lower, respectively, than those of SHV-1, and the catalytic efficiency (k_{cat}/K_m ratio) against penicillins was more than 10-fold higher for SHV-1 (k_{cat}/K_m ratio, 20 to 84 $\mu M^{-1} \cdot s^{-1}$) than for SHV-55 (k_{cat}/K_m ratio, 2 to 5 $\mu M^{-1} \cdot s^{-1}$). However, the enzyme activity and catalytic efficiency against extended- and broad-spectrum cephalosporins were higher for SHV-55 (k_{cat}, 7 to 24 s^{-1}, and k_{cat}/K_m ratio, 0.2 to 1 $\mu M^{-1} \cdot s^{-1}$) than for SHV-1 (note, however, that the values for monobactam were undeterminable), although the catalytic efficiencies of the two enzymes against cephalothin were similar (k_{cat}/K_m ratios, 3.2 and 4.4 $\mu M^{-1} \cdot s^{-1}$). This result may be due to the amino acid substitutions in SHV-55 causing conformational modifications in the active site. Fifty percent inhibitory concentrations (IC_{50}s) indicated that SHV-55 was nine-fold more susceptible to the inhibitor activity of clavulanate than SHV-1 (IC_{50}s of clavulanate, 0.02 versus 0.17 μM).

In conclusion, these results confirmed the extended-spectrum activity of the SHV-55 enzyme, which is important due to the magnitude of extended- and broad-spectrum SHV β-lactamases described to date and not biochemically characterized, in spite of the ease of sequencing genes (http://www.lahey.org/studies).

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TABLE 1. Kinetic constants of SHV-55 and SHV-1 β-lactamases[a]

Antibiotic	SHV-1[b]				SHV-55			
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ratio ($\mu M^{-1} \cdot s^{-1}$)	IC_{50}(μM)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ratio ($\mu M^{-1} \cdot s^{-1}$)	IC_{50} (μM)
Clavulanic acid				0.17				0.02
Penicillin G	23 ± 0.42	1,937 ± 82	84.3 ± 2.0		5 ± 0.51	23 ± 0.76	5.3 ± 0.42	
Amoxicillin	31 ± 1.29	1,044 ± 10	33.3 ± 1.1		10 ± 0.14	23 ± 0.17	2.5 ± 0.02	
Ticarcillin	11 ± 3.40	220 ± 49	20.4 ± 1.7		6 ± 0.02	8 ± 0.00	1.5 ± 0.00	
Piperacillin	24 ± 0.53	1,490 ± 96	62.5 ± 2.7		8 ± 0.37	27 ± 1.53	3.7 ± 0.03	
Cephalothin	40 ± 1.46	128 ± 33	3.2 ± 0.8		9 ± 0.68	38 ± 3.94	4.4 ± 0.78	
Cefuroxime	80 ± 0.59	<0.1	ND[c]		23 ± 0.53	7 ± 0.19	0.3 ± 0.00	
Ceftazidime	142 ± 3.18	<0.1	ND		58 ± 7.40	9 ± 0.21	0.2 ± 0.02	
Cefotaxime	257 ± 20.65	<0.1	ND		21 ± 0.13	24 ± 0.34	1.1 ± 0.01	
Aztreonam	ND	<0.1	ND		5 ± 0.62	<0.1	ND	
Cefepime	149 ± 7.30	<0.1	ND		149 ± 2.61	30 ± 3.10	0.2 ± 0.02	

[a] Values (except IC_{50}s) are means ± standard deviations.
[b] Data are from reference 6.
[c] ND, not determinable.

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Chapter 2. Class A β-lactamases: function meets structure

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Role of SHV β-lactamase variants in resistance of clinical *Klebsiella pneumoniae* strains to β-lactams in an Algerian hospital

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Three clinical *Klebsiella pneumoniae* strains, KpARG74, KpARG220 and KpARG185, isolated from a hospital in Algeria, carried the novel β-lactamases SHV-98, SHV-99 and SHV-100, respectively, and co-expressed TEM-1 and either CTX-M-3 or CTX-M-15. In contrast, transformed cells possessing the genes for these novel β-lactamases, i.e. EcDH5α-SHV-98, EcDH5α-SHV-99 and EcDH5α-SHV-100, respectively, carried unique sequence features of *bla*$_{SHV}$ gene variants, enabling oxyimino-cephalosporin susceptibility and confirming that none of the transformants exhibited extended-spectrum β-lactamase (ESBL) properties. SHV-100 is apparently functional, despite differing from the SHV-1 sequence by duplication of 13 amino acids. The SHV-99 enzyme differed from the parental SHV-1 by the amino acid substitution Asp104→Gly, which is an important position in the development of the ESBL phenotype in TEM β-lactamases. This is the first time, to our knowledge, that this mutation has been reported in clinically occurring isolates. Thus, kinetic characterization of the SHV-99 enzyme was performed. The SHV-99 enzyme showed higher affinity (K_m of 196 μM), catalytic activity (k_{cat} of 0.5 s^{-1}) and catalytic efficiency (k_{cat}/K_m of 0.003 μM^{-1} s^{-1}) than SHV-1 β-lactamase against aztreonam. These results showed that the neutral glycine at residue 104 increased the affinity of the enzyme to aztreonam, but was unable to develop the ESBL phenotype in SHV enzymes. As the emergence of new threatening combinations of resistance determinants among nosocomial pathogens is further possible, this study has highlighted the need to reverse the spread of initial mutations.

INTRODUCTION

Klebsiella pneumoniae is an opportunistic pathogen with clinical importance among members of the family *Enterobacteriaceae* responsible for various community and hospital infections (Paterson & Bonomo, 2005). β-Lactamase production is the most frequent antibiotic resistance mechanism in this pathogen. In Algerian hospitals, β-lactamases are also emerging, with CTX-M enzymes being the most described extended-spectrum β-lactamases (ESBLs) (Messai *et al.*, 2008; Ramdani-Bouguessa *et al.*, 2006).

K. pneumoniae is intrinsically resistant to amoxicillin due to the production of a chromosomal enzyme, the SHV-1 β-lactamase (Babini & Livermore, 2000). However, the SHV β-lactamase family confers resistance to β-lactamase inhibitors/β-lactam combinations or to expanded-spectrum cephalosporins and monobactams, when one to five amino acid substitutions occur in the structural gene (http://www.lahey.org/studies). More frequent substitutions related to clavulanic acid resistance are in Met69 and Ser130, and those associated with the ESBL phenotype are at positions Gly238 and Glu240 (Paterson & Bonomo, 2005).

In this work, phenotypes expressed by three clinical *K. pneumoniae* strains producing SHV enzymes, first isolated in an Algerian hospital, are reported. Molecular characterization demonstrated that SHV-98 and SHV-99 have one novel substitution and that SHV-100 has an amino acid duplication when compared with SHV-1. Biochemical characterization of the SHV-99 enzyme is also described as

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Abbreviations: ESBL, extended-spectrum β-lactamase; IC$_{50}$, 50 % inhibitory concentration.

The GenBank/EMBL/DDBJ accession numbers for the gene sequences of SHV-98, SHV-99 and SHV-100 are AM941844, AM941845 and AM941846, respectively.

this is the first time, to our knowledge, that the mutation Asp104→Gly has appeared in clinical SHV β-lactamase-producing isolates.

METHODS

Bacterial isolates. During the months of February, May and June 2005, three *K. pneumoniae* isolates (KpARG74, KpARG220 and KpARG185) were collected from male patients attending the Centre Hospitalo-Universitaire Mustapha Pacha, a 1800-bed facility located in Algiers, Algeria. These strains were collected from distinct clinical departments (orthopaedics, neurosurgery and intensive care unit, respectively) and from several biological sources (pus, cerebrospinal fluid and urine, respectively).

Antimicrobial susceptibility testing. The MICs of antibiotics tested against clinical isolates and respective transformants were each determined by an agar dilution method according to the French Society of Microbiology (CA-SFM; Bonnet *et al.*, 2010). The MICs of the β-lactam antibiotics tested were each determined alone and four antibiotics were tested in combination with fixed concentrations of β-lactamase inhibitors: amoxicillin, cefotaxime and ceftazidime were tested with 2 μg clavulanic acid ml^{-1} and piperacillin was tested with 4 μg tazobactam ml^{-1}. Clinical isolates that were not susceptible to one or more extended-spectrum β-lactams (cefotaxime, ceftazidime and/or ceftriaxone) and showing synergy between these antibiotics and clavulanic acid were inferred to produce ESBLs.

β-Lactamase characterization. β-Lactamases were characterized by isoelectric focusing of ultrasonicated bacterial extracts and β-lactamase genes (bla_{TEM}, bla_{SHV}, $bla_{\text{OXA-1}}$, *ampC* and bla_{CTX}) were detected and identified by PCR and sequencing using previously described protocols (Caniça *et al.*, 1997; Mendonça *et al.*, 2007).

β-Lactamase gene transfer experiments. The $bla_{\text{SHV-99}}$ gene was cloned into plasmid pBK-CMV and transferred into electrocompetent *Escherichia coli* DH5α Δ*ampC* by electroporation as described previously (Mendonça *et al.*, 2008). Transformants were selected on LB agar supplemented with 30 μg kanamycin ml^{-1} and 50 μg amoxicillin ml^{-1}. To confirm the presence of the inserted genes and to determine their orientation, PCR was performed as described previously (Mendonça *et al.*, 2008).

Biochemical characterization of the SHV-99 enzyme. After electroporation of the SHV-encoding plasmid (pBK-SHV-99), the β-lactamase was extracted from an overnight culture by sonication and purified by ion exchange and gel filtration according to previously described protocols (Labia *et al.*, 1973). SHV-99 total protein concentration was estimated by the BCA protein assay kit (Pierce). The purity of enzyme extracts was estimated by SDS-PAGE. The kinetic constants of purified extracts of SHV-99 were obtained by a computerized microacidimetric method using a 702 SM Titrino pH-stat apparatus (Metrohm) (Mendonça *et al.*, 2008). The complete hydrolysis time-courses were analysed and kinetic progress curves were fitted by non-linear least-squares regression. These parameters were determined and compared to those of the SHV-1 enzyme, which had been previously purified and characterized (Mendonça *et al.*, 2008), for eight β-lactams.

RESULTS AND DISCUSSION

Antimicrobial susceptibility testing revealed that the clinical isolates were resistant to the penicillins, cephalosporins and monobactam tested. β-Lactamase inhibitors only partially restored activity of the penicillins when tested in combination (Table 1).

Genotypic characterization showed that clinical isolates KpARG74, KpARG220 and KpARG185 had the $bla_{\text{SHV-98}}$, $bla_{\text{SHV-99}}$ and $bla_{\text{SHV-100}}$ genes, respectively. These genes were cloned and transferred to an isogenic system to evaluate their phenotypic contributions. The SHV-98-, SHV-99- and SHV-100-producing transformants exhibited a β-lactam resistance phenotype similar to that of the clinical isolates with respect to penicillins. However, in contrast to the clinical isolates, EcDH5α-SHV-98, EcDH5α-SHV-99 and EcDH5α-SHV-100 were susceptible to oxy-imino-cephalosporins, thus confirming that none of the transformants exhibited ESBL properties. Indeed, SHV-type β-lactamases contributed poorly to the resistance profile of the clinical isolates, which was mostly reflected by the expression of a CTX-M-type enzyme. Both clinical isolates and transformants were susceptible to imipinem and ciprofloxacin. Only the transformants were susceptible to gentamicin and trimethoprim, in contrast to the clinical isolates (Table 1).

Isoelectric focusing of KpARG74, KpARG220 and KpARG185 showed two bands that were not identified in the transformants, one cofocusing with TEM-1 (pI 5.4) and the other with CTX-M-3 or CTX-M-15 β-lactamases (pI 8.9 and 8.4, respectively); the presence of CTX-M-3 or CTX-M-15 β-lactamases explains the ESBL phenotype of the clinical isolates, with CTX-M-15 β-lactamase disseminated worldwide (Messai *et al.*, 2008; Paterson & Bonomo, 2005; Ramdani-Bouguessa *et al.*, 2006). Additionally, β-lactamases SHV-98, SHV-99 and SHV-100 produced by both clinical isolates and transformants had pIs of 7.6, 7.8 and 7.2, respectively.

Sequencing enabled us to identify the novel amino acid substitution Ser271→Ile in SHV-98, the substitution Asp104→Gly in SHV-99 and the amino acid duplication (n=13, SESQLSGRVGMIE) between positions 36 and 48 in the SHV-100 β-lactamase. As shown by MIC data, SHV-100 is apparently functional despite the insertion. As repeated attempts to detect the presence of these SHV-encoding genes in plasmid DNA failed, it is presumed that they have a chromosomal location (data not shown).

In class A β-lactamases, Ambler position 104 shows some amino acid variability (Guo *et al.*, 1999; Ambler *et al.*, 1991); however, previous investigators have shown that amino acid substitution at this position is important in the development of the ESBL phenotype in TEM β-lactamases (Petit *et al.*, 1995). To evaluate the role of this substitution, which has not to our knowledge been previously reported in naturally occurring isolates, in SHV enzymes the kinetic parameters of the purified SHV-99 enzyme (purity rate $\geqslant$98 %) were determined for eight β-lactams (Table 2). SHV-99 showed comparable K_m values for penicillins to SHV-1 (K_m of 5–13 μM and 11–31 μM, respectively). For cefalotin, kinetic parameters were slightly different for the two enzymes: the SHV-99 enzyme had diminished affinity

Table 1. MICs of antibiotics for the clinical *K. pneumoniae* KpARG74, KpARG220 and KpARG185 isolates, SHV-1-, SHV-98-, SHV-99- and SHV-100-producing transformants and the recipient *E. coli* EcDH5α Δ*ampC*

Strains: 1, *E. coli* EcDH5α Δ*ampC*; 2, *E. coli* EcDH5α-SHV-1 (pBK-SHV-1); 3, *K. pneumoniae* KpARG74 (SHV-98/TEM-1/CTX-M-3); 4, *E. coli* EcDH5α-SHV-98 (pBK-SHV-98); 5, *K. pneumoniae* KpARG220 (SHV-99/TEM-1/CTX-M-15); 6, *E. coli* EcDH5α-SHV-99 (pBK-SHV-99); 7, *K. pneumoniae* KpARG185 (SHV-100/TEM-1/CTX-15); 8, *E. coli* EcDH5α-SHV-100 (pBK-SHV-100). MIC values for *E. coli* EcDH5α Δ*ampC* and *E. coli* EcDH5α-SHV-1 are from Mendonça *et al.* (2008).

Antibiotic	1	2*	3	4*	5	6*	7	8*
Amoxicillin	8	2.048	>4.096	1.024	>4.096	>4.096	>4.096	2.048
Amoxicillin + CLA†	8	8	8	⩽0.5	>64	8	16	8
Ticarcillin	4	1.024	>4.096	4.096	>4.096	>4.096	>4.096	>4.096
Piperacillin	2	64	>64	>64	>64	>64	>64	64
Piperacillin + TAZ‡	⩽16	⩽16	⩽16	⩽16	256	⩽16	⩽16	⩽16
Cefalotin	8	16	64	8	>1.024	32	>1.024	8
Cefuroxime	4	4	>256	128	>256	8	>256	8
Ceftazidime	⩽0.5	⩽0.5	4	⩽0.5	64	1	16	⩽0.5
Ceftazidime + CLA†	⩽0.5	⩽0.5	⩽0.5	⩽0.5	1	⩽0.5	⩽0.5	⩽0.5
Ceftriaxone	⩽0.25	⩽0.25	>512	⩽0.25	>512	⩽0.25	512	⩽0.25
Cefotaxime	0.06	0.06	>256	⩽0.25	>256	0.06	256	0.06
Cefotaxime + CLA†	⩽0.25	⩽0.25	⩽0.25	⩽0.25	1	⩽0.25	⩽0.25	⩽0.25
Aztreonam	⩽0.5	⩽0.5	64	⩽0.5	512	⩽0.5	32	⩽0.5
Imipenem	⩽0.5	⩽0.5	⩽0.5	⩽0.5	⩽0.5	⩽0.5	⩽0.5	⩽0.5
Ciprofloxacin	⩽0.5	⩽0.5	⩽0.5	⩽0.5	⩽0.5	⩽0.5	⩽0.5	⩽0.5
Gentamicin	⩽2	⩽2	>8	⩽2	>8	⩽2	>8	⩽2
Trimethoprim	⩽0.125	⩽0.125	>64	⩽0.125	>64	⩽0.125	>64	⩽0.125

**E. coli* DH5α-SHV-1, *E. coli* DH5α-SHV-98, *E. coli* DH5α-SHV-99 and *E. coli* DH5α-SHV-100 were the transformants producing SHV-1, SHV-98, SHV-99 and SHV-100, respectively.

†CLA, Clavulanic acid at a fixed concentration of 2 μg ml^{-1}.

‡TAZ, Tazobactam at a fixed concentration of 4 μg ml^{-1}.

(K_m, 2.5-fold), catalytic activity (k_{cat}, 3.5-fold) and catalytic efficiency (k_{cat}/K_m, 9-fold) compared to SHV-1, with no impact on the MIC value. Neither enzyme exhibited significant enzymic activity against oxyimino-cephalosporins. Nevertheless, the Asp104→Gly mutation of SHV-99 generated a considerably higher affinity (K_m of 196 μM), catalytic activity (k_{cat} of 0.5 s^{-1}) and catalytic efficiency (k_{cat}/K_m of 0.003 $\mu\mathrm{M}^{-1}\ \mathrm{s}^{-1}$) against aztreonam than the parental enzyme, whose values were not determinable because the hydrolysis rate was too low. Fifty per cent

Table 2. Kinetic constants and IC_{50} values of SHV-99 and SHV-1 β-lactamases

Values for kinetic enzyme parameters are means ± SD. ND, Not determinable because the hydrolysis rate was too low.

Antibiotic	SHV-1*				SHV-99			
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu\mathrm{M}^{-1}\ \mathrm{s}^{-1}$)	IC_{50} (μM)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu\mathrm{M}^{-1}\ \mathrm{s}^{-1}$)	IC_{50} (μM)
Clavulanic acid				0.17				0.02
Tazobactam				0.11				0.03
Penicillin G	23 ± 0.42	1937 ± 82	84.2 ± 2.0		12 ± 0.11	778 ± 61	62.3 ± 4.4	
Amoxicillin	31 ± 1.29	1044 ± 10	33.7 ± 1.1		11 ± 0.26	563 ± 8	49.6 ± 1.8	
Ticarcillin	11 ± 3.40	220 ± 49	20.0 ± 1.7		5 ± 0.93	58 ± 2	13.0 ± 2.4	
Piperacillin	24 ± 0.53	1490 ± 96	62.1 ± 2.7		13 ± 1.43	563 ± 13	43.5 ± 6.5	
Cefalotin	40 ± 1.46	128 ± 33	3.2 ± 0.8		102 ± 11.38	37 ± 2	0.37 ± 0.04	
Ceftazidime	142 ± 3.18	<0.1	<0.001		136 ± 4.09	<0.1	<0.001	
Cefotaxime	257 ± 20.65	<0.1	<0.001		183 ± 0.72	<0.1	<0.001	
Aztreonam	ND	<0.1	<0.001		196 ± 0.60	0.5 ± 0.001	0.003	

*The kinetic constants for SHV-1 are from Mendonça *et al.* (2008).

inhibitory concentrations (IC_{50}) indicated that SHV-99 was susceptible to clavulanic acid (IC_{50} of 0.02 µM) and tazobactam (IC_{50} of 0.03 µM), as observed with SHV-1 (IC_{50} values of 0.17 and 0.11 µM, respectively) (Table 2). The possibility that this novel β-lactamase is an inhibitor-resistant SHV enzyme, examples of which have been found by other workers, is therefore excluded (Mendonça *et al.*, 2008).

The hydrophilic, negatively charged side-chain of residue Asp104, together with Tyr105, is located on the left side of the active site pocket, being one of the active site loops of serine β-lactamases. This residue is hydrogen-bonded to Asn132, which may therefore stabilize the catalytic Ser130 in the conserved Ser-Asp-Asn (SDN) loop from positions 130 to 132 (Kuzin *et al.*, 1999). The increased affinity of the enzyme to aztreonam and, given its location in the catalytic pocket, the interaction with residue 104 seems to be the first step in the substrate binding and recognition of oxyimino-β-lactams (Bethel *et al.*, 2006).

Variations in the MICs of penicillins and cefalotin reveal that substitutions at position 104 are tolerated in both SHV and TEM enzymes (Bethel *et al.*, 2006; Guo *et al.*, 1999; Petit *et al.*, 1995; Knox, 1995). However, according to our results, the substitution of an Asp by a neutral Gly at residue 104 in SHV has a poor impact on the properties of the enzyme, at least in the absence of any other substitutions. These are interesting data since the structurally equivalent position in TEM β-lactamases is known to have more relevance (Guo *et al.*, 1999; Petit *et al.*, 1995; Knox, 1995). Our kinetic study confirmed that the Asp104→Gly substitution alone is unable to develop the ESBL phenotype in SHV enzymes, as suggested by phenotypic characterization, unlike in TEM β-lactamases (Petit *et al.*, 1995). The impact of this mutation might be more relevant if additional ESBL-conferring mutations are present (Bethel *et al.*, 2006).

CTX-M-type β-lactamases are the ESBLs most commonly produced by isolates in Algerian hospitals (Messai *et al.*, 2008; Ramdani-Bouguessa *et al.*, 2006), as in isolates from our study. However, their co-production with other β-lactamases is frequent, such as SHV and/or TEM, conferring resistance phenotypes which are additive, with the respective consequences at clinical and therapeutic levels (Paterson & Bonomo, 2005; Ramdani-Bouguessa *et al.*, 2006). In parallel, the importance of enzymes that do not contribute to β-lactamase inhibitor resistance or to the ESBL phenotype is an open issue. However, as already demonstrated, non-ESBL enzymes may facilitate the evolution to ESBLs under selection pressure (Hammond *et al.*, 2008).

The easy horizontal and vertical spread of β-lactamases worldwide will therefore dictate future research. Thus, considering the diversity of the enzymic characteristics of β-lactamases, the biochemical characterization of novel enzymes is essential to understand their individual contribution to resistance phenotypes of pathogens with an impact on public health (Bush & Jacoby, 2010). This characterization will also have an important role as the emergence of new threatening combinations of resistance determinants among nosocomial pathogens is further possible. Overall, this study has highlighted the need to reverse the spread of initial mutations.

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SECTION III. CONCLUDING REMARKS

The introduction into clinical practice of a large collection of β-lactam antibiotics, namely oxyimino-β-lactams and carbapenems — as a response to the increased prevalence of β-lactamases in certain bacteria, such as *E. coli* and *K. pneumoniae* — has lead to the emergence of an even larger variety of β-lactamases designed to confer resistance to those agents. At present, more than 860 β-lactamases are identified in clinically important bacteria (http://www.lahey.org/Studies/). The most prevalent β-lactamases, and the most clinically important in Gram-negative bacteria, are represented in Table 5 (Section I: Chapter 4).

The work presented in this PhD thesis answer several issues, such as how mechanisms of resistance to oxyimino-β-lactams and carbapenem antibiotics emerged and disseminated into *Enterobacteriaceae* and *A. baumannii* clinical isolates? How did they spread into community environments? What are the risk factors? Or which specific amino acid substitutions promote or improve the antibiotic resistance and what is their function in determining resistance?

The answers throught the results are branched into two chapters in the thesis: the first chapter, entitled "β-lactamases: impact in the antibiotic resistance, dissemination and co-resistance," provide updated molecular epidemiology data regarding extended-spectrum- and plasmid-mediated AmpC β-lactamase-producing *Enterobacteriaceae* (Paper I and II, respectively) and class D β-lactamase-producing carbapenem-resistant *A. baumannii* (Paper III); the chapter 2, entitled "Class A β-lactamases: function meets structure", reports the correlation of structure and function of five novel clinical important β-lactamases, namely three IRS (SHV-72, SHV-84 and SHV-107), one ESBL (SHV-55) and one parental SHV (SHV-99) enzyme. Overall, the results presented here contributed to the knowledge of molecular epidemiology of β-lactamases and biochemical diversity of antibiotic resistance mechanisms in Gram-negative clinical isolates

At the end of each Paper I to VIII (Section II: Results), a discussion was presented about the results enclosed in that particular paper. This Section III (Concluding Remarks) will focus on an overall discussion with the presentation of the main results of the different Chapters.

Several strategies for controlling the spread of antibiotic resistance have been suggested and implemented, where surveillance has an important role. Indeed, antibiotic resistance and MDR in invasive *E. coli* and *K. pneumoniae* in several

European countries under surveillance in EARS-Net, shows increasing trends (EARS-Net, 2010). The frequency of resistance to third-generation cephalosporins reported for both pathogens has increased significantly during the last four years in over half of the EARS-Net reporting countries (Figure 15 and 16) (EARS-Net, 2010).

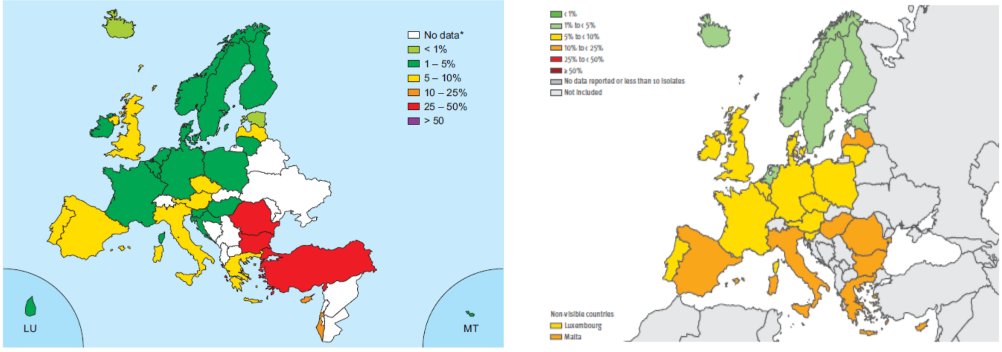
2006
2009

Figure 15 – Proportion of *E. coli* invasive isolates resistant to third-generation cephalosporins in 2006 (EARSS, 2007) and in 2009 (EARS-Net, 2010), in Europe.

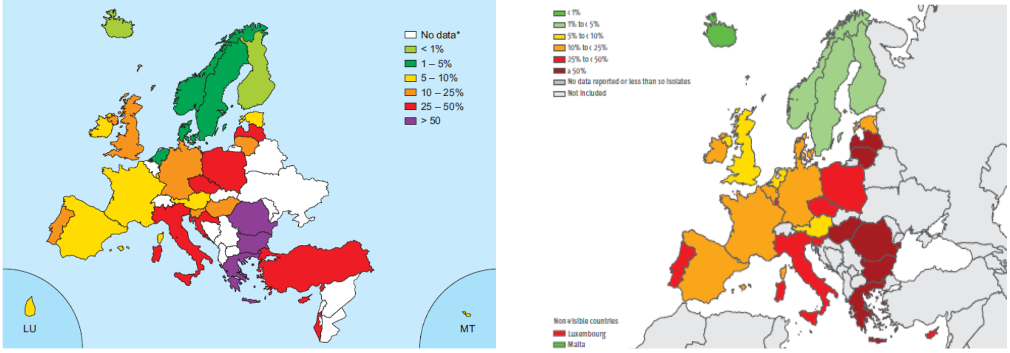
2006
2009

Figure 16 - Proportion of *K. pneumoniae* invasive isolates resistant to third-generation cephalosporins in 2006 (EARSS, 2007) and in 2009 (EARS-Net, 2010), in Europe.

In this thesis, in Paper I to III, the trends of resistance to third-generation cephalosporins related to Portugal are associated with an increase in the number and diversity of antibiotic resistance mechanisms that has been observed, namely the emergence of ESBLs from CTX-M family in *Enterobacteriaceae*, both in clinical and community settings. In addition, carbapenemases were also demonstrated in *A. baumannii*.

Although ESBLs from the SHV, TEM and GES families are important, because they confer β-lactamase-mediated resistance to oxyimino-β-lactams in *Enterobacteriaceae*, the ability of CTX-M enzymes to spread between different pathogens has led to it becoming the most significant problem nowadays (Cantón & Coque, 2006; Cantón *et al*, 2008b; Livermore *et al*, 2007). The work presented in this thesis detects and reinforces this view for Portugal, since CTX-M enzymes were found in all studies performed, both in *Enterobacteriaceae* and *A. baumannii* isolates (Section II: Chapter 1).

In the Iberian Peninsula, β-lactamases of the CTX-M family were first reported in 2000, with the identification of isolates of *E. coli* producing CTX-M-9, recovered in 1996-1998 (Sabaté *et al*, 2000; Simarro *et al*, 2000). In Portugal, the first isolate carrying a CTX-M enzyme, CTX-M-14, was collected from a healthy subject in 2003 (Machado *et al*, 2004), but CTX-M-15 has been described as being the most prevalent (Machado *et al*, 2007; Mendonça *et al*, 2007; Mendonça *et al*, 2009a). The plasmid-mediated dissemination of $bla_{\text{CTX-M-15}}$, with the $bla_{\text{OXA-1 or OXA-30}}$, $bla_{\text{TEM-1}}$, and *aac(6')-Ib-cr* genes, in association with the intercontinental *E. coli* clone O25:H4-ST131, was also identified (Machado *et al*, 2006; Mendonça *et al*, 2007; Nicolas-Chanoine *et al*, 2008).

Despite recent studies — one of which including *K. pneumoniae* isolates, collected in 1999 (Mendonça *et al*, 2009a) — the spread and evolution of oxyimino-β-lactam resistance mechanisms, in *Enterobacteriaceae* isolates collected from Portuguese healthcare facilities (with or without the expression of ESBLs), is not sufficiently understood. Therefore, in Paper I of this thesis, a retrospective study was performed, using a total of 220 *Enterobacteriaceae* collected consecutively at a 600-bed tertiary-level hospital, over two periods (1999 and 2004-2008). Overall, this study documents the emergence of bla_{ESBL} genes in clinical isolates: in the first period, β-lactamases were all non-ESBL, and included TEM-1, SHV-1 and CMY-2-types, but during the second period, there was a significant increase in the number of ESBLs found in different species, namely *E. coli*, *K. pneumoniae* and *P. mirabilis*, from 8 in 2004 to 58 in 2008, with CTX-M-type enzymes accounting for 94.8% of the ESBL-positive isolates in 2008. This findings highlight the emergence of this family of β-lactamases in Portugal, as reported in other countries (Cantón & Coque, 2006; Livermore *et al*, 2007), since at least 2004. It is noteworthy that in a surveillance study performed with isolates collected between 2004 and 2006 the rate of CTX-M enzymes among ESBL-producing *E. coli* isolates was already over 65% (Mendonça *et al*, 2007).

The most widespread CTX-M variant, CTX-M-15, was also detected in this study as the most prevalent β-lactamase of this family (80.5%), which is consistent with previous studies performed in Portugal and worldwide (Bonnet, 2004; Cantón *et al*, 2008b; Livermore *et al*, 2007; Machado *et al*, 2007; Mendonça *et al*, 2007; Mendonça *et al*, 2009a). However, a local and transient epidemic clone, *K. pneumoniae* producing CTX-M-3, was identified, which increased the CTX-M frequency among ESBL producers, in the tertiary hospital, to 87.9% in 2007. This was the first time that $bla_{\text{CTX-M-3}}$, which encodes an enzyme initially reported in 1995 in *Citrobacter freundii* and *E. coli* isolates from Praski Hospital in Poland (Gniadkowski *et al*, 1998), has been described in Portugal.

Moreover, in Paper I were identified various risk factors contributing to the emergence of β-lactamase-mediated nonsusceptibility to oxyimino-β-lactams in *Enterobacteriaceae* isolates, in the studied hospital, namely ages of ≥65 years old, nosocomial infection and production of CTX-M-type enzymes. Long-term hospitalizations leading to nosocomial infections and exposure to antibiotics have also been described by others as risk factors for the acquisition of ESBL-producing isolates (Ben-Ami *et al*, 2009; Lavigne *et al*, 2007). We also found that nonsusceptibility to oxyimino-β-lactams, aminoglycosides, and quinolones was associated with CTX-M-producing isolates, and that this could be related to the dissemination of a single clone or mobile genetic elements, as also identified in other countries (Nicolas-Chanoine *et al*, 2008; Ramdani-Bouguessa *et al*, 2006).

In Paper I, we also demonstrated that resistance to oxyimino-β-lactam antibiotics was not only attributed to the production of ESBLs. Overall, 24.1% of the 220 *Enterobacteriaceae* tested were not ESBL-producers, including three PMAβ-producing isolates. Isolates producing these enzymes are generally less frequent than those producing ESBLs, but they are of increasing significance worldwide (Jacoby, 2009; Philippon *et al*, 2002). Hence, it was crucial to understand the real dissemination of PMAβs among Portuguese hospitals and community environments.

Accordingly, the investigation of the presence of PMAβ in 2750 clinical *Enterobacteriaceae* isolates lacking inducible chromosomal *ampC* genes, collected from 28 Portuguese hospitals and other health settings and in different periods (1999 and 2004-2009), was carried out (Paper II). In this study, the prevalence of PMAβs was 2.8% out of the 2570 clinical isolates: 9.9% of which in 1999 and 90.1% in the second period (2004-2009). This value is higher than that found by Mulvey *et al* (2005) (0.09%) and Pitou *et al* (2007) (0.16%), although Hopkins *et al* (2006) (24%) and Potz *et al* (2006) (16.9%) described higher values. A high diversity of β-lactamases was encountered, namely DHA-1, CMY-2, CMY-39, MIR-1, MIR-3, FOX-5, and two novel PMAβ enzymes (CMY-2-type). The sequences of the two CMY-type encoding genes differed from *bla*$_{\text{CMY-2}}$ by several amino acid substitutions and were designated CMY-46 (Ala49Thr, Arg105Ser, Arg126Thr, His133Thr, Thr143Ala, Gln193Lys, Pro208Ala, Ala236Val, His242Arg) and CMY-50 (Val32Ile, Gln35Glu, Gln102Arg, Arg105Ser, Asp124Glu, Arg126Thr, His133Arg, Lys164Gln, Asn194Ser, Asp198Asn, Ala253Glu, Arg261Cys, Val348Ala).

The PMAβ enzymes are frequently found in *Enterobacteriaceae* producing ESBL and/or Qnr and Aac(6′)-Ib-cr determinants (Pai *et al*, 2007; Park *et al*, 2007; Poirel *et al*, 2006; Wang *et al*, 2008). Overall, in this study, we found a co-expression of PMAβs with ESBLs and PMQR determinants of 50.7% and 78.9%, respectively. Indeed, this study shows the association between QnrB4-type and DHA-1 determinants, especially in *K. pneumoniae* isolates, and between DHA-1 and SHV-12, the most detected ESBL after CTX-M-type. Among genes encoding the Qnr families, and contrarily to recent studies in the Iberian Peninsula (Ferreira *et al*, 2010; Lavilla *et al*, 2008), *qnrA* and *qnrD* genes were not detected, but the *qnrB, qnrS* and *qnrC* genes were detected in 67.6%, 7.0% and 2.8% of isolates, respectively. To our knowledge, this is the first report of QnrC outside of China (Wang *et al*, 2009). In summary, this study (Paper II) provides data not only on the prevalence of PMAβs in clinical *Enterobacteriaceae* isolates lacking inducible chromosomal *ampC* genes, but also regarding the occurrence of PMQR determinants in Portuguese healthcare facilities and their association with ESBLs, knowledge that was missing prior to this study.

As mentioned above, CTX-M-15-type enzymes were also detected in *A. baumannii* isolates, a ubiquitous pathogen capable of colonization and causing both community and healthcare-associated infections (CDC, 2004; Munoz-Price & Weinstein, 2008). However, ESBLs are not yet the main problem associated with this specie.

The extensive use of antimicrobial chemotherapy to treat bacterial infections, particularly carbapenems, has contributed to the emergence of CHDLs, and further dissemination of multidrug-resistant *A. baumannii* (MDR-Ab) strains (Perez *et al*, 2007). In Portugal, despite reports of three particular isolates producing IMP-5 (Da Silva *et al*, 2002; Da Silva *et al*, 2010) and, more recently, OXA-23 (Grosso *et al*, 2011), imipenem resistance observed in *A. baumannii* clinical isolates has been mainly associated with the production of OXA-24/40 and linked to the spread of a particular multidrug-resistant clone (Da Silva *et al*, 2004; Quinteira *et al*, 2007). However, there are more recent data available in Paper III regarding the molecular epidemiology of MDR-Ab in Portugal and in an international context, as well as its relation with carbapenemase production. Of the 127 MDR-Ab isolates recovered in nine Portuguese hospitals (each with between 100 and 743 beds), mainly between April 2009 and April 2010, 77.2% expressed OXA-23, 18.1% OXA-24, 27.6% TEM-1, 2.4% CTX-M-15-type and 0.8% TEM-110 genes. This study reports the first appearance of the epidemic strain ST118 (OXA-23- or OXA-24-producer), in both community and Portuguese health care facilities, even in military patients, as well as one ST92 isolate co-producing OXA-23 and OXA-24.

ST118, identified previously in Italy, France, UK and Norway (Hamouda *et al*, 2010; Jolley *et al*, 2004; Mugnier *et al*, 2010), has disseminated since 2009, in all but one of nine hospitals, suggesting importation from elsewhere. ST98 and ST92, which co-existed before 2009 (Grosso *et al*, 2011), seemed to have been replaced by ST118. The identification of a pandrug-resistant (PDR) ST118, and carbapenem-resistant ST92 and ST118 isolates, both in community and health care facilities, demonstrates the menace of *A. baumannii*-associated infections. Furthermore, this study also demonstrated that efficacy of colistin (one of the last-resort antibiotics for treating MDR-Ab infections) is increasingly been compromised. This resistance might be due to point mutations in the PmrA/B two-component system, affecting the expression of genes implicated in lipid A modification and thereby influencing susceptibility to colistin (Beceiro *et al*, 2011).

The results obtained in this thesis highlight different strategies for bacterial spread of resistance, namely mediated by β-lactamases, that can occur either through clonal spread or horizontal gene transfer (HGT). As also Courvalin (2005) reported, HGT can include plasmid epidemics or the dissemination of other mobile genetic elements (MGEs) (Courvalin, 2005).

The best example of HGT is the dissemination of CTX-M-producing isolates. The higher rates of this β-lactamase, among total ESBL enzymes, are associated with high mobilization of the encoding genes (Barlow *et al*, 2008). CTX-M-15 appears to have the best dissemination capacity of all of the CTX-M family, probably due to successful genetic rearrangements (Smet *et al*, 2010). Indeed, a number of genetic mechanisms have apparently been involved in acquisition of CTX-M genes. Insertion sequences IS*26*, IS*Ecp1* and IS*CR1,* in association with class 1 integron structures, seem to have played a prominent role in these processes (Arduino *et al*, 2002; Eckert *et al*, 2006; Lartigue *et al*, 2004; Nordmann *et al*, 2008; Poirel *et al*, 2005; Poirel *et al*, 2008). Moreover, IS*Ecp1* elements constitute an alternative promoter region leading to increased expression of the $bla_{\text{CTX-M}}$ gene (Karim *et al*, 2001), which is clinically relevant, but only weakly expressed in its natural reservoirs (Karim *et al*, 2001; Poirel *et al*, 2003a). Other elements might be involved in the mobilization of $bla_{\text{CTX-M}}$ genes, such as IS*26*, which has been observed upstream of $bla_{\text{CTX-M}}$ genes (Literacka *et al*, 2009; Saladin *et al*, 2002), and IS*903*-like elements, identified downstream of $bla_{\text{CTX-M-14}}$, $bla_{\text{CTX-M-17}}$ and $bla_{\text{CTX-M-19}}$ genes (Cao *et al*, 2002; Chanawong *et al*, 2002; Poirel *et al*, 2003a).

In this thesis, 91.5% of PMAβ-producing isolates (Paper II) were found positive for the class 1 integrase gene, *intI1*. The IS*26* element was identified in 46.0% of DHA-1-producing isolates. The IS*Ecp1 and* IS*903* elements were found upstream and downstream, respectively, of the $bla_{\text{CTX-M-15}}$ genes, which is consistent with what was reported by Mendonça *et al* (2007), where there was an IS*Ecp1* element upstream of all detected $bla_{\text{CTX-M}}$ genes. Given the importance of MGEs, in the mobilization of β-lactamase genes, further investigation into the presence and dissemination of these elements is imperative. In fact, there is a need to characterize the genetic background of new β-lactamase-encoding genes in order to understand their contribution in the HGT of resistance genes and, consequently, to resistance phenotypes of pathogens with impact on public health. Hence, in Paper II, we characterized the genetic environment of the novel CMY-46 and CMY-50-encoding genes. Three antimicrobial resistance cassettes were encountered: an intact *ampC*-*ampR* in both new bla_{CMY} genetic regions, implying that the respective enzymes expressed are inducible (Lindquist *et al*, 1989;

Miriagou *et al*, 2004); a truncated mercury resistance operon, which is of great concern, since mercury resistance may help to promote antibiotic resistance through indirect selection (Baker-Austin, 2006); and a *sul1*-type class 1 integron, containing *dfrA1*, and *aadA2* gene cassettes.

In this thesis (Chapter 1 of Section II), the spread of antibiotic resistance is found to be associated with highly promiscuous plasmids. Plasmid analysis, by conjugation and/or electroporation, indicates that horizontal transfer of these elements is a very efficient mechanism involved in the spread of β-lactamase-mediated resistance in Portuguese hospitals and community environments, namely through those organisms expressing ESBLs (Paper I), PMAβs (Paper II) and/or CHDLs (Paper III). In order to describe the dissemination and follow the evolution of these resistance plasmids, it will be also useful to determine incompatibility groups by a PCR-based replicon typing method (Carattoli *et al*, 2005), after investigation of the localization of the *bla* gene by S1 nuclease digestion, followed by PFGE and Southern blot hybridization (Barton *et al*, 1995).

Once one of these MGEs is acquired, clonal spread is a likely mechanism for the perpetuation of resistance. As seen in Paper I, *K. pneumoniae* was the predominant specie among ESBL-producing isolates in 2007, but not in 2008 or the previous years; this coincided with the high frequency of CTX-M-3-producer *K. pneumoniae* isolates in 2007, suggesting that a micro-epidemic clone emerged in the hospital setting in 2006, both for *K. pneumoniae* and for *E. coli*, which then disappeared in 2008, meanwhile revealing the first observation of CTX-M-3 in Portugal. Otherwise, the persistent *E. coli* CTX-M-15-producer clone, also reported in other Portuguese hospitals (Mendonça *et al*, 2007), was widespread over diverse hospital wards and across several years. CTX-M-3 and CTX-M-15 epidemic clones crossed the hospital-community barrier, contributing to the dissemination of β-lactamase-mediated resistance to oxyimino-β-lactams.

Concerning PMAβ-producers, the study performed in Paper II, showed heterogeneity of profiles among PMAβ-producers, with some epidemic local clones, mainly DHA-1-producers, as in other countries (Naseer *et al*, 2010; Woodford *et al*, 2007). Regarding CMY-producers, the majority of isolates was genetically unrelated, and was distributed over different health institutions, with a higher prevalence among *E. coli* isolates collected in the period of 1999.

In Paper III, as mentioned above, ST98, considered as endemic in Portugal (Da Silva *et al*, 2010), and ST92, both single-locus variant of ST118, were simultaneously present before 2009, as was also reported by others (Grosso *et al*, 2011). However, since 2009 they seemed to have been replaced by epidemic strain ST118, suggesting importation and spread of this ST from elsewhere.

The studies performed in this thesis have therefore clearly shown that specific *bla*-positive clones are able to persist in clinical settings for long periods, resulting in a complex endemic situation.

Resistance to β-lactam antibiotics and inhibitors is rapidly developing, because of single amino acid substitutions and the facilitated spread of β-lactamase genes on plasmids and other MGEs. Hence, β-lactamases offer one of the best examples of protein diversification and evolution as a mechanism of rapid adaptation of bacterial populations to changing environments. In the present dissertation, five novel SHV β-lactamases, conferring different phenotypes, were characterized in terms of their structure and functional properties.

Three new IRS enzymes (SHV-72, SHV-84 and SHV-107), conferring increased resistance to amoxicillin plus clavulanic acid, were characterized (Paper IV, Paper V and Paper VI, respectively); furthermore, the role of the Lys234Arg and Thr235Ala substitutions was investigated (Paper IV and Paper VI, respectively). Overall, with the identification of these β-lactamases, the number of natural occurring SHV enzymes that confer resistance to inhibitors was increased to seven (http://www.lahey.org/Studies).

For the clinically isolated SHV-72 enzyme (Ile8Phe, Ala146Val, Lys234Arg), K_m values for penicillins were not significantly changed and IC_{50}s for clavulanic acid increased 10-fold compared to those of SHV-1 (Paper IV). Interestingly, the mutations observed in SHV-72 did not significantly affect K_m values and did not decrease catalytic activities against penicillins, as noted in other IRS enzymes (Drawz & Bonomo, 2010), namely in SHV-84 (Paper V) and SHV-56 (Dubois *et al*, 2008), where the Lys234Arg substitution appears alone or with Leu35Gln, respectively. Consequently, in SHV-72, mutations other than Lys234Arg may confer increased affinity of the enzyme to penicillins, as well as better catalytic activity against those antibiotics. No effect was observed on the MIC of imipenem in SHV-72-producing *K. pneumoniae* in spite of the presence of substitution Ala146Val (Poirel *et al*, 2003b), since this substitution was described before in SHV-38 as contributing to an elevated carbapenem-hydrolytic activity (Poirel *et al*, 2003b). However, the authors did not present data explaining how the amino acid substitution alters the behavior of the SHV-38 enzyme towards imipenem. Nevertheless, the future characterization of this substitution by itself could explain the increased affinity of the SHV-72 enzyme for penicillin. Simulations of molecular dynamics show that the Lys234Arg substitution induces movement of the Ser130 oxygen away from Ser70. We propose that the mechanism of inhibitor resistance in SHV-72 and SHV-84 may be due to movement of Ser130 based on its role in both hydrolysis and terminal inactivation by cross-linking with Ser70.

One of the other new IRS enzymes, SHV-107 (Paper VI), combines the amino acid substitutions Leu35Gln, which is frequently encountered alone in the SHV-11 enzyme (Mendonça *et al*, 2009b) and known to have no significant impact on kinetic constants (Nüesch-Inderbinen *et al*, 1997), and Thr235Ala, in comparison to the SHV-1 sequence. The latter substitution is the first natural substitution reported at position 235 in class A enzymes, located in a conserved element (Lys234-Thr/Ser235-Gly236). We observed that the substitution Thr235Ala affected SHV-107 kinetic constants against β-lactams with no dramatic consequences to the resistance to penicillins; however, catalytic efficiency for those antibiotics was lower for SHV-107 than for SHV-1. Likewise, previous enzymatic studies of a TEM-1 Ser235Ala mutant, obtained by site-directed mutagenesis, showed results against penicillins similar to the parental TEM-1 enzyme, even though the mutant had greatly reduced cephalosporinase activity (Dubus *et al*, 1994b; Imtiaz *et al*, 1993b). In Paper VI, the simulation of molecular dynamics suggested that the Thr235Ala substitution affects the accommodation of clavulanate in the binding site and therefore its inhibition activity. The results obtained in this thesis, regarding the biochemical characterization of IRS enzymes, permit the inference that the conserved motif Lys234-Thr/Ser-Gly236 present in class A β-lactamases, is a hot-spot for β-lactamase inhibition, meaning that new compounds can be designed to address this structural feature.

Since 1992, over thirty-five inhibitor resistant TEM (IRT) and eleven complex mutant TEM (CMT) enzymes were identified. However, only seven inhibitor resistant mutants from the SHV β-lactamase family (IRS) have been detected clinically so far, mainly in *K. pneumoniae* isolates (Drawz & Bonomo, 2010). The emergence of IRS enzymes raises the question of the possible appearance of CMS (Complex Mutant SHV) enzymes capable of conferring ESBL plus IRS phenotype. However, no CMS has been encountered so far, which is surprising since SHV enzymes are usually more susceptible to inactivation by clavulanate than TEM enzymes, due to differences in the enzyme's active sites (Bonomo & Rice, 1999; Drawz & Bonomo, 2010). The emergence of complex mutant β-lactamases by mutational events may be associated with a selection process within specific sites of infection. Since most IRT and CMT enzymes have been recovered from isolates from urine samples, where penicillin-β-lactamase inhibitors reach higher concentrations, the same could happen with CMS enzymes. The massive use of β-lactam plus β-lactamase inhibitor combinations in patients from the community and hospital environments also facilitates the appearance of these type of enzymes (Cantón *et al*, 2008a).

In Paper VII, a new ESBL, SHV-55, was also characterized in terms of its kinetics properties. This enzyme, carrying the amino acid substitutions Tyr73Phe (as in SHV-28) and Gly238Ser and Glu240Lys (both found in SHV-5), differed from SHV-5 only in the signal peptide region (Gutmann *et al*, 1989). Even though MIC evaluation tests reveal SHV-55 conferring a higher level of resistance to ceftazidime than to cefotaxime (Mendonça *et al*, 2006), kinetic studies showed higher affinity, enzymatic activity and catalytic efficiency towards cefotaxime than to ceftazidime. Overall, these results confirmed that both mutations in combination, Gly238Ser and Glu240Lys, lead to an ESBL phenotype (Chaïbi *et al*, 1999; Knox, 1995).

The importance of β-lactamases not contributing to the β-lactamase inhibitor resistance or to ESBL phenotype is an open issue. However, as already demonstrated, non-ESBL enzymes may facilitate the evolution to ESBL under selection pressure (Hammond *et al*, 2008). Accordingly, the biochemical characterization of the SHV-99 enzyme (Paper VIII) was performed in order to clarify the importance of the Asp104Gly mutation in SHV, since it is the first time that this particular mutation appears in clinical SHV β-lactamase producing isolates. Our kinetic study showed that the Asp104Gly substitution alone is unable to develop the ESBL phenotype in SHV enzymes, as suggested by phenotypic characterization, unlike in TEM β-lactamases (Petit *et al*, 1995). The increased affinity of the enzyme to aztreonam, and given the location in the catalytic pocket, seems to suggest that interaction with residue 104 is the first step for substrate binding and recognition of oxyimino-β-lactams (Bethel *et al*, 2006).

Two other novel enzymes, SHV-98 and SHV-100, which we detected in Paper VIII, possess the amino acid substitution Ser271Ile and amino acid duplication (n=13, SESQLSGRVGMIE) between positions 36 and 48, respectively. EcDH5α-SHV-98 and EcDH5α-SHV-100 transformants were susceptible to oxyimino-cephalosporins and to amoxicillin plus clavulanic acid, confirming that none of the transformants exhibit ESBL or IRS properties. Indeed, these SHV-type β-lactamases contribute poorly to the resistance profile of the clinical isolates.

This is the second time that a new SHV has derived from the parental enzyme by an insertion sequence in a clinical strain, instead of nucleotide substitution(s) (http://www.lahey.org/Studies). However, in the first case, a tandem duplication in the omega loop (163DRWET167), has lead to SHV-16, conferring an extended-spectrum phenotype (Arpin *et al*, 2001). Since the peptide signal of the SHV protein ends in Ambler position 25, as compared to SHV-1 (Péduzzi *et al*, 1989), in SHV-100 the duplication occurs very near to the beginning of the mature protein. As showed by MIC data, SHV-100 is apparently functional despite the insertion, i.e. this duplication seems to have no impact on β-lactamase functions.

In summary, the work performed in this thesis allows for the elucidation on the dynamics of β-lactamases in Gram-negative bacteria in Portugal. The emergence of ESBL-producing *Enterobacteriaceae*, as well as the appearance of new epidemic clones, namely in carbapenem-resistant MDR- and PDR-Ab isolates, is of great concern. Molecular characterization of the genetic context of β-lactamases together with structural and functional data, is essential not only for understanding the emergence of new resistance mechanisms, but also to control its spread. In fact, the results obtained within this thesis reinforce the need for developing control strategies and interventions, in order to reduce both the development of antimicrobial resistant bacteria, and the spread of such infections from hospital to the community.

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